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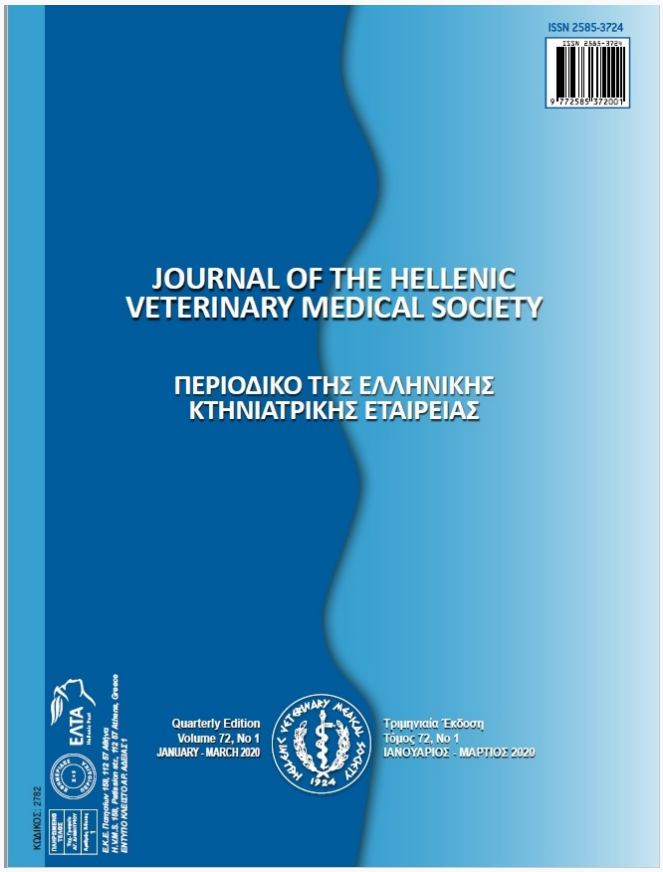

The whole issue

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				Number of specialist veterinarians active in Greece Αριθμός ειδικευμένων κτηνιάτρων εργαζόμενων στην Ελλάδα
1		ECAR	European College of Animal Reproduction	2
2		ECAWBM	European College of Animal Welfare and Behavioural Medicine	2
3		ECAAH	European College of Aquatic Animal Health	3
4		ECBHM	European College of Bovine Health Management	3
5		ECEIM	European College of Equine Internal Medicine	0
6		ECLAM	European College of Laboratory Animal Medicine	0
7		ECPHM	European College of Porcine Health Management	3
8		EPVS	European College of Poultry Veterinary Science	4
9		ECSRHM	European College of Small Ruminant Health Management	12
10		ECVAA	European College of Veterinary Anaesthesia and Analgesia	1
11		ECVCN	European College of Veterinary Comparative Nutrition	0
12		ECVCP	European College of Veterinary Clinical Pathology	1
13		ECVD	European College of Veterinary Dermatology	3
14		ECVDI	European College of Veterinary Diagnostic Imaging	2
15		ECVECC	European College of Veterinary Emergency and Critical Care	0
16		ECVIM-ca	European College of Veterinary Internal Medicine-companion animals	0
17		ECVN	European College of Veterinary Neurology	2
18		ECVO	European College of Veterinary Ophthalmology	0
19		ECVP	European College of Veterinary Pathology	0
20		ECVPH	European College of Veterinary Public Health	5
21		ECVPT	European College of Veterinary Pharmacology and Toxicology	1
22		ECZM	European College of Zoological Medicine	1
23		ECVS	European College of Veterinary Surgery	1
24		EVDC	European Veterinary Dentistry College	0
25		EVPC	European Veterinary Parasitology College	3

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History of the Hellenic Veterinary Medical Society

The Hellenic Veterinary Medical Society (HVMS) is one of the oldest Scientific Societies in our Country. It was founded in 1924 and its first scientific journal was published in 1926. Prompter, Founder and Animating Spirit of HVMS was the General Ioannis D. Petridis (1870-1947), first President and for many years Honorary President of the HVMS. Among the 49 founding members of the HVMS there was also the memorable professor Konstantinos Livadas, the founder of the Veterinary School of the Aristotelian University in Thessaloniki. In spite of the disagreements, the HVMS contributed greatly to the foundation of Veterinary School.

During that time there was only one Scientific Society in Greece, the Medical Society of Athens, which was founded in 1835 and published its first scientific journal in 1922. The HVMS dealt not only with scientific but also with professional topics, like the establishment of the invoices for the veterinarians' payment, taxes, insurance etc. Also, at that period, the accession of the Veterinary Branch in the Hygienists' Pension and Self Insurance Treasury (TSAY) was achieved.

The first post-war assembly of the HVMS took place in the private medical office of Petros Kiappe, on Peta Street in Athens. With its post-war first president Konstantinos Melanidis, the HVMS has been working by implementing its old memorandum of association and has been located in the premises of the Veteri- nary Microbiological Institute of Votanicos, from where all mem- bers of the Governing Board and the Editorial Board of the Journal of the HVMS, were coming from. There, the first «nucleus» of the Library of the HVMS, has been created. That is the reason, this second period of the HVMS successor of the «Petridis period», used to be called «Votanikos period, 1944-1965».

Because HVMS's income was very small, it will remain homeless for many years. Looking for a meeting place the HVMS will find positive response from several services and societies (State Veterinary Offices, Greek Chemical Society, Hellenic Agricultural Society, Medical Society of Athens, Insti- tute of Agricultural Studies, State Veterinary Service of Athens, National Organization of Greek Handwork), which during the following years are going to offer its premises, while in the mid 1958 and for a short period, depending on its financing capabil- ities, the HVMS will rent its own room.

In 1944, the HVMS writes down its first post-war Member Book and in 1948 has already acquired its first 74 regular mem- bers. Also, HVMS is actively working with scientific subjects during regular meetings and public seminars, analyzing current veterinary issues, members' proposals and so on. On 29th May 1947 Mr Petridis presented in the Academy of Athens an issue for veterinary science and its contribution to the progress of the agricultural production and safeguard of Public Health. Also, it should be pointed out, that because there was no professional body, the HVMS is also dealing with issues related to the exe- cution of the veterinary profession.

Furthermore, the role of the HVMS has been determinative on the decision making of the Ministry of Agriculture on veter- inary legislation, on the organization of the Veterinary Service in the Ministry of Agriculture as well as on livestock topics. In the decade of 30s the Supreme Veterinary Advisory Council was created mainly dealing with scientific issues and other aims like promotion, publicity and consolidation of the veterinary science and the veterinary profession in our country and internationally.

The Hellenic Veterinary Medical Society publishes a quarterly scientific journal called Journal of the Hellenic Veterinary Medical Society (J Hellenic Vet Med Soc), as well as other scientific publications, organizes Congresses, Symposiums, Meetings, Lectures etc and generally and almost exclusively it has undertaken for life the Continuing Education of the Greek veterinarians and the students of the two Veterinary Schools.

Nowadays, the Hellenic Veterinary Medical Society is governed by a 9 member Governing Board which is elected every 3 years and has 3 branches:

- **Branch of Companion Animals**
- **Branch of Food Hygiene and Public Health**
- **Branch for Farm Animals**

The HVMS collaborates with the Supreme Educational Foundations, the Technological Educational Institutes, the Veterinary Services, and the Veterinary Associations as well as with Scientific Societies and the Greek and Foreign Chambers.

- **The HVMS is member of the:**
- **Worldwide Veterinary Society**
- **Worldwide Veterinary Society for Companion Animals**
- **Federation of European Veterinary Societies for Compan- ion Animals (founding member)**
- **Veterinary Society of the Balkan and the Black Sea (found- ing member)**

The HVMS has a total of 1220 members many of which have been distinguished in the scientific field (University Profes- sors, Researchers), in the Public Administration, in the Army as well as in the Professional Veterinary Societies and Chambers, in Greece and abroad.

Since 29 May 2001, having signed the contract and since 15 December 2002 the date on which the official opening celebration took place, the Hellenic Veterinary Medical Society is housed in its private premises in a beautiful and majestic one-floor apartment, on the 7th floor of a building in the centre of Athens at 158, Patission street, of 265m2 area, including main lobby (14m2), secretary (13m2), lecture room (91m2), the President's office (22m2), the Governing Board meeting room & library (44m2), the kitchen (18m2), two big baths, a storage room and a large veranda. Al the actions performed for possessing this new private office for the HVMS were performed during the presidency of Dr Theodoros Cl. Ananiadis and the following Governing Board:

President:	Theodoros Cl. Ananiadis†
Vice-President:	Veniamin Albalas
General Secretary:	Athanassios E. Tyrpenou
Spec. Secretary:	Konstantinos Chandras
Treasurer:	Olga Sabatakou
Member:	Emmanuel Archontakis
Member:	Apostolos Rantsios

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Review article
Ανασκόπηση

Hereditary myelopathies in dogs

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ABSTRACT: The purpose of the current review is to summarize data regarding hereditary myelopathies in dogs. Canine degenerative myelopathy (DM) is a progressive disease prevalent in senior (≥8 years old), large breed-dogs, predominating in German shepherd dog. Neurolocalization indicates a thoracolumbar, upper motor neuron, lesion; it can progress to the thoracic limbs and later to lower motor neurons of all limbs resembling human amyotrophic lateral sclerosis (ALS). Tentative diagnosis is based on ruling out other progressive myelopathies. Clinical similarities between ALS and DM made superoxide dismutase 1 gene (SOD1) a viable candidate gene as an etiopathogenic factor. A E40K missense mutation of the SOD1 has been linked to DM. A genetic test for DM exists, which will aid breeding programs to eliminate the disease. Exercise and physiotherapy are important to slow the progression of DM. Long-term prognosis is poor as dogs become non-ambulatory within 4-6 months from the time of diagnosis. Dystrophic myelopathies include Afgan hound myelopathy/hereditary necrotizing myelopathy in Kooikerhondje dogs, leukoencephalomyelopathy and neuroaxonal dystrophy in Rottweilers and spongiform leukoencephalomyelopathy. A similar myelopathy is Jack Russell and Fox terrier hereditary ataxia. Their etiology is suspected to be hereditary and they appear at a young age. They are diagnosed solely postmortem with histopathological examination. There is no etiologic treatment and the prognosis is poor except for Rottweiler neuroaxonal dystrophy and Jack Russel ataxia due to the extremely slow progression of symptoms. Syringomyelia is characterized by the formation of fluid filled cavities within the spinal cord and outside the central canal that may communicate with the central canal and it is caused mainly due to Chari-like malformation (CLM). The most important clinical sign is neuropathic pain and is localized in the cervical region of the spinal cord. Progression of the disease varies, there are severe disabilities due to pain or it can be an incidental finding. CT and MRI are the preferable ways to detect the lesions. For CLM, therapy includes surgical and medical management. Drugs can be divided into analgesics (non-steroidal anti-inflammatory, gabapentin, pregabalin, tramadol), drugs reducing cerebrospinal fluid (CSF) production (omeprazole, acetazolamide, furosemide) and corticosteroids. Medical therapy diminishes the severity of clinical signs but never succeeds full resolution.

Keywords: *degenerative disease, myelodystrophy, myelopathy*

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INTRODUCTION

The etiology of myelopathies is multifactorial. Clinical signs depend on the location, size, and the rate of lesion development (Dewey and da Costa, 2016). The current review focuses on the most important hereditary myelopathies in dogs. Summarized data of the hereditary myelopathies are found in Tables 1 and 2.

Table 1. Summarizing data regarding hereditary myelopathies

Hereditary myelopathies	Etiology	Affected breeds	Lesion localization	Therapy	Prognosis
Degenerative myelopathy	Proteins generated from the mutation of SOD1 gene (E40K)	GSD, PWC, other large- and mixed-breed	T3-L3 spinal cord segments	Physio-therapy	Poor
Dystrophic myelopathies					
Afgan hound myelopathy	Autosomal recessive mode of inheritance	Afgan hound, Kooikerhondje	C6-T2 or T3-L3 spinal cord	None	Poor
Rottweiler leukoencephalopathy	Suspected inherited disorder	Rottweiler, Rottweiler crossbreed	Cervical spinal cord, brainstem, cerebellum	None	Poor
Rottweiler neuroaxonal dystrophy	Inherited disorder	Rottweiler	Cervical spinal cord	None	Guarded
Spongiform leukoencephalopathy	Suspected genetically inherited enzyme deficiency	Dalmatian, Miniature, Poodle, Border Terriers	Cervical spinal cord, brain	None	Poor
Jack Russel/Fox-terrier ataxia	Autosomal recessive mode of inheritance (CAPN1 gene, KCNJ10 gene)	Jack Russell terrier, Smooth-coated Fox terriers Ibizan hound, Parson Russell terriers	Cervical spinal cord, brainstem	None	Guarded to poor
Syringomyelia (Hydromyelia)	CLM, spinal cord/brain malformation, spinal cord trauma, obstructive inflammatory/neoplastic lesions	CKCS, any breed	Cervical spinal cord	Surgical Medical	Good to poor (depends on syrinx size)

CKCS, Cavalier King Charles Spaniel;CLM, Chiari-like malformation;GSD, German Shepherd;PWC, Pembroke Welsh Corgi; SOD1, Superoxidase dismutase 1

Table 2. Major clinicopathological findings of the hereditary myelopathies

Hereditary myelopathies	Anatomical region affected	Clinicopathological findings
Degenerative myelopathy	T3-L3 spinal cord segments	Dorsal portion of lateral and dorsal funiculi of the white matter: axon degeneration, myelin loss
Dystrophic myelopathies		
Afgan hound myelopathy	C6-T2, T3-L3 spinal cord	Dorsal and ventro-medial white matter: myelin loss, vessel proliferation
Rottweiler leukoencephalopathy	Cervical spinal cord	Dorsolateral funiculi of the white matter: demyelinating lesions
	Brainstem/Cerebellar	Demyelinating lesions
Rottweiler neuroaxonal dystrophy	Cervical spinal cord	Dorsal horn grey matter: dystrophic axons
Spongiform leukoencephalopathy	Cervical spinal cord, brain	Spongiform change of the white matter, diminished myelin content, absence of microgliosis, Rosenthal fibers (astrocytic inclusion bodies)
Jack Russel/Fox-terrier ataxia	Cervical spinal cord	Dorsal and ventral funiculi
Syringomyelia (Hydromyelia)	Cervical spinal cord	Fluid-filled dilatation +/- communication with spinal canal

DEGENERATIVE MYELOPATHY (DM)

Degenerative myelopathy (DM) in dogs was first described by Averill in 1973 as a specific degenerative spinal cord disease (Kathmann et al 2006; Granger and Neeves, 2015).

Canine degenerative myelopathy is a slow, progressive neurodegenerative disease prevalent in several dog breeds (Griffiths and Duncan, 1975; Barclay and Haines, 1994; Coates et al. 2007; Awano et al. 2009; Holder et al. 2014; March et al. 2009; Miller et al. 2009). It has been recognized for > 35 years as a spontaneously occurring, adult-onset spinal cord disorder (Awano et al. 2009). Early neuroanatomical localization indicates a lesion between the 3rd thoracic (T3) and the 3rd lumbar (L3) spinal cord segments (Kathmann et al. 2006; Coates et al. 2007; Awano et al. 2009; Miller et al. 2009) and in lumbosacral spinal cord (Kathmannet al. 2006). Initially the disease is thought to be breed-specific to German Shepherds (GSD), thus named German shepherd myelopathy (Braund and Vandevelde, 1978; Longhofer et al. 1990; Barclay and Haines, 1994; Coates et al. 2007; Awano et al. 2009; March et al. 2009; Holder et al. 2014). However, other large breeds, including Old English sheepdog, Belgian shepherd, Collie, Giant Schnauzer, Labrador retriever, Borzoi, Soft-coated Wheaten terrier, Mastiff (Kathmann et al. 2006), Rhodesian Ridgeback, Boxer, Chesapeake Bay Retriever (Long et al. 2009; Granger and Neeves, 2015), Hovawart, Bernese Mountain dog, Kuvasz (Kathmann et al. 2006), Pug and Cavalier King Charles spaniel (Granger and Neeves, 2015), along with mixed-breed dogs have been reported to develop the disease. Pembroke Welsh Corgi (PWC) is a breed commonly affected by DM (Coates et al. 2007; Long et al. 2009; Miller et al. 2009; Granger and Neeves, 2015). DM is confirmed histologically in 3 closely related Siberian husky dogs, suggesting that hereditary factors may be involved in the pathogenesis (Bichsel et al. 1983). Interestingly, there is a feline case report with a presumed histopathological diagnosis of degenerative myelopathy due to the histopathological similarities identified in the thoracolumbar segments and the cause of the lesion that is not apparent (Mesfin et al. 1980).

There is no sex predilection in DM (Kathmann et al. 2006; Awano et al. 2009; Granger and Neeves, 2015), although in affecting PWC there is a predominance of females (Coates et al. 2007). Most dogs are at least 8 years of age before the onset of clinical signs (Griffiths and Duncan, 1975; Kathmann et al. 2006; Awano et al. 2009; Crisp et al. 2014; Holder et al. 2014; Granger and Neeves, 2015), but young dogs can be affected as well (Coates et al. 2007; Polizopoulou et al. 2008). Interestingly, there are reported cases in 2 juvenile German Shepherds (6 and 7 months old) that present symptoms compatible to DM, which is confirmed histologically (Longhofer et al. 1990). On the contrary, in DM-affected PWC, there is a slightly older age distribution (median age 11 years) compared with previous studies (Coates et al. 2007; March et al. 2009). The initial clinical sign is a spastic and general proprioceptive ataxia and paresis of the pelvic limbs (Longhofer et al. 1990; Barclay and Haines, 1994; Kathmann et al. 2006; Awano et al. 2009; March et al. 2009; Crisp et al. 2014; Granger and Neeves, 2015). The asymmetric/symmetric weakness frequently progresses to paraplegia (Polizopoulou et al. 2008; Awano et al. 2009). At this stage of the disease, the preservation of spinal reflexes indicate an upper motor neuron paresis (Kathmann et al. 2006; Awano et al. 2009) although the patellar reflex can be decreased or abolished because of lesions in the dorsal nerve root ganglia (Miller et al. 2009). Pain sensation, bladder control and the panniculus reflex are normal (Griffiths and Duncan, 1975). Hyporeflexia of the myotatic and withdrawal reflexes occur in the advanced disease stage (Kathmann et al. 2006; Awano et al. 2009). In dogs that are not euthanized, the disease progresses and thoracic limbs may be affected (Barclay and Haines, 1994; Awano et al. 2009; Miller et al. 2009; Crisp et al. 2014). In advanced DM cases, dogs may also exhibit signs of lower motor neuron disease, including flaccid tetraparesis, generalized muscle atrophy and hyporeflexia of all limbs (Kathmann et al. 2006; Awano et al. 2009; Miller et al. 2009). The disease duration can exceed 3 years however; most dog owners elect euthanasia within 6 months to a year of diagnosis and after they become paraplegic (Awano et al. 2009). Clinical progression of DM in PWC is similar to that of DM in GSD and other described breeds, however, it is characterized by a longer duration (19 months versus 6 months) (Coates et al. 2007). Many affected dogs may survive with supportive nursing care until respiratory failure develops, which has been reported to occur 3 years or later after the disease onset (Nakamae et al. 2015).

Tentative antemortem diagnosis is based upon ruling out other diseases causing progressive myelopathy (Coates et al. 2007; Granger and Neeves, 2015). Common differentials include spinal cord compres-

sion, inflammatory disease and neoplasia (Griffiths and Duncan, 1975; Jones et al. 2005; Coates et al. 2007). Hip dysplasia and lumbosacral stenosis can often be confused with DM, but the neurologic deficits are different if a careful examination is performed (Griffiths and Duncan, 1975; Coates et al. 2007). It is worth remembering that hip dysplasia can often co-exist with DM and exaggerate the signs seen in the latter disease (Griffiths and Duncan, 1975). Initial tentative diagnosis is based on signalment (older large-breed dogs) and onset of clinical sigs (slow progression) (Kathmann et al. 2006). The proposed diagnostic investigation includes advanced diagnostic imaging techniques (computed tomography-CT, magnetic resonance imaging-MRI), along with cerebrospinal fluid (CSF) analysis (Jones et al. 2005; Kathmann et al. 2006; Coates et al. 2007). Results of CSF analysis are usually unremarkable although occasionally increased protein concentration is noted, especially when samples are collected via a lumbar tap (Kathmann et al. 2006). Morphologic characteristics such as spinal stenosis, focal attenuation of the subarachnoid space, spinal cord deformity, small spinal cord and paraspinal muscle atrophy are common in DM dogs detected via computed tomography combined with myelography (Jones et al. 2005).

Clinical similarities between amyotrophic lateral sclerosis (ALS) and DM lead to the investigation of superoxide dismutase 1 (SOD1), a gene potentially involved in DM pathogenesis (Awano et al. 2009; Holder et al. 2014; Ivansson et al. 2016). In humans, ALS is a disease typically seen between the ages of 45 and 60, where degeneration of upper and lower motor neurons culminates in paralysis and death (Holder et al. 2014). Studies suggest SOD1 activity is unaffected by the presence of SOD1 mutations. Instead, proteins encoded by the mutation version of the gene demonstrate an increased propensity to form aggregates. The relationship of this finding to the pathogenesis of neuronal dysfunction seen in ALS and DM is to be elucidated (Holder et al. 2014). However, while SOD1 mutation homozygosis is a predisposing risk factor, some affected animals do not have the mutation (Awano et al. 2009; Chang et al. 2013). Furthermore, carrier dogs might not develop the disease (Awano et al. 2009; Chang et al. 2013).

There is a study including at risk-PWC dogs homozygous for the SOD1 risk allele, detecting genetic modifier loci that are differentiated between dogs that developed the disease early and dogs that did not develop the disease even at an advanced age (Ivansson et al. 2016). The study results suggest that the mutant SOD1 proteins accumulate in reactive astrocytes during the early phase of DM pathology, which may contribute to subclinical neurodegeneration (Kobatake et al. 2016).

Recently, a novel missense mutation, associated with DM, has been described in the Bernese mountain dog (T18S) (Capucchio et al. 2014; Crisp et al. 2014). Both E40K and T18S mutations in cSOD1 are not loss of function mutations, but form enzymatically active dimers that are prone to aggregation in vitro (Crisp et al. 2014). A genetic test for DM has been developed and is expected to aid in planning breeding programs designed to slowly eliminate DM (Long et al. 2009; Holder et al. 2014). Dogs showing consistent clinical signs and being homozygous for the mutation, are raising the index of suspicion of DM whereas those with heterozygous or homozygous wild-type genotypes are considered more likely to be affected by other disease processes (Holder et al. 2014).

A polyclonal antibody against cSOD1 is generated to further characterize the mutant SOD1 protein and its involvement in DM pathogenesis (Nakamae et al. 2015). Definite diagnosis of DM is determined postmortem by histopathological examination of the spinal cord (Coates et al. 2007; March et al. 2009). Neuromuscular histopathological findings include axon loss, which begins in the thoracolumbar spinal cord and later may involve the cervical and lumbar segments (Barclay and Haines, 1994; Coates et al. 2007; March et al. 2009). Lesions are restricted to the white matter (Barclay and Haines, 1994) and described as discontinuous, bilateral and asymmetric (Longhofer et al. 1990; Coates et al. 2007; March et al. 2009). All white matter funiculi are affected but a predominance of lesions in the dorsal portion of the lateral funiculus and dorsal funiculus have been reported (March et al. 2009; Miller et al. 2009). The degenerative changes involve both the axons and the myelin sheaths (Griffiths and Duncan, 1975; March et al. 2009) with dilation of the myelin sheath, axonal swelling and concurrent fragmentation and phagocytosis of axonal and myelin debris (March et al. 2009). Both motor and sensory tracks are affected (Johnston et al. 2000). They occur at the distal portions of the fiber tracks (Griffiths and Duncan, 1975; Johnston et al. 2000). In the dorsal columns, the rostral portion is affected (Griffiths and Duncan, 1975). Histologically, there are vacuolar spaces which represent lost myelin and axonal degen-

eration which appears as large eosinophilic circular granular bodies in sections stained with hematoxylin and eosin (Barclay and Haines, 1994). Together, these result in a spongy appearance of the white matter histologically (Barclay and Haines, 1994).

Studying the brain of DM-affected dogs reveals neuronal degeneration and loss in the red, lateral vestibular and dentate nuclei, changes that may be related to the origin of the damage and subsequent Wallerian degeneration (Kathmann et al. 2006).

Affected dogs present lesions in the posterior and lateral columns (Awano et al. 2009). In all DM affected dogs, the surviving spinal cord neurons contain cytoplasmic inclusions which are stained with anti-SOD1 antibodies, appearing as well-defined dark clumps immunohistologically (Awano et al. 2009; Granger and Neeves, 2015). Homozygosity for the A allele is associated with DM in 5 dog breeds: Pembroke Welsh Corgi, Boxer, Rhodesian ridgeback, German shepherd and Chesapeake Bay retriever (Awano et al. 2009). In contrast, homozygosity for G allele and heterozygotes, no staining or diffuse light staining similar to the background staining is found in cells of the spinal cords (Awano et al. 2009).

Several authors suggest that exercise and physiotherapy are important to slow the progression of DM (Kathmann et al. 2006; Granger and Neeves, 2015) by virtue of optimizing muscular tone and blood circulation. Walking or swimming should be done on a daily or every other day schedule (Polizopoulou et al. 2008). In a study performed in DM-affected dogs, daily, controlled physiotherapy prolongs survival time to an average of 255 days (Kathmann et al. 2006). A physiotherapy protocol can include daily gait exercise, massage, passive joint movement and hydrotherapy and it seems to be the most important factor in preserving ambulatory status in dogs with DM (Kathmann et al. 2006). Thus, even in dogs with severe neurological deficits at the time of diagnosis, physiotherapy may result in longer survival time even in comparison with dogs with minor neurological deficits that do not receive physiotherapy (Kathmannet al. 2006).When the affected animals are left untreated, non-ambulatory paraplegia is usually established within 6 months after the initial diagnosis (Kathmann et al. 2006; Polizopoulou et al. 2008).

Long-term prognosis is poor (Coates et al. 2007). Dogs lose the ability to ambulate within 4-6 months from the time of diagnosis (Coates et al. 2007). Thoracic limb function and urinary and fecal continence usually are spared until end-stage disease (Coates et al. 2007).

DYSTROPHIC MYELOPATHIES

Afgan hound myelopathy/ Hereditary necrotizing myelopathy in Kooikerhondje dogs

This is a rapidly progressive myelopathy with an autosomal recessive mode of inheritance (Averill and Bronson, 1977). It appears in young (3-13 months old) Afgan hounds (Averill and Bronson, 1977; Dewey and da Costa, 2016). Neuroanatomically, it is a C6-T2 or a T3-L3 myelopathy (Dewey and da Costa, 2016). Affected dogs present symmetrical paraparesis and become paraplegic or even tetraparetic/tetraplegic within 1-3 weeks (Dewey and da Costa, 2016). It can affect the respiratory function lethally (Dewey and da Costa, 2016). Diagnostic tests are normal except for CSF analysis that may reveal increased total protein concentration in some cases (Dewey and da Costa, 2016). Diagnosis is established postmortem with the histopathological examination of the spinal cord. Lesions include symmetrical vacuolization with extensive myelin loss and vessel proliferation in the dorsal and ventro-medial spinal cord of the white matter from caudal cervical to lower lumbar segments, bilaterally (Averill and Bronson, 1977; Cummings and de Lahunta, 1978). There is no treatment and the prognosis is poor (Dewey and da Costa, 2016). A similar disorder, which is called, hereditary necrotizing myelopathy is reported in Kooikerhondje dogs (Mandigers et al. 1993).

Rottweiler leukoencephalomyelopathy

Leukoencephalopathy (LEM) is a progressive, non-painful, central nervous system (CNS) white matter disorder characterized by abnormal myelin synthesis and/or maintenance (Dewey and da Costa, 2016). Its etiology remains unknown although it is suspected to be an inherited disease (Wouda and van Nes, 1986; Dewey and da Costa, 2016). Leukoencephalopathy with brain stem and spinal cord involvement is a neurodegenerative disease in humans with clinical features and MRI findings similar to the histopathological lesions of LEM in Rottweiler dogs (Hirschvogel et al. 2013). Leukoencephalopathy in humans is caused by mutations in the *DARS2* gene, which encodes a mitochondrial aspartyl-tRNA synthetase (Hirschvogel et al. 2013). Due to the phenotypic similarities between human leukoencephalomyelopathy with brain stem and cervical involvement and lactate elevation

(LBSL) patients and LEM-affected Rottweiler dogs, the DARS2 gene is investigated as a candidate for canine LEM in a case, but no mutation in the gene is found (Hirschvogel et al. 2013). More advanced DNA sequencing, such as the use of next-generation technologies for whole-exome or whole-genome resequencing, may enable the identification of the causative mutation of Rottweiler LEM (Hirschvogel et al. 2013). Young adult Rottweiler and Rottweiler crossbred dogs are most commonly affected (Gamble and Chrisman, 1984; Dewey and da Costa, 2016). The neuroanatomical localization of the disease is the cervical spinal cord (Dewey and da Costa, 2016). Clinical signs include progressive ataxia, proprioceptive deficits which are more prominent in the pelvic limbs while hypermetria is more pronounced in the thoracic limbs (Dewey and da Costa, 2016; Hirschvogel et al. 2013). Tetraparesis worsens slowly over maximum a year (Dewey and da Costa, 2016).

Diagnostic investigation involves signalment, history, clinical signs and MRI (Dewey and da Costa, 2016). CSF analysis, including measurement of proteins and total nucleated cells count (TNCC), CSF cytology and measurement of lactate concentrations in the CSF and serum are unremarkable (Yin et al. 2001; Löbert, 2003). Radiological examination of the cervical region of the spinal cord is normal (Dewey and da Costa, 2016). MRI findings are extremely rare, they have been described in only one case, showing bilateral symmetrical hyper intensities in the region of both lateral funiculi on transverse T2-weighted images and isointense on T1-weighted plain images (Hirschvogel et al. 2013). Due to the lack of a genetic test for this disorder, postmortem histopathological examination is required to confirm the diagnosis (Hirschvogel et al. 2013). Symmetrical demyelinating lesions are seen in the spinal cord, especially in the cervical region, brain stem and cerebellum. Demyelination is more prominent in the dorsolateral funiculi of the cervical spinal cord and explains both paresis and ataxia, as the ascending proprioceptive tracts and descending upper motor neuron (UMN) tracts traverse this region (Wouda and van Nes, 1986; Slocombe et al. 1989; Christman, 1991). Despite the cerebellar involvement, these dogs typically do not exhibit symptoms of cerebellar dysfunction (Davies and Irwin, 2003; Dewey and da Costa, 2016). There is no effective treatment against leukoencephalomyelopathy and prognosis is poor (Dewey and da Costa, 2016). There have been no reports of LEM in the literature since 1989 (Slocombe et al. 1989), which could suggest a true decrease in incidence, a lack of information about the disease or exclusion of carrier animals from breeding (Davies and Irwin, 2003).

Rottweiler neuroaxonal dystrophy

Neuroaxonal dystrophy is a degenerative, inherited UMN disease that affects young adult Rottweiler dogs (Davies and Irwin, 2003). Clinical signs appear at a young age (<12 months old), earlier than in leukoencephalomyelopathy (Christman, 1992; Boersma et al. 1995). They include forelimb hypermetria, progressive sensory ataxia, proprioceptive dysfunction (Cork et al. 1983; Evans et al. 1988; Boersma et al. 1995; Davies and Irwin, 2003). Cerebellovestibular signs (head tremor, nystagmus) develop later in the course of the disease however strength is maintained (Christman, 1992; Davies and Irwin, 2003). Neuroanatomically, neuroaxonal dystrophy affects the cervical region of the spinal cord as in leukoencephalomyelopathy, however, the cerebellovestibular signs (frequent in the former disease) are lacking (Cork et al. 1983). Cerebellar atrophy was observed in the autopsy of two cases (Cork et al. 1983).

Definite diagnosis is based on postmortem histopathological examination of the spinal cord. Dystrophic axons appear swollen, eosinophilic, homogeneous or granular and contain variable amount of ubiquitin-immunoreactive deposits (Moretto et al. 1993). Characteristic axonal spheroids are most prominent in the dorsal horn grey matter, the nucleus of the dorsal spinocerebellar tract, the vestibular nucleus, and the nucleus gracilis, the lateral cuneate and cuneate nuclei (Cork et al. 1983; Chrisman, 1992). This distribution of lesions explains the predominance of sensory abnormalities observed clinically. Ultrastructurally, spheroids appear to be swellings of distal axons which are filled with accumulations of smooth membrane-bound vesicles, membranous lamellae, dense bodies, and other organelles (Cork et al. 1983). An inherited disorder of axonal transport is suspected as the underlying defect in this disorder (Cork et al. 1983; Siso et al. 2001). Siso et al. (2001) mention the presence of abnormal expression and accumulation of proteins, which participate in the trafficking, docking and fusion of the synaptic vesicle to the plasma membrane. Similar findings have been observed in isolated dystrophic axons of aged dogs, indicating severe disruption of axonal transport in dystrophic axons in canine neuroaxonal dystrophy (Siso et al. 2001). Neuropathological changes are similar to those identified in human neuroaxonal dystrophy (Cork et al. 1983).

There is no therapy for neuroaxonal dystrophy (Boersma et al. 1995). The short term prognosis is not as poor as in leukoencephalomyelopathy because the progression of the disease is very slow and the affected dogs can be acceptable pets for several years (Christman 1992; Boersma et al. 1995).

Spongiform leukoencephalomyelopathy

It has been reported in young Dalmatians, Miniature Poodles and Border Terriers (Martin-Vaquero et al. 2012). It is a rare disorder called fibrinoid leukodystrophy, and is characterized by lesions in both the brain and the spinal cord. Even though the spinal cord is affected, the predominant clinical signs are generalized tremors (Martin-Vaquero et al. 2012). Histopathological findings include a spongiform change affecting the white matter of the brain and spinal cord with diminished myelin content and absence of microgliosis (Martin-Vaquero et al. 2012). This diminished myelin staining can indicate disruption of normal function or diminished formation (Martin-Vaquero et al. 2012). A prominent histopathological finding is the detection of Rosenthal fibers, which are astrocytic inclusion bodies, located in the white matter of the brain and the cervical region of the spinal cord (Richardson et al. 1991). Their origin is not clear, but it is believed that they represent a primary astrocytic abnormality (Borrett and Becker, 1985). It is not yet known whether the astrocytic abnormality is due to a genetically inherited enzyme deficiency (Richardson et al. 1991).

There is no curative treatment, the disease progresses and the prognosis is poor (Dewey and da Costa, 2016).

JACK RUSSELL AND FOX-TERRIER ATAXIA

It is a rare, presumably inherited, progressively degenerative axonopathy that was first diagnosed in Jack Russell terriers and Smooth-coated Fox terriers in Sweden and England (Björck et al. 1957; Hartley, 1973). As these dogs have common ancestry, affected dogs are suspected to have the same disease (Rohdin et al. 2015). However, segregation analysis indicated different mode of inheritance in the two dog breeds despite the similarities between the diseases (Björck et al. 1957; Hartley, 1973; Wessmann et al. 2004). It has also been described in Ibizan hounds and Parson Russell terriers. In the latter, a missense mutation in the CAPN1 gene is found and is strongly associated with a phenotypically similar inherited ataxia (Forman et al. 2013). Additionally, another missense mutation in the KCNJ10 gene is significantly associated with a similar ataxic neurological disease in 14 dogs belonging in the Russell group of terriers (Gilliam et al. 2014) and in ataxic Smooth-haired Fox Terriers and Toy Fox Terriers (Rodhin et al. 2015). Identification of this gene in a homozygous state in ataxic dogs of different, albeit related, breeds support an earlier assertion that this mutation is causal for a neurodegenerative disease that is inherited in an autosomal recessive mode (Gilliam et al. 2014).

Clinical signs appear at a young age (2-9 months), and worsen progressively (Wessmann et al. 2004). They include symptoms of cervical myelopathy; pelvic limb ataxia that progresses to all four limbs ataxia, dysmetric gait and occasional intention tremor suggesting cerebellar dysfunction than myelopathy. However the involvement of the spinocerebellar tract of the cervical spinal cord is a possible explanation for these clinical signs (dysmetric spastic gait and intention tremor). Rodhin et al. (2010) mention clinical evidence of brain stem involvement (behavioral changes, bilateral decreased menace response), that is confirmed histopathologically. Wessmann et al. (2004) report seizures occurring simultaneously with ataxia while others develop respiratory distress. Additionally, they find that the brain lesions are detected only in Jack Russell Terriers and not in Fox Terriers (Wessmann et al. 2004).

The disease can be clinically diagnosed with a high certainty because of the breed, the age of clinical onset and the nature of the typical clinical signs (Wessmann et al. 2004). Routine laboratory tests in blood and CSF in addition to radiological examination of the spinal cord are normal (Wessmann et al. 2004). Abnormal brain stem auditory- evoked potentials (BAEPs) are recorded in affected dogs and can support clinical diagnosis. Diagnosis is confirmed postmortem with histopathological examination of the affected region (Hartley, 1973). Lesions are characterized by bilateral myelopathy (Hartley, 1973; Summers et al.1995), and more precisely in the dorsal and ventral funiculi of the cervical region of the spinal cord (Summers et al.1995). Regarding the lesions, they are located in the central auditory pathways, the dorsal part of the lateral lemniscus, the ventromedial sulcus of the ventral funiculus and the trapezoid body (Hartley, 1973; Summers et al. 1995). Rarely, lesions are found in the spinal ganglia of the lumbar region or the cauda equina (Hartley, 1973).

There is no curative therapy for this disease. Al-

though it is not lethal, most dogs are euthanized after their owner's request in their 1st year of life (Hartley, 1973). However some cases with mild gait abnormalities have survived for years (Wessmann et al. 2004).

SYRINGOMYELIA (HYDROMYELIA)

Syringomyelia is a fluid- filled dilatation within the spinal cord that can or cannot communicate with the central canal and is usually not lined by ependymal cells (Lee et al. 1985; Norman, 1987; Wisoff and Epstein, 1989; McClarty et al. 1990; Kirberger et al. 1997; Churcher and Child, 2000). In the vast majority of cases, syringomyelia is the result of Chari-like malformation (CLM) (Oldfield et al. 1994; Rusbridge et al. 2000). A lot of breeds have been identified to be at risk for Chiari-like malformation, however, the Cavalier King Charles Spaniel (CKCS) is the predominantly affected breed (McGrath, 1960; Geib and Bistner, 1967; Churcher and Child, 2000; Rusbridge and Knowler,2004). Furthermore, syringomyelia can be caused by other spinal cord or brain malformation, spinal cord trauma, inflammatory of neoplastic processes that obstructs the normal flow of CSF, or idiopathic with no underlying etiology (Schmahl and Kaiser, 1984; Child et al. 1986; Cauzinille and Kornegay, 1992; Kirberger et al. 1997; Rusbridge and Knowler, 2004).

Clinical signs in CKCS with CLM may appear between 6 months and 3 years of age (Rusbridge et al. 2006). Depending on the underlying etiology dogs may be presented at any age but those with more severely clinical signs tend to be younger than 2 years old (Rusbridge and Knowler, 2004). The most important clinical sign in syringomyelia is neuropathic pain (Rusbridge et al. 2000; Todor et al. 2000; Nakamura et al. 2004). Although lesion localization may be difficult, it usually involves the cervical spinal cord (Rusbridge et al. 2006). In CLM, there is cervical hyperesthesia appearing clinically with persistent scratching of the neck and shoulder without skin contact (phantom scratching) (Churcher and Child, 2000; Rusbridge et al. 2000). Scoliosis associated with syringomyelia has also been identified mostly in young animals (Fourneaux et al. 1973; Child et al. 1986; Chuma et al. 1997; Rusbridge et al. 2000). Scoliosis secondary to syringomyelia is the result of muscular imbalance due to paraspinal muscle atrophy (Tagaki et al. 2005). Progression of the disease varies (Rusbridge et al. 2006). In some dogs, there is only scratching with mild pain, and other neurological disorders (ataxia, paresis) appear slowly and progressively or never (Rusbridge et al. 2006). However other dogs are severely disabled due to pain that progressed within 6 months from the first symptom observed (Rusbridge et al. 2006). In others, syringomyelia is an incidental finding (Lu et al. 2003).

The age of clinical onset varies, depending on the etiology of the disease, the different rate of fluid accumulation and the ensuing spinal cord damage and dysfunction (Rusbridge et al. 2006). However, signs of pain are not well correlated with the size of the syrinx, ie, humans or animals with wider or longer syrinxes are not necessarily in more pain than those with smaller syrinxes (Lu et al. 2003; Nakamura et al. 2004). On the other hand, there are other reports showing a relationship between the degree of pain and the width of the syrinx; dogs with larger syrinxes are in pain and dogs with smaller syrinxes are not in pain (Rusbridge et al. 2007). Furthermore, Rusbridge et al. (2007) examined not only the width of the syrinx but also the localized damage in the spinal cord, indicating that the asymmetry of the syrinx, is important in the pathogenesis of neuropathic pain. There have reported other neurological disorders such as thoracic limb weakness, muscle atrophy (ventral horn cell damage), pelvic limb ataxia and weakness, white matter damage with involvement of the lumbar spinal cord by the syrinx (Rusbridge et al. 2000). Hydrocephalus has also been reported in syringomyelia cases (Schmahl and Kaiser, 1984; Itoh et al.1996).

Diagnostic imaging and especially CT and MRI are the preferable way to detect the cavitary lesions (Kirberger et al. 1997; Churcher and Child, 2000; Rusbridge et al. 2000). Mid-sagittal images provide excellent definition of intramedullary cavitation and allow quantitative investigation of the malformation (Churcher and Child, 2000). Sometimes, myelography can be helpful, when the contrast medium enters the cavity within the spinal cord (Kirberger et al. 1997). CSF analysis is usually normal however sometimes it reveals mild inflammation. It is important to perform CSF analysis in order to rule out other diseases (Kirberger et al. 1997).

For Chiari-like malformation (CLM), therapy can be either surgical and/or medical. However, therapy of CLM is beyond the scope of this review and thus only therapy reflecting the elimination of symptoms due to syringomyelia is going to be discussed.

Medical treatment includes analgesics, drugs that reduce CSF production and corticosteroids (Rus-

bridge et al. 2006). In mild cases, pain can be controlled with nonsteroidal anti-inflammatory drugs (Rusbridge et al. 2000). For the relief of scratching, or more severe cases, gabapentin (10 mg/kg b.w.,orally, TID) is useful in order to eliminate the neuropathic pain (Levendoglu et al. 2004). Pregabalin (2 mg/kg b.w., orally, BID), which is considered more effective than gabapentin, and tramadol (2-4 mg/kg b.w., orally,TID/QID) may also be administered. There are some reports on medications that decrease CSF flow and include omeprazole, acetazolamide, furosemide but, clinical data on their use and effectiveness are lacking (Lindvall-Axelsson et al. 1992; Itoh et al. 1996; Javaheri et al. 1997; Churcher and Child, 2000). Regarding acetazolamide, long-term treatment has adverse effects like abdominal pain, weakness, lethargy and thus it is contradicted for long-term use (Vogh, 1980; Shinnar et al. 1985; Carrion et al. 2001). Corticosteroids are administered (0.5 mg/kg b.w., orally, BID), to decrease CSF production (Rusbridge et al. 2000). Although corticosteroids may be effective in limiting signs and progression, most dogs require continuous therapy and develop adverse effects such as immunosuppression, weight gain and skin changes (Rusbridge et al. 2000). Medical therapy can diminish the severity of clinical signs but they never succeed full resolution (Rusbridge et al. 2000). Regarding surgical therapy, syringotomy can improve clinical signs (Cauzinille and Kornegay, 1992; Churcher and Child, 2000). In another report, they performed laminectomy combining with acetazolamide and the dog partially ameliorated, without deteriorating 12 months post surgically (Itoh et al.1996).

Syringomyelia is usually presented with pain. The degree of pain is evaluated clinically and is not necessarily compatible with the severity of the lesions. Pain management can be sufficient to improve pet's quality of life in most of the cases.

CONCLUSIONS

- Degenerative myelopathy is a progressive myelopathy of the thoraco-lumbar spinal cord segments, commonly seen in old German shepherd dogs.
- Physiotherapy can prolong the ambulatory status of the DM-affected dogs.
- Afgan hound myelopathy, Rottweiler leukoencephalopathy, Rottweler neuroaxonal dystrophy, Spongiform leukoencephalopathy and Jack-Russel/ Fox-terrier ataxia are non-painful, hereditary myelopathies affecting the cervical region of the spinal cord.
- Syringomyelia is commonly caused by Chiari-like malformation (CLM).
- Syringomyelia can become painful depending on the length and the size of the intramedullary fluid-filled syrinx.
- Therapy for syringomyelia caused by CLM includes surgical and medical management.

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REFERENCES

Averill DR, Bronson RT (1977) Inheriting necrotizing myelopathy of afghan hounds, J Neuropathol Exp Neurol 36 (4): 734-747.

Awano T, Johnson GS, Wade CM, Katz ML, Johnson GC, Taylor JF, Perloski M, Biagi T, Baranowska I, Long S, March PA, Olby NJ, Shelton GD, Khan S, O'Brien DP, Lindblad-Toh K, Coates JR (2009) Genome-wide association analysis reveals a SOD1 mutation in canine degenerative myelopathy that resembles amyotrophic lateral sclerosis. PNAS 106 (8): 2794-2799.

Barclay KB, Haines DM (1994) Immunohistochemical evidence for immunoglobin and complement deposition in spinal cord lesions in degenerative myelopathy in German shepherd dogs. Can Vet J Res 58: 20-24.

Bichsel P, Vandevelde M, Lang J, Kull-Hächler S(1983) Degenerative myelopathy in a family of Siberian Husky dogs. J Am Vet Med Assoc 183 (9): 998-1000.

Björck G, Dyrendahl S, Olsson SE (1957) Hereditary ataxia in Smooth-Haired Fox Terriers. Vet Rec 69: 871-876.

Boersma A , Zonnevylle H, Sánchez MA, Espiñeira MD (1995) Progressive ataxia in a Rottweiler dog. Vet Q 17: 108-109.

Borrett D, Becker LE (1985) Alexander's disease: a disease of astrocytes. Brain 108: 367-385.

Braund KG, Vandevelde M (1978) German Shepherd dog myelopathy- a morphologic and morphometric study. Am J Vet Res 39(8): 1309-1315.

Capucchio MT, Spalenza V, Biasibetti E, Bottero MT, Rasero R, Dalmasso A, Sacchi P (2014) Degenerative myelopathy in German Shepherd dog: comparison of two molecular assays for the identification of the SOD1:c.118G>A mutation. Mol Biol Rep 41: 665-670.

Carrion E, Hertzog JH, Medlock MD, Hauser GJ, Dalton HJ (2001) Use of acetazolamide to decrease cerebrospinal fluid production in chronically ventilated patients with ventriculopleural shunts. Arch Dis Child 84: 68-71.

Cauzinille L, KornegayJN (1992) Acquired syringomyelia in a dog. J Am Vet Med Assoc 201 (8): 1225-1228.

Chang H-S, Kamishina H, Mizukami K, Momoi Y, Katayama M, Rahman MM, Uddin MM, Yabuki A, Koyiama M, Yamato O (2013) Genotyping assay for the Canine Degenerative Myelopathy- Associated c.118G>A (p.E40K) Mutation of the SOD1 Gene using conventional and Real-Time PCR Methods: A high prevalence in the Pembroke Welsh Corgi Breed in Japan. J Vet Med Sci 75(6): 795-798.

Child G, Higgins RJ, Cuddon PA (1986) Acquired scoliosis associated with hydromyelia and syringomyelia in two dogs. J Am Vet Med Assoc 189 (8): 909-912.

Christman CL (1991) Hemiplegia, hemiparesis, quadriplegia,quadriparesis, ataxia of all four limbs, andepisodic weakness. In: Problems in Small Animal Neurology, 2nd ed, Lea & Febiger, Philadelphia:pp 337-396.

Chrisman CL (1992) Neurological diseases of Rottweilers: Neuroaxonal dystrophy and leukoencephalomalacia. J Small Anim Pract 33: 500-504.

Chuma A, Kitahara H, Minami S, Goto S, Takaso M, Moriya H (1997) Structural scoliosis model in dogs with experimentally induced syringomyelia. SPINE 22 (6): 589-595.

Churcher RK, Child G (2000) Chiari 1/Syringomyelia complex in a Cavalier King Charles Spaniel. Aust Vet J 78 (2): 92-95.

Clemmons RM (1992) Degenerative myelopathy. Vet Clin North Am Small Anim Pract 22 (4): 965-971.

Coates JR, March PA, Oglesbee M, Ruaux CG, Olby NJ, Berghaus RD, O'Brien DP, Keating JH, Johnson GS, Williams DA (2007) Clinical characterization of a familial degenerative myelopathy in Pembroke Welsh Corgi Dogs. J Vet Intern Med 21: 1323-1331.

Cork LC, Troncoso JC, Price DL, Stanley EF, Griffin JW (1983) Canine Neuroaxonal Dystrophy. J Neuropathol Exp Neurol 42 (3): 286-296.

Crisp MJ, Beckett J, Coates JR, Miller TM (2014) Canine Degenerative Myelopathy: Biochemical characterization of superoxide dismutase 1 in the first naturally occurring non-human amyotrophic lateral sclerosis model. Exp Neurol available in PMC, 1-20.

Cummings JF,De Lahunta A (1978) Hereditary myelopathy of Afghan Hounds, a myelinolytic disease. Acta Neuropath 42: 173-181.

Davies DR, Irwin PJ (2003) Degenerative neurological and neuromuscular disease in young Rottweilers. J Small Anim Pract 44: 388-394.

Yin W, Tibbs R, Aoki K, Badr A, Zhang J (2001) Metabolic alterations in cerebrospinal fluid from double hemorrhage model of dogs. Neurol Res 23(1): 87-92.

Dewey CW, Da Costa RC (2016) Myelopathies: Disorders of the Spinal Cord. In: Practical guide to canine and feline neurology, 3rd ed, Wiley Blackwell, Singapore: 358-369.

Evans MG, Mullaney TP, Lowrie CT (1988) Neuroaxonal dystrophy in a Rottweiler pup. J Am Vet Med Assoc192: 1560-1562.

Forman OP, De Risio L, Mellersh CS (2013) Missense mutation in CAPN1 is associated with spinocerebellar ataxia in the Parson Russell Terrier dog breed. PLoS One8 (5) e64627.

Furneaux RW, Doige CE, Kaye MM (1973) Syringomyelia and Spina Bifida Occulta in a Samoyed Dog. Can Vet Jour 14 (2): 317-321.

Gamble DA, Chrisman CL (1984) A leukoencephalomyelopathy of Rottweiler dogs. Vet Pathol 21: 274-280.

Geib LW, Bistner SI (1967) Spinal cord dysraphismin a dog.J Am Vet Med Assoc 150: 618-620.

Gilliam D, O'Brien DP, Coates JR, Johnson GS, Johnson GC, Mhlanga-Mutangadura T, Hansen L, Taylor JF, Schnabel RD (2014) A homozygous KCNJ10 mutation in Jack RussellTerriers and related breeds with spinocerebellar ataxia with myokymia, seizures or both. J Vet Intern Med28: 871-877.

Granger N, Neeves J (2015) Developing objective measures of gait abnormalities in dogs with degenerative myelopathy. Vet Rec 176: 290.

Griffiths IR, Duncan ID (1975) Chronic degenerative radiculomyelopathy in the dog. J Small Anim Pract 16: 461-471.

Hartley WJ (1973) Ataxia in Jack Russell Terriers. Acta Neuropath 26: 71-74.

Hirschvogel K, Matiasek K, Flatz K, Drögemüller M, Drögemüller C, Reiner B, Fischer A (2013) Magnetic Resonance Imaging and genetic investigation of a case of Rottweiler leukoencephalomyelopathy. Vet Res 9: 57.

Holder AL, Price JA, Adams JP, Volk HA, Catchpole B (2014) A retrospective study of the prevalence of the canine degenerative myelopathy associated superoxidase dismutase 1 mutation (SOD1:c.118G>A) in a referral population of German Shepherd Dogs from the UK. Canine Genet Epidemiol 1: 10.

Itoh T, Nishimura R, Matsunaga S, Kadosawa T, Mochizuki M, Sasaki N(1996) Syringomyelia and hydrocephalus in a dog. J Am Vet Med Assoc 209 (5): 934-936.

Ivansson EL, Megquier K, Kozyrev SV, Murén E, Baranowska Körberg I,Swofford R, Koltookian M, Tonomura N, Zeng R, Kolicheski AL, Hansen L, Katz ML, Johnson GC, Johnson GS, Coates JR, Lindblad-Toh K (2016) Variants within the SP110 nuclear body protein modify risk of canine degenerative myelopathy. PNAS: 3091-3100.

Javaheri S, Corbett WS, Simbartl LA, Mehta S, Khosla A (1997) Different effects of omeprazole and Sch 28080 on canine cerebrospinal fluid production. Brain Res 754:321-324.

Johnston PEJ, Barrie JA, McCulloch MC, Anderson TJ, Griffiths IR (2000) Central nervous system pathology in 25 dogs with chronic degenerative radiculomyelopathy. Vet Rec 146: 629-633.

Jones JC, Inzana KD, Rossmeisl JH, Bergman RL, Wells T, Butler K (2005) CT-myelopathy of the thoraco-lumbar spine in 8 dogs with degenerative myelopathy. J Vet Sci 6: 341-348.

Kathmann I, Cizinauskas S, Doherr MG, Steffen F, Jaggy A (2006) Daily controlled physiotherapy increases survival time in dogs with suspected degenerative myelopathy. J Vet Intern Med 20: 927-932.

Kirberger RM, Jacobson LS, Davies JV, Engela J (1997) Hydromyelia in a dog. Vet Radiol Ultrasound 38 (1): 30-38.

Kobatake Y, Sakai H, Tsukui T, Yamato O, Kohyama M, Sasaki J, Kato S, Urushitani M, Maeda S, Kamishina H (2016) Localization of a mutation SOD1 protein in E40K- heterozygous dogs: Implications of anon-cell-autonomous pathogenesis of degenerative myelopathy. JNeurolScihttp://dx.doi.org/10.1016/j.jns.2016.10.034

Lee BCP, Zimnicrman RD, Manning JJ, Dcck MDF (1985) MR imaging of syringomyclia and hydromyelia. AJNR 6: 221-228.

Levendoglu F, Ogun CO, Ozerbil O, Ogun TC, Ugurlu H (2004) Gabapentin is a first line drug for the treatment of neuropathic pain in spinal cord injury. Spine 29:743-751.

Lindvall-Axelsson M, Nilsson C, Owman C, Winbladh B (1992) Inhibition of cerebrospinal fluid formation by omeprazole. Exp Neurol 115: 394-399.

Löbert V (2003) Etablierung von laktat- und pyruvatmessung im plasma und liquor cerebrospinalis zur diagnostik von mitochondrialen erkrankungen beim hund. Inaugural-dissertation. University of veterinary medicine Hannover: Department of Small Animal Medicine and Surgery.

Long SN, Henthorn P, Serpell J, Van Winkle T, Coates JR (2009) Degenerative myelopathy in a Chesapeake Bay Retrievers. J Vet Intern Med 23: 396-416.

Longhofer SL, Duncan ID, Messing A (1990) A degenerative myelopathy in young German shepherd dogs. J Small Anim Pract 31: 199-203.

Lu D, Lamb CR, Pfeiffer DU, Targett MP (2003) Neurological signs and results of magnetic resonance imaging in 40 cavalier King Charles Spaniels with Chiari type 1 like malformations. Vet Rec 153: 260-263.

McClarty B, Suthcrland S. Bertram EG (1990) The Spine. In: Kucharczyk W MRI: Central Nervous System. Raven Press, Philadelphia: 19.

Mc Grath JT (1960) Neurologic Examination of the Dog. In: Neurological examination of the dog with clinicopathologic observations. 2nd Ed, Lea & Febiger, Philadelphia: 200-211.

Mandigers PJJ, Van Nes JJ, Knol BW, Ubbink GJ, Gruys E (1993) Hereditary necrotizing myelopathy in Kooiker dogs. Res Vet Sience 54: 118-123.

March PA, Coates JR, Abyad RJ, Williams DA, O' Brien DP, Olby NJ, Keating JH, Oglesbee M (2009) Degenerative myelopathy in 18 Pembroke Welsh Corgi dogs. Vet Pathol 46: 241-250.

Martin-Vaquero P, Da Costa RC, Simmons JK, Beamer GL, Ja¨ derlund KH, Oglesbee MJ (2012) A novel spongiform leukoencephalomyelopathy in Border Terrier Puppies. J Vet Intern Med 26: 402-406.

Mesfin GM, Kusewitt D, Parker A (1980) Degenerative myelopathy in a cat. J Am Vet Med Assoc 176 (1): 62-64.

Miller AD, Barber R, Porter BF, Peters RM, Kent M, Platt SR, Schatzberg SJ (2009) Degenerative myelopathy in two boxer dogs. Vet Pathol 46: 684-687.

Moretto G, Sparaco M, Monaco S, Bonetti B, Rizzuto N (1993) Cytoskeletal changes and ubiquitin expression in dystrophic axons of Seitelberger's disease. Clin neuropathol 12: 34-37.

Nakamae S, Kobatake Y, Suzuki R, Tsukui T, Kato S, Yamato O, Sakaki H, Urushitani M, Maeda S, Kaminisha H (2015) Accumulation and aggregate formation of mutatnt superoxidase 1 in canine degenerative myelopathy. Neuroscience 303: 229-240.

Nakamura M, Chiba K, Nishizawa T, Maruiwa H, Matsumoto M, Toyama Y (2004) Retrospective study of surgery-related outcomes in patients with syringomyelia associated with Chiari I malformation: Clinical significance ofchanges in the size and localization of syrinx on pain relief. JNeurosurg 3 (l): 241-244.

Norman D (1987) MR of the spine. In: Brant-Zawadzki M, Norman D, eds. Magnetic restinance imaging of the central nervous system. Raven Press,New York: 311-314.

Oldfield EH, Muraszko K, Shawker TH,Patronas NJ (1994) Pathophysiology of syringomyelia associated with Chiari 1 malformation of the cerebellar tonsils. Implications for diagnosis and treatment. Neurosurg 80: 3-15.

Polizopoulou ZS, Koutinas AF, Patsikas MN, Soubasis N (2008) Evaluation of a proposed therapeutic protocol in 12 dogs with tentative degenerative myelopathy. Acta veterinaria Hungarica 56 (3): 293-301.

Richardson JA, Tang K, Burns DK (1991) Myeloencephalopathy with Rosenthal fiber formation in a miniature poodle. Vet Pathol 28: 536-538.

Rohdin C, Lüdtke L, Wohlsein P, Jäderlund KH (2010) New aspects of hereditary ataxia in smooth-haired fox terriers. Vet Rec 166 (18): 557-560.

Rohdin C, Gilliam D, O'Leary CA, O'Brien DP, Coates JR, Johnson GS, Hultin Jäderlund K (2015) A KCNJ10 mutation previously identified in the Russell group of terriers also accurs in Smooth-haired Fox Terrierswith hereditary ataxia and in related breeds. Acta Veterinaria Scandinavica 57 (26): 1-4.

Rusbridge C, MacSweeny JE, Davies JV, Chandler K, Fitzmaurice SN, Dennis R, Cappello R, Wheeler SJ (2000) Syringohydromyelia in Cavalier King Charles Spaniels. J Am Anim Hosp Assoc 36 (1): 34-41.

Rusbridge C, Knowler SP (2004) Inheritance of occipital bone hypoplasia (Chiari type I malformation) in Cavalier King Charles Spaniels. J Vet Intern Med 18: 673-678.

Rusbridge C, Greitz D, Iskandar BJ (2006) Syringomyelia: current concepts in pathogenesis, diagnosis and treatment. J Vet Intern Med 20: 469-479.

Rusbridge C, Carruthers H, Dube M-P, Holmes M,Jeffery ND (2007) Syringomyelia in Cavalier King Charles Spaniels: the relationship between syrinx dimension and pain. J Small Anim Pract 48: 432-436.

Schmahl W, Kaiser E (1984) Hydrocephalus, Syringomyelia and Spinal cord angiodysgenesis in a Lhasa-apso dog. Vet Pathol 21: 252-254.

Shinnar S, Gammon K, Bergman EW Jr, Epstein M, Freeman JM (1985) Management of hydrocephalus in infancy: Use of acetazolamide and furosemide to avoid cerebrospinal fluid shunts. J Pediatr 107: 31-37.

Siso S, Ferrer I, Pumarola M (2001) Juvenile neuroaxonal dystrophy ina Rottweiler: accumulation of synaptic proteins in dystrophic axons. Acta Neuropathol 102: 501-504.

Slocombe RF, Mitten R, Mason TA (1989) Leykoencephalomyelopathy in

Australian Rottweiler dogs. Austr Vet J 66 (5): 147-150.

Summers BA, Cummings JF, De Lahunta A (1995) Textbook of Veterinary Neuropathology. MO: Mosby, St Louis: pp 321-322.

Tagaki S, Kadosawa T, Ohsaki T, Hoshino Y, Okumura M, Fujinaga T (2005) Hindbrain decompression in a dog with scoliosis associated with syringomyelia. J Am Vet Med Assoc 226 (8): 1359- 1363.

Todor DR, Harrison TM, Millport TH (2000) Pain and syringomyelia: review. Neurosurg Focus 8: 1-6.

Vogh BP (1980) The relation of choroid plexus carbonic anhydrase activity to cerebrospinal fluid formation: Study of three inhibitors in cat with extrapolation to man. J Pharmacol Exp Ther 213: 321-331.

Wessmann A, Goedde T, Fischer A, Wohlsein P, Hamann H, Distl O, Tipold A (2004) Hereditary Ataxia in the jack Russell terrier - Clinical and Genetic Investigations. J Vet Intern Med 18: 515-521.

Wisoff JH, Epstein F (1989) Management of hydromyelia. Neurosurgery 25: 562-571.

Wouda W, van Nes JJ (1986) Progressive ataxia due to central demyelination in Rottweiler dogs. Vet Q 8 (2): 89-97.

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Review article
Ανασκόπηση

Essential oils as alternatives to chemical feed additives for maximizing livestock production

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ABSTRACT: This review is aimed at providing basic and current knowledge about possible mechanisms and nutritional applications of essential oils (EOs) for food animals. Public concern on the excessive use of antibiotics in livestock production has started extensive research to find safe and efficient options. EOs extracted from aromatic plants are known to have a range of biologically active properties that can be applied to modern animal production. Primarily, EOs possess anti-inflammatory, anti-microbial, and digestion enhancing effects as they improve digestive enzymes, improve feed conversion ratio, modulate ruminal fermentation, add antioxidant properties, and underpin animal immunity. The dietary supplementation of EOs demonstrated as a simple and proficient approach to enhance the performance of livestock. However, mechanisms involved in enhancing animal performance, modulating ruminal fermentation, and microflora are still unclear. Moreover, limited information is available regarding interactions among feed, EOs, and gut ecosystem of animals. EOs could be used as nutraceuticals with possible commercial applications in modern animal nutrition such as antimicrobials, antioxidants, growth promoters, and immunomodulators, alternatives to chemical feed additives. This knowledge encourages further investigations about EOs to realize their full potential and build up their standard use in livestock production.

Keywords: Essential oils; poultry; ruminants; antibiotic resistance; pigs

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INTRODUCTION

For a long time, dietary supplementation of chemical feed additives started to increase animal growth, performance, and efficiency. Antibiotics supplemented in the animal diet at a sub-therapeutic level is intensifying livestock production, reducing morbidity and mortality but also associated with the development of antimicrobial resistance that may present a risk to human health. Correspondingly, natural feed additives extracted from herbs, plants, and spices such as EOs have been evaluated and considered as a substitute to chemical feed additives in livestock production for improving animal production and health. EOs are complex mixture of different components, hence chances of development of resistance in microbes are less as compared to the single synthetic compound. In terms of biological activity and effects, each constituent of EO possesses its characteristic properties. EOs hold the potential of possible therapeutic exploitation in different ways in animal production. They represent a wide range of biologically active compounds like phenolic and terpenoids which possess a variety of functions with health-related benefits and nutrigenomics implications on the development of the gut and immunity (Christaki et al., 2020). In terms of ruminant nutrition, EOs enhance animal performance, manipulate rumen fermentation such as increase protein metabolism, reduce ammonia and methane production, improve volatile fatty acids (VFA) proportions and target some ruminal microorganisms like methane-producing archaea and hyper-ammonia producing (HAP) bacteria (Campolina et al., 2020; Hart et al., 2019; Silva et al., 2020; Tapki et al., 2020; Zhou et al., 2020). They also possess remarkable effects on monogastric animals like improve digestive secretions, body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), meat, and egg qualities (Ismail, El-Gogary, and El-Morsy, 2019; Lee et al., 2020; Masood et al., 2020; Sariözkan et al., 2020; Yalçin et al., 2020). They also exhibit antioxidant properties, stimulate blood circulation, reduce the pathogens count, enhance digestion and nutrient uptake, and relieve the animals from disease and environmental stress. However, due to the intricacy of the animal body systems and EOs composition, the dosage level and effects of EOs on different animal species and systems seem to be difficult to predict (Puvača et al., 2020). To date, only a few studies evaluated EOs with a known chemical composition in modulating their effects and function, while the mode of actions of underlying these functions has not been completely clarified yet (Simitzis, 2017; Zeng et al., 2015). Moreover, the chemical composition of EOs depends on species, topographical location, harvesting stage, parts of the plant, and extraction methods (Puvača et al., 2019). Source of inconsistency also relies on origin and type of EOs, the dosage level of EO supplemented in animal feed, the amount of FI, formulation, and digestibility of basal diet, and environmental conditions (Brenes and Roura, 2010; Dudareva, Pichersky, and Gershenzon, 2004). This review clarifies the current advancements in the utilization of EOs to possibly benefits in food animal production. Mode of action is summarized, including impacts on animal performance, control of pathogens, ruminal fermentation, and microflora.

ANTIMICROBIAL EFFECTS OF EOS

Plant and plant extracts have traditionally played a vital role in the wellbeing and healthcare of humans and animals as therapeutic agents for the treatment of many illnesses. Due to essence, flavor, antimicrobial, and preservative properties, plant secondary metabolites have been used by mankind since early history (Giannenas et al., 2020; Akram et al., 2019a; Jalal et al., 2019). EOs and their components are hydrophobic, a feature that allows them to penetrate the lipidic layer of bacteria resulting in the disturbance of cell osmotic pressure by interrupting membrane integrity and ion transport process (Florou-Paneri et al., 2019). EOs or their components sensitize the cell wall, causing significant membrane damages leading to the integrity collapse of membranes and biosynthetic machinery of the bacterial cell resulting in the leakage of vital cellular contents and death of bacterial cells. In detail, rapid dissipation of proton motive sources (hydrogen and potassium ion gradients) and depletion of the intracellular adenosine triphosphate (ATP) pool is seen through the declination of ATP synthesis and the increased hydrolysis. It results in the slowing down of bacterial growth by increasing permeability of the membrane and decreasing trans-membrane electric potential in the bacterial cell. When the bacterial cell tolerance level is passed, extensive loss of cell substances leads to cell death. Furthermore, the presence of hydroxyl group (OH) attached to a phenyl ring and its capability to discharge its proton are viewed as critical factors in disturbing normal ion transport across the cytoplasmic membrane and in deactivating microbial enzymes (Burt, 2004; Ultee et al., 2002). The previously described mode of action is more potent against gram-positive than gram-negative bacte-

ria. The external cell wall of gram-negative bacteria is hydrophilic and hydrophobic components of EOs cannot easily infiltrate into the membrane. However, molecules of EOs with low molecular weight can interrupt the membrane integrity by passing the bacterial cell wall through diffusion with the assistance of membrane proteins or layer of lipopolysaccharides (Akram et al., 2019b; Giannenas et al., 2018).

EOs possess antimicrobial activity due to terpenoids and phenolic compounds (Florou-Paneri et al., 2019). Thyme and oregano inhibited the growth of pathogenic strains like *Salmonella enteritidis, Salmonella choleraesuis, Salmonella typhimurium,* and *Escherichia coli* (Peñalver et al., 2005), which is attributed to the phenolic components such as thymol and carvacrol. Moreover, Abdullah et al., (2015) studied the effects of clove bud oil and rosemary oil for their antimicrobial effects against multidrug-resistant strains such as *Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumannii,* and *Enterococcus feacalis.* EOs have also antimicrobial properties against zoonotic enteropathogenic strains like *Salmonella spp.* and *Escherichia coli O157:H7* (Guo et al., 2020; Olaimat et al., 2019), which shows that EOs can be used as alternatives to antibiotics in animal nutrition and production. Furthermore, EOs possess antimicrobial effects against gram-positive bacteria such as *Fusobacterium necrophorum, Trueperella pyogenes, Staphylococcus aureus,* and *Listeria monocytogenes* (Cho et al., 2020; Paiano et al., 2020) and gram-negative bacteria like *Escherichia coli* (Al-Mnaser and Woodward, 2020). In addition, EOs could be used against mastitis-causing bacteria (Amat et al., 2017; Zhu et al., 2016), respiratory pathogens (Akbari et al., 2018), and urinary tract infection (Ebani *et al.*, 2018). However, EOs showed effectiveness against viruses like *Melissa officinalis* EO was found effective against *Avian influenza virus* (Pourghanbari et al., 2016), while ajwain oil and *Artemisia arborescens* EOs showed antiviral activity against *Japanese encephalitis virus* (Roy, Chaurvedi, and Chowdhary, 2015) and *Herpes simplex virus type I and II* respectively (Sinico et al., 2005). Additionally, Govindarajan et al., (2016) observed that antilarval activity of the EO isolated from *Plectranthus barbatus* against larvae of the malaria vector *Anopheles subpictus*, the dengue vector *Aedes albopictus,* and the Japanese encephalitis vector *Culex tritaeniorhynchus.* Application of EOs in animal feed for health management, improvements in productivity and quality has proved a viable strategy, which is also the consumers' demand. The effects of EOs against bacteria, viruses, fungi, and protozoa are illustrated in Table 1. Dietary supplementation of EOs is an appropriate strategy of introducing natural antimicrobials in the body of animals that are entered, circulated in the body, and retained in tissues, which may provide help to prevent the lipid oxidation and microbial spoilage at their localized sites.

Table 1: Summary of studies testing antimicrobial activity of essential oils or their components against pathogenic microbes.

Essential oil or components	Species/group of microorganisms	References
Cinnamon	*Escherichia* coli and *Staphylococcus aureus*	Zhang et al., 2016
	Listeria monocytogenes	Abdollahzadeh et al., 2018
	Agrobacterium tumefaciens	Lee et al., 2020
	Escherichia coli	Kosariet al., 2020
	Bovine mastitis in organic dairy farming: *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hyicus, Staphylococcus xylosus* and *Escherichia coli*	Zhu et al., 2016
	Pseudomonas aeruginosa	Elcocks et al., 2020
Thyme	*Listeria monocytogenes*	Sarengaowaet al., 2019
	Escherichia coli (E. coli) O157:H7	Guoet al., 2020
	Streptococcus mutans	Abdel Hameed et al., 2020
	Staphylococcus aureus	Mohammed et al., 2020
	Streptococcus pyogenes	Maqbulet al., 2020
	Aspergillus flavus	Khaliliet al., 2015
Thyme & Cinnamon	*Salmonella* Species	Al-Nabulsiet al., 2020
	Salmonella species	Olaimatet al., 2019
Thyme & Oregano	*Listeria monocytogenes*	Cho et al., 2020

Pine oil	*Escherichia coli* O157:H7, *Listeria*, and *Campylobacter* species	Wells et al., 2015
Eucalyptus	Multi-drug resistant *Acinetobacter baumannii* *Escherichia coli*	Knezevicet al., 2016 Kareem et al., 2020
Eugenol	Verotoxin producing *Escherichia coli*	Ezzeldeenet al., 2015
Tea tree oil	Multi-drug resistant *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*	Oliva et al., 2018
MEO	Environmental mastitis: *Staphylococcus aureus, Staphylococcus chromogenes, Staphylococcus sciuri, Staphylococcus warneri, Staphylococcus xylosus* and *Escherichia coli*	Fratini et al., 2014
	Bovine respiratory pathogen: *Mannheimia haemolytica*	Amat et al., 2017
	Bovine endometritis: Escherichia coli, Fusobacterium necrophorum, Trueperella pyogenes, Staphylococcus aureus	Paiano et al., 2020
	Urinary tract infection: Multidrug-resistant strains of *Escherichia coli*, *Enterococcus* spp., *Candida albicans* and *Candida famata*	Ebaniet al., 2018
	Leishmania, Plasmodium and Trypanosoma species	Le et al., 2018
	Arthropod disease vector: female *Ixodesricinus*	Kulmaet al., 2017
Oregano	*Salmonella* species	Mohan and Purohit, 2020
Oregano & Rosemary	*Escherichia coli* O157:H7	Diniz-Silva et al., 2020
Oregano &Carvacrol	*Escherichia coli O23:H52*	Al-Mnaser and Woodward, 2020
Melissa oil	*Avian influenza A virus (H9N2)*	Pourghanbari et al., 2016
Ajwain oil	*Japanese encephalitis virus*	Roy et al., 2015
Plectranthus barbatus oil	Larvicides against malaria, dengue and Japanese encephalitis mosquito vectors	Govindarajanet al., 2016
Marrubium vulgare oil	Bovine reproduction system pathogen: *Trichomonas vaginalis*	Akbari et al., 2018
Arisaemafargesii	Larvicidal activity against *Aedes mosquitoes*	Huang et al., 2020
Myristica fragrans	*Escherichia coli*, *Staphylococcus aureus*	Kiarsiet al., 2020

MEO = mixture of essential oils

EFFECTS OF EOS ON THE DIGESTIVE SYSTEM OF MONOGASTRIC ANIMALS

Dietary supplementation of EOs has positive effects on animal health, gut microflora, intestinal morphometrics, enzymatic activity, and growth performance parameters that have been studied comprehensively in Table 2. In general, EOs seem to stimulate beneficial bacteria, inhibit pathogenic microbes, regulate enzyme activities and execute beneficial effects on gut villi with inducing positive effects on BWG, FCR, and FI (Abbasi et al., 2019; Barbarestani et al., 2020; Park and Kim, 2018; Yang et al., 2019). Beneficial microbes like *Lactobacilli species (sp.)* trigger the local intestinal immune system by releasing the peptides of low molecular weight, which increase the resistance against diseases (Muir et al., 2000). Furthermore, a high number of *Lactobacilli sp.* decrease the pathogenic microbes through developing the colonization resistance by modifying the receptors used by pathogens (Rinttilä and Apajalahti, 2013; Adil and Magray, 2012). EOs further showed improvements in averaged daily gain, growth performances, carcass quality and reduced cholesterol level in broilers, quails, and pigs (Attia, Bakhashwain, and Bertu, 2017; Fathi et al., 2020; Mercati et al., 2020; Placha et al., 2019; Wade et al., 2018). Moreover, they helped poultry in fighting against diseases such as Newcastle disease, Infectious bursal disease, and avian influenza and coccidiosis (Ahmadian et al., 2020; Lee et al., 2020). EOs also increased the immunity and antioxidant capacity in heat stress periods (Eler *et al.*, 2019; Sariözkan et al., 2020). In layer hen, along with improving growth performance characteristics, EOs improve egg quality and shell related parameters (Abo Ghanima et al., 2020; Beyzi et al., 2020; Yalçin et al., 2020). As documented in the literature, EOs exhibit antimicrobial activity against *Escherichia coli* (Park and Kim, 2018), *Clostridium perfringens* (Cho et al., 2014), *Salmonella typhimurium* (Ahmed et al., 2013), and prevent the adhesion, colonization, and proliferation in the gut of broiler. The increased number of beneficial bacteria and decreased number of pathogenic bacteria maintain the proper bacterial balance in the intestine seem to improve the intestinal absorptive capacity by improving the ability of epithelial cells to regenerate villi (Pathak et al., 2017).

Table 2: Effects of essential oils/components on digestive system and growth performance parameters in mono-gastric animals.

Essential oil and components	Dosage level	Observations	References
Broilers			
Thyme	1.0-2.0 g/kg	Improvement in FCR and Immunity during HS	Attia et al., 2017
	0.5-1 g/kg	Increase in BWG, FI and improvement in FCR	Pournazariet al., 2017
	300 mg/kg	Increase of digestive enzyme activities, intestinal morphometrics and immunity	Yang et al., 2018
	100 mg/kg	Increased BWG, improved FCR, livability and profit	Wade et al., 2018
	5 g/kg	Improvement of BWG, FI, FCR. Beneficial effects on cholesterol , immunity and antioxidant status	Ismail et al., 2019
	0.1 %	Increased meat quality and antioxidant status in breast muscle	Plachaet al., 2019
	150-200 mg/kg	Improved growth performance and immune responses in HS	RafatKhafaret al., 2019
	300 mg/kg	Reduced adverse effects of HS	Sariözkanet al., 2020
Sumac and Thyme	1, 2 & 3 %	Reduce fat content and improve disease responsiveness, antiviral effects against ND and AI.	Ahmadian et al., 2020
Thyme and Carvacrol	60 and 120 mg/kg	Showed anticoccidial effects	Lee et al., 2020
Thyme oil and Black cumin oil	250 and 100 mg/kg	Positive effects on intestinal health	Aydin and Yildiz, 2020
Thyme and Peppermint	100 mg/kg each	Improved ADG, FCR, carcass yield and decreased cholesterol level	Hassan, 2019
	NS	Increased BW, WG and immune response	Witkowskaet al., 2019
Oregano	150 mg/kg	Increased ADG, ADFI and antioxidant status	Riet al., 2017
	200-600 mg/kg	Improved performance and meat quality as increased breast meat redness and reduced yellowness	Cázares-Gallegos et al., 2019
	NS	Beneficial effect on the growth performance	Hn et al., 2019
	300-900 mg/kg	Improved performance, carcass yield and immunity	Eleret al., 2019
Oregano and Thyme	4% each	Improvement of WG, immune parameters and intestinal morphology	Parvizi et al., 2020
MEO	0.03%	Improvement of total tract retention of DM, increase of LAB and reduction of E.coli	Park and Kim, 2018
	0.01%	Improvement of BW, FCR and LAB	Ruben et al., 2018
	NS	Improved immunity and showed antiviral effects against ND and IBD	El-Shall et al., 2020
Cinnamon	500 mg/kg	Increased villi height and immunity, reduction of *Salmonella* and *Clostridium* counts	Pathak et al., 2017
	NS	improved the immune status, antioxidant ability and cecal microbiota	Yang et al., 2019
Rosemary	300 mg/kg	Improvement in FCR, immunity and concentration of Se in liver and breast muscles of broiler	Mohammadi et al., 2019
	0.5-1.0 g/kg	Beneficial effect on lipid profile, immunity, antioxidant status	El-Gogary, 2020
Rosemary, Thymus & Satureja	300 ppm	Improved immune responses, antioxidants and intestinal microflora	Abbasiet al., 2019
Rosemary & Thyme	5-10 g/kg each	Significant effect on live BW, FI and dressing percentage	Tayeb et al., 2019
Lavender oil	600 mg/kg	Increased growth performance, intestinal morphometrics, villi height, antioxidant status and gut bacteria balance, reduced E. coli	Barbarestaniet al., 2020
Laying hen			
Oregano	50-250 mg/kg	No effect on FI, FCR, egg production and egg shell characteristics	Cufadar, 2018

	50-200 mg/kg	Enhanced immune response	Miglioriniet al., 2019
Thyme	300 mg/kg	Improvement in antioxidant status during HS period	Beyziet al., 2020
	2%	Showed hypolipidemic and antioxidative effects along with improved immunity without effecting performance and egg quality	Yalçinet al., 2020
Rosemary & Cinnamon	300 mg/kg each	Significant better egg production and weight, Haugh unit, FI, FCR, blood cholesterol, immunity, and antioxidant parameters	Abo Ghanimaet al., 2020
Quil			
Thyme	200-400 ppm	Improved FCR	Dehghaniet al., 2018
	150-450 mg/kg	Increased BW, ADG, FI and antioxidant status	Gumuset al., 2017
Eucalyptus	0.1%	Enhanced productive performance, eggshell quality, immunocompetence and reduces number of broken eggs	Fathiet al., 2020
Cinnamon and Ginger oil	0.5-0.1 ml/kg	Improvements in ADG and FCR	Ahmed et al., 2019
MEO	0.33-1.0 ml/L	Improved BW, FCR, villi height and intestinal health	Masood et al., 2020
	600-900 g/ton	Improvements in growth hormone, growth performance and intestinal histomorphology	Maty and Hassan, 2020
Pigs			
Thymol &Carvacrol	30 mg/kg	Improvement of ADG, apparent digestibility of DM, crude protein, gross energy and enzymatic activity in intestine. Increased LAB	Xu et al., 2018
MEO	100 mg/kg	Enhanced growth performance and decrease diarrhea prevalence through increases in antioxidative capacity.	Tian and Piao, 2019
Plant EO	NS	Improvement of BW, growth performance, immunity and antioxidant status	Su et al., 2018
	50-200 ppm	Improvements in regulation of growth and intestinal health	Su et al., 2020
Oregano	NS	Increased antioxidant action and can be used as antimicrobial agent to prevent antimicrobial resistance	Mercatiet al., 2020
	2000 ppm	Increased carcass performance and consumer acceptability.	Janacua-Vidaleset al., 2019
	400 g/ton	Increased Bifidobacterium and Bacillus species to improve immune status	Pu et al., 2020

MEO = Mixture of essential oils, FCR = feed conversion ratio, HS = heat stress, BWG = body weight gain, FI = feed intake, ND = Newcastle disease, AI = avian influenza, ADG = average daily gain, BW = body weight, WG = weight gain, ADFI= average daily feed intake, DM = dry matter, LAB = lactic acid producing bacteria, IBD = infectious bursal diseas, NS = not specified

EFFECTS OF EOS ON RUMEN FERMENTATION

Ruminant animals are producing high-quality protein from low-quality feed resources due to their symbiotic relationship with ruminal microflora. The efficiency of rumen metabolism is also associated with environment-polluting waste products. Inefficiency in rumen fermentation leads to energy and protein losses in the form of methane and ammonia gas production. Methane is the main constituent of greenhouse gas which plays 21 times more potential role in global warming than carbon dioxide (Bodas et al., 2012). Moreover, 2-12% of gross energy intake dissipates into enteric methane mitigation in ruminants depending upon feed intake and type of diet (Benchaar and Greathead, 2011). It can be therefore determined that a decrease in methane emission with the dietary supplementation of EOs is favorable both for the animals and the environment. EOs also possess a significant influence on protein metabolism and reduce ammonia production by inhibiting the deamination of amino acids (AA), possibly through the suppression of HAP at the level of adhesion and colonization (Benchaar and Greathead, 2011; McIntosh et al., 2003).

Several EOs (oregano, cinnamon, eucalyptus, rosemary, clove oil, garlic oil, and peppermint oil) have already been tried *in vitro* and *in vivo* in animals

to reduce methane and ammonia production (Baraz et al., 2018; Cobellis et al., 2015, 2016; Hamdani et al., 2019; Tomkins et al., 2015). EOs do not affect rumen fermentation at low doses, whereas, these compounds inhibit the target microbial species as well as rumen microbes at high doses. EOs might selectively discourage the methanogens and HAP bacteria at low doses, but the high concentration of EOs overwhelm all the microorganisms (Cobellis et al., 2016; Wallace, 2004). Mitigation of methane and ammonia occurs at high doses and it is frequently associated with a decrease in dry matter (DM) degradability, feed digestion, total VFA production and rumen fermentation (Vendramini et al., 2016; Cobellis et al., 2016; Hristov et al., 2013). EOs (oregano, cinnamon, eucalyptus, and rosemary) both individually and in combination reduced methane and ammonia production (Cobellis et al., 2016). Zhou et al., (2020) also revealed that supplementation of oregano EO at 13-130 mg/liter potentially reduced the methane production. Various investigations demonstrated that the composition and inclusion level of EOs could affect the ruminal N metabolism. Cinnamon bark inhibited the ammonia production by 43.9% and 59.3% reduced by the combination of cinnamon, oregano, and rosemary leaves (Cobellis et al., 2015). Patra and Yu, (2012) reported that EOs of oregano and clove decreased the ammonia production more potentially *in vitro* when compared with garlic, eucalyptus and, peppermint EOs. Multifaceted relations happen among EOs, feed, and host, thus correlation of the results from feed degradability, rumen fermentation features, and microbiome dynamics could provide more information for the development of effective mitigation technologies.

Total VFAs production is little affected (Patra and Saxena, 2010) or decrease due to high concentrations of EOs in the diet (Baraz et al., 2018). Dietary supplementation of clove and thyme EOs at 2ml per day in sheep increased the total VFA concentrations (Abeer et al., 2019). Some EOs and their major constituents shift molar proportions of VFA i.e. decrease in acetic acid and an increase in the propionic acid proportion which is nutritionally favorable (Ribeiro et al., 2019; Silva et al., 2020).

Variations in results among *in vitro*, *in situ*, or *in vivo* studies can be attributed to numerous variables such as diet (forage: concentration ratio), pH (more potent action at low pH) time (adaptation period), and EOs composition. The lack of effects of EOs on rumen metabolism in long-term studies as compared to short-term studies could be due to adaptation of ruminal microbes to EOs and the obvious difficulty in predicting the dose rate of dietary supplementation of EOs. Long term exposure of EOs may lead to adjustments in rumen micro-organisms, and it is convincible that some EO compounds are subjected to degradation by rumen microbial populations (Abdallah Sallam et al., 2011). Cardozo et al., (2004) examined the effects of cinnamon, garlic, and anise oils at different doses such as 7.5 mg/kg and 0.22 mg/liter of DM on continuous culture. They noticed the progression in the VFA profile during the initial six days, however no effects from that point because of microbial adaptation to EOs. EOs containing phenolic compounds as an active compound exert more pronounced antimicrobial effects than others (Patra and Yu, 2012). Although EOs in high doses could exert positive effects in vitro on rumen fermentation, these doses result in negative implications on feed palatability, digestion, and animal productivity, when applied *in vivo* (Yang et al., 2010; Beauchemin and McGinn, 2006). At the same time, the levels of EOs that have elicited favorable fermentation responses in vitro are far too high for *in vivo* applications due to their possible toxic effects and high cost.

Besides, very few data available on the effects of EOs on DM intake, milk production, composition, and body growth of ruminants. Oregano increased the rumen fermentation, FI, DM digestibility, and feed efficiency along with reduction in methane production and ammonia nitrogen (Tapki et al., 2020; Zhou et al., 2020). Moreover, the addition of a mixture of essential oils (MEO) in the diet increased the average daily gain, live weight, FCR, and nutrient digestibilities (Giller et al., 2020). They also increased gut health, immunity, and prevented the animals from diarrhea and other diseases (Campolina et al., 2020; Liu et al., 2020). In addition, EOs increased milk production, milk fat, and carcass characteristics (Hart et al., 2019; Silva et al., 2020; Wang et al., 2020). Supplementation of EOs could increase conjugated linoleic acid, a health-promoting fatty acid in milk fat by suppressing the bacteria involved in biohydrogenation (Bayat et al., 2015). Rivaroli et al., (2016) recommends MEO (oregano, garlic, lemon, rosemary, thyme, eucalyptus, and sweet orange) at 3.5 g/day in feedlot animals to decrease the lipid oxidation. Table 3 shows effects on growth performance parameters along with effects on methane production, total VFA concentrations, and rates (i.e., acetate to propionate ratio), animal health, performance, and quality characteristics of animal products.

Table 3: Effects of essential oils or their components on rumen characteristics and performance of ruminants.

Essential oil or components	Dosage level	Observations	References
Cattle			
Oregano	100-150 mg/L	Improved FE, growth performance, health status. Reduced diarrhea incidents and lower farm costs	Tapkiet al., 2020
	13-130 mg/L	Increased DM, NDF and ADF digestibility. Decreased AN, MP and alter VFA concentration	Zhou et al., 2020
	4 g/day	Alter ruminal microbiota	Zhou et al., 2019
	50 mg/kg	No effect on RF, ND, MP, MY and MF	Benchaar, 2020
	10 g/day	Improved FE	Bosco Stivaninet al., 2019
BEO	1 g/day	Immunity improvement and a decrease morbidity of neonatal diarrhea in pre-weaning phase	Campolinaet al., 2020
	3.5 g/day	No effect on carcass quality. EOs can be added in low amount without affecting meat quality	Rivaroliet al., 2020
	150 mg/kg	Increased NDF digestibility and N utilization	Teobaldoet al., 2020
	150 mg/kg	Increased NDF and OM digestibility, MY and MF. Reduced A:P ratio	Silva et al., 2020
	4 g/day	Improved ADG, DM intake, FE	Souza et al., 2019
	1000 mg/day	No effect on rumen microbiota	Schärenet al., 2017
	1 g/day	Increased MY and reduced MP	Hart et al., 2019
	1 g/day	Increased MY and FE	Elcoso et al., 2019
MEO	25 g/day	Increased FI. No effects on milk composition and antioxidant capacity	Gilleret al., 2020
	1 g/day	Improved carcass quality	Wang et al., 2020
	NS	Improvements of ADG, FCR, ND, calf growth, ruminal development, gut health, and immunity	Liu et al., 2020
	50-100 ul/L	Improved DM digestibility and microbial protein yield. Reduced MP	Davoodiet al., 2019
	1 g/day	Improved meat quality attributes	Pukrop et al., 2019
	25 mg/kg	Improved MY, udder health and immunity	Salem et al., 2019
	100 µl/L	Reduced MP, increased microbial protein synthesis and RF	Kurniawatiet al., 2020
	3.5 g/animal/ day	Decrease in the lipid oxidation.	Rivaroli et al., 2016
Thyme	100 ul/L	Decreased AN, VFA concentration and MP	Baraz et al., 2018
	100 g/day	Increased MY and reduced MP	Hamdaniet al., 2019
Clove & Rosemary	2 g and 4 g / animal/day	No effect on carcass quality. Affect oxidation	de Oliveira Monteschioet al., 2017
Coriander oil	14 mL/cow/ day	Increase of FI, ND and MY. Decreasein ruminal AN concentration	Matloupet al., 2017
Cashew and caster	2 g/day	No effect on FI and N digestibility. Alter ruminal pH	Coneglianet al., 2019
Buffaloes			
Ajwain oil	1-2 ml/day	Increased DM intake, ADG and protein metabolism	Pawaret al., 2019
	0.05%	Reduced MP, A:P ratio and improved ND	Wadhwa and Bakshi, 2019
Eucalyptus	20-120 ul/40ml	Reduced MP	Singh et al., 2019
	NS	No effect on FI, ND, Ruminal pH, temperature and BUN. Increase of total VFA concentration. Decrease of ruminal AN, protozoal, proteolytic bacteria, MP and A:P ratio	Thao et al., 2015
	2 mL/day	No effect on FI, ND, N utilization, total VFA concentration. Decrease of MP and A:P ratio. Reduction of protozoal population	Thao et al., 2014
Sheep			
Thyme	1.25g/kg	Increased RF and N metabolism. Decreased A:P ratio	Ribeiro et al., 2019

Clove	2 ml/day	Improved ND and carcass characteristics	El-Essawyet al., 2019
Clove and Thyme	2 ml/day each	Increased MY, MF, VFA and antioxidant capacity. Reduced cholesterol	Abeer et al., 2019
Orange peel	300-450 mg/kg	Increase of FI, antioxidant status and MF	Kotsampasiet al., 2018
Rosemary	0.3-0.6 ml/day	No effect on DMI, and growth. Increase of PUFA and sensorial attributes in meat	Smetiet al., 2018
Garlic oil	62.5 mg/L	No effect on ADG, performance, FCR, ND, calcium and phosphorus blood concentration. Improvement of TDN and digestible CP conversion ratio	El-Katcha, Soltan, and Essi, 2016
MEO	1.6 mL/day	No effect on ruminal pH, VFA concentration, MP, A:P ratio and blood profile. Decrease of ruminal AN	Khateri et al., 2017
Chavil EO	250-750 mg/kg	Decreased saturated fatty acids and increased antioxidant capacity of meat	Parvaret al., 2018
Functional EO	2-6 g/day	Decreases FI without negatively affecting nutrient fermentation and usage	Michailoffet al., 2020
Red pepper EO	0.14-0.42%	Improved carcass characteristics	Bertoloniet al., 2020
Goat			
Callistemon viminalis oil	100-200 mg/kg	Improvement of DM intake, ND, N utilization and biochemical parameters	Mekuikoet al., 2018
Rosemary	600 mg/kg	No effect on DM, OM, CP and NDF digestibility. Increase of MY, MF and protein contents	Smetiet al., 2015
	100-400 mg/kg	No effect on ADG, hematological parameters. Increased immunity	Shokrollahiet al., 2015
Oregano & linseed	3% and 0.6 %	Improvements of carcass quality and antioxidation. No effects on performance parameters	Rotondiet al., 2018
Juniper	0.4-2 ml/kg	No effect on FI, LWG, ruminal pH, VFA concentration, fecal pH. Increase of FE and antioxidant status	Yesilbaget al., 2017
MEO	2 mg/kg	Increased ADG and improved phenotypes (cashmere fiber traits, carcass weight, and meat quality)	Lei et al., 2019
Fennel EO	100-1000 ug	Decreased MP, AN	Cheshmehgachiet al., 2019

MEO = mixture of essential oils, BEO = blend of essential oils, FE= feed efficiency, DM = dry matter, NDF = neutral detergent fiber, ADF = acid detergent fiber, AN = ammonia nitrogen, MP = methane production, VFA = volatile fatty acids, RF = rumen fermentation, ND = nutrient digestibility, MY = milk yield, MF = milk fat, N = nitrogen, A:P = acetate to propionate, ADG = average daily gain, FI = feed intake, FCR = feed conversion ratio, BUN = blood urea nitrogen, PUFA = poly unsaturated fatty acids, TDN = total digestible nutrients, CP = crude protein, OM = organic matter, NS = not specified

ANTIOXIDANT EFFECTS OF EOS

The most important purpose of EOs is to minimize the pathogenic microbes and decrease the phenomenon of lipid oxidation. Oxidation of lipids and free radical production are natural processes that influence the membrane integrity, interrupt the cell transport channels and function of cell organelles. Lipid content of membrane particularly phospholipids is more inclined to oxidative damage, which is related to the level of unsaturation of fatty acids (UFAs). Polyunsaturated fatty acids (PUFA) are responsible for keeping up cell membrane respectability including fluidity and permeability. Hydroperoxides (ROOH) formation occurs because of reaction between peroxy radicals and polyunsaturated FAs resulting in the formation of non-radical aromatic compounds that adversely affect the carbohydrates, protein, lipids, and vitamin contents and limit the nutritional value and shelf life of animal products. The EOs as an antioxidant have various modes of action to reduce lipid oxidation. One of the possible mechanisms of action is that they block the chain initiation, start the hydrogen abstraction, act as free radical scavenger and terminators, bind the transition metal ions, and stop the formation of singlet oxygen (Tongnuanchan and Benjakul, 2014). Several EOs possess phenolic compounds up to 85% of their composition. In phenolic compounds, carvacrol, eugenol, and thymol are the active components that act

as primary oxidants and effective free radical scavengers (Bakkali et al., 2008). Antioxidants work in three stages: initiation, propagation, and termination. The presence of hydroxyl group (-OH) in antioxidant compounds usually acts as a hydrogen donor, inactivates the free radicals generated from the lipid oxidation. They scavenge the free radicals by donating electrons to them, this feature makes them potentiated anti-oxidant that prevents other compounds from oxidizing (Coma, 2012). It results in the development of new radicals, which are unable to extract the hydrogen (H) atoms from unsaturated FAs (Coma, 2012). Hence, these subsequent radicals can react with similar radicals or free radicals leading to the formation of non-radical species (Jayasena and Jo, 2014). In this way, phenolic compounds can counteract lipid oxidation, act as oxidative chain inhibitors, and protect the animal products from oxidative damage.

Animal diet can play an important role to inhibit the free radical production in organisms and their derived products. The addition of EOs in the diet of animals is a simple and efficient approach to incorporate natural anti-oxidant compounds into lipidic layers of membrane (Table 4). In this way, they can inhibit lipid oxidation more effectively and prevent oxidative losses of animal products compared to postmortem addition (Decker and Park, 2010; Govaris et al., 2004).

Table 4: Summary of studies testing antioxidant activity of essential oils or their components in food processing

Essential oil	Dosage level/ concentration applied	Product	Effect	SP	SL	References
Oregano	0.2%	Rabbit meat	+		+	Cardinaliet al., 2015
	0.125-3.0 ml/kg	Rainbow trout	NE			Dileret al., 2017
	2000 ppm	Pig meat	+	+		Janacua-Vidaleset al., 2019
	NS	Pig meat	+	+		Mercatiet al., 2020
	200-600 mg/kg	Broiler meat	+	+		Cázares-Gallegos et al., 2019
Oregano & linseed	3% and 0.6 %	Goat meat	+	+		Rotondiet al., 2018
Rosemary	200-400 mg/kg	Lamb meat	+		+	Ortuñoet al., 2014
	300 mg/kg	Broiler meat	+	+		Mohammadi et al., 2019
Rosemary and Thyme	5-10 g/kg each	Broiler meat	NE			Tayeb et al., 2019
Rosemary and Cinnamon	300 mg/kg each	Layer meat and egg	+	+		Abo Ghanimaet al., 2020
Thyme and Peppermint	100 mg/kg	Broiler meat	+	+		Hassan, 2019
Thyme	0.125%	Fresh chicken sausage	+		+	Sharma et al., 2017
	0.1%	Broiler meat	+	+		Plachaet al., 2019
	300 mg/kg	Layer meat and egg	+			Beyziet al., 2020
	2%	Layer meat and egg	+	+		Yalçinet al., 2020
	150-450 mg/kg	Quil meat	+	+		Gumuset al., 2017
	600 mg/kg	Broiler meat	+	+		Onel and Aksu, 2019
Thyme & Clove	4 MIC and 2 MIC respectively	Minced beef	+		+	(Zengin and Baysal, 2015
Clove	0.25%	Fresh chicken sausage	+		+	Sharma et al., 2017
	2ml/d	Sheep meat	+	+		El-Essawyet al., 2019
Basil EO	0.062, 0.125 and 0.25%	Beef burger	+	+	+	Sharafati Chaleshtori et al., 2015
	2 and 4%	Cattle meat	+	+	+	Falowoet al., 2019
	0,25, 0.50, 0.75%	Mutton nuggets	+	+	+	Kumar et al., 2018
Sage oil	0.05, 0.075, 0.1 µL/g	Pork fresh sausages	+	+		Šojićet al., 2018
Chavil EO	250-750 mg/kg	Sheep meat	+	+		Parvaret al., 2018
MEO	750-2000 mg/kg	Broiler meat	+	+		(Mountzouris et al., 2020)
	250-750 ml/1000 L	Broiler meat	+	+		Tekceet al., 2020
	25 g/day	Cattle meat	+	+		Giller et al., 2020
	1 g/d	Cattle meat	+	+		Pukrop et al., 2019
	2 mg/kg	Goat meat	+	+		Lei et al., 2019

MEO = blend of oils, SP = sensory properties, SL = shelf life, NE = no effect

CONCLUSION

The growing pressure on the livestock industry is to limit the application of chemical feed additives particularly antimicrobial agents as growth promoters have started a new investigation to discover the safe and effective substitutes. A variety of different biologically active agents including EOs proved themselves as multifunctional feed supplements for animals. The EOs and their constituents possess the remarkable potential to influence the gut-microbiota, rumen fermentation and avoid lipid oxidation results in the improvements in growth performance parameters and quality characteristics of animal products. Whereas, their potential and efficacy in livestock production have not yet been determined to be steady and indisputable and some concerns should be investigated before their business application. For instance, an ideal concentration of EOs according to their chemical composition and type, ought to be established, since their application at high doses can impose undesirable effects on living organisms. Dietary supplementation of EOs is safe to use but their mode of action, pharmacokinetics, and pharmacodynamics are still unclear. Simultaneously, the good effects of dietary supplementation of EOs ought to be legitimized the extra expense of their application. A further demonstration of the above inquiries is needed for the regular application of EOs in animal production. In this way, it may be possible to formulate animal feed that optimizes animal efficiency. EOs besides being a promising approach as drug candidates in modern medicine, their dietary supplementation in food (soft drinks and food confectionary) and feed industry (growth promoters, antimicrobials, and antioxidants) can also be action-oriented approach in modern nutrition.

CONFLICT OF INTEREST

None declared by the authors.

REFERENCES

Abbasi MA, Ghazanfari S, Sharifi SD & Gavlighi HA (2019) The effect of rosemary, thymus and satureja essential oils, vitamin E and vegetable oils on immune system and intestinal microflora of broiler chicken. J Vet Res 74:153-166.

Abdallah Sallam SM, Mohamed Abdelgaleil SA, da Silva Bueno IC, Abdelwahab Nasser ME, Araujo RC & Abdalla AL (2011). Effect of some essential oils on in vitro methane emission. Arch Ani Nut 65:203-14.

Abdel Hameed RG, Mostafa MH & El-Malt MA (2020). Evaluation of the Antimicrobial Effect of Thyme Extract on Streptococcus Mutans. Al-Azhar Dental J Girls 7:313-318.

Abdollahzadeh E, Ojagh SM, Hosseini H, Ghaemi H & Irajian G (2018) Quantitative and Qualitative Evaluation of Antibacterial Activity of Cinnamon Essential Oil and ZnO Nanoparticles against Listeria monocytogenes. Fish Sci Technol 7:49-55.

Abdullah BH, Hatem SF, Jumaa W (2015). A comparative study of the antibacterial activity of clove and rosemary essential oils on multidrug resistant bacteria. UK J Pharm Biosci 3:18-22.

Abeer M, Ahlam R & Marwa H (2019) Impact of Anise, Clove, and Thyme essential oils as feed supplements on the productive performance and digestion of Barki ewes. Australian J Basic Appl Sci 13:1-13.

Abo Ghanima MM, Elsadek MF, Taha AE, El-Hack A, Mohamed E, Alagawany M, Ahmed BM, Elshafie MM & El-Sabrout K (2020) Effect of Housing System and Rosemary and Cinnamon Essential Oils on Layers Performance, Egg Quality, Haematological Traits, Blood Chemistry, Immunity, and Antioxidant. Animals 10:245

Adil S, Magray SN (2012). Impact and manipulation of gut microflora in poultry: a review. J Anim Vet Adv 11:873-877.

Ahmadian A, Seidavi A & Phillips CJC (2020) Growth, Carcass Composition, Haematology and Immunity of Broilers Supplemented with Sumac Berries (Rhus coriaria L.) and Thyme (Thymus vulgaris). Animals 10:513

Ahmed EM, Attia AI, Ibrahem ZA & Abd El-Hack ME (2019) Effect Of Dietary Ginger And Cinnamon Oils Supplementation On Growing Japanese Quail Performance. Zagazig J Agri Res 46:2037-2046

Ahmed ST, Hossain ME, Kim GM, Hwang JA, Ji H, Yang CJ (2013). Effects of Resveratrol and Essential Oils on Growth Performance, Immunity, Digestibility and Fecal Microbial Shedding in Challenged Piglets. Asian-Australas J Anim Sci 26:683-690.

Akbari Z, Dastan D, Maghsood AH, Fallah M & Matini M (2018) Investigation of In vitro Efficacy of Marrubium vulgare L. Essential Oil and Extracts Against Trichomonas vaginalis. Zahedan J Res Med Sci 20:e67003.

Akram MZ, Fırıncıoğlu SY, Jalal H & Doğan SC (2019b) The Use of Essential Oils in Active Food Packaging: A Review of Recent Studies. TURJAF 7:1799-804.

Akram MZ, Salman M, Jalal H, Asghar U, Ali Z, Javed MH & Khan M (2019a) Evaluation of dietary supplementation of Aloe vera as an alternative to antibiotic growth promoters in broiler production. Turkish J Vet Res 3:21-26.

Al-Mnaser AA & Woodward MJ (2020) Sub-lethal Concentrations of Phytochemicals (Carvacrol and Oregano) Select for Reduced Susceptibility Mutants of Escherichia coli O23:H52. Polish J Microbiol 69:121-125.

Al-Nabulsi AA, Osaili TM, Olaimat AN, Almasri WE, Ayyash M, Al-Holy MA, Jaradat ZW, Obaid RS & Holley RA (2020). Inactivation of Salmonella spp. in tahini using plant essential oil extracts. Food Microbiol 86:103338.

Amat S, Baines D & Alexander TW (2017). 249 Antimicrobial activities of commercial essential oils against the bovine respiratory pathogen Mannheimia haemolytica and analysis of their chemical composition and cytotoxicity on bovine turbinate cells. J Anim Sci 95:122-123.

Attia YA, Bakhashwain AA & Bertu NK (2017) Thyme oil (Thyme vulgaris L.) as a natural growth promoter for broiler chickens reared under hot climate. Italian J Ani Sci 16:275-282.

Aydin ÖD & Yildiz G (2020) The effects of thyme oil and black cumin oil in broiler feeding on growth performance, intestinal histomorphology, and cecal volatile fatty acids. Turk J Vet Anim Sci 44:17-25.

Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008). Biological effects of essential oils - A review. Food Chem Toxicol 46:446-475.

Baraz H, Jahani-Azizabadi H & Azizi O (2018) Simultaneous use of thyme essential oil and disodium fumarate can improve in vitro ruminal microbial fermentation characteristics. Vet Res Forum 9:193-198.

Barbarestani SY, Jazi V, Mohebodini H, Ashayerizadeh A, Shabani A & Toghyani M (2020) Effects of dietary lavender essential oil on growth performance, intestinal function, and antioxidant status of broiler chickens. Livest Sci 233:103958.

Bayat AR, Kairenius P, Stefański T, Leskinen H, Comtet-Marre S, Forano

E, Chaucheyras-Durand F, Shingfield KJ (2015). Effect of camelina oil or live yeasts (Saccharomyces cerevisiae) on ruminal methane production, rumen fermentation, and milk fatty acid composition in lactating cows fed grass silage diets. J Dairy Sci 98:3166-3181.

Beauchemin KA, McGinn SM (2006). Methane emissions from beef cattle: Effects of fumaric acid, essential oil, and canola oil. J Anim Sci 84:1489-1496.

Benchaar C (2020) Feeding oregano oil and its main component carvacrol does not affect ruminal fermentation, nutrient utilization, methane emissions, milk production, or milk fatty acid composition of dairy cows. J Dairy Sci 103:1516-1527

Benchaar C, Greathead H (2011). Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Anim. Feed Sci. Technol., Special Issue: Greenhouse Gases in Animal Agriculture - Finding a Balance between Food and Emissions 166:338-355.

Bertoloni AV, Polizel DM, Júnior MV, Oliveira GB, Miszura AA, Barroso JP, Martins AS, Sardinha LA, Limede AC, Ferreira EM & Pires AV (2020) Brazilian red pepper leaves essential oil (Schinus terebinthifolius) in diets for feedlot lambs. Brazilian Journal of Vet Res Ani Sci 57:e157580-e157580

Bodas R, Prieto N, García-González R, Andrés S, Giráldez FJ, López S (2012). Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim. Feed Sci. Technol., Special Issue: Plant Bioactive Compounds in Ruminant Agriculture - Impacts and Opportunities 176:78-93.

Bosco Stivanin SC, Vizzotto EF, de Paris M, Zanela MB, Passos LT, Angelo ID & Fischer V (2019) Addition of oregano or green tea extracts into the diet for Jersey cows in transition period. Feeding and social behavior, intake and health status. Plant extracts for cows in the transition period. Ani Feed Sci Technol 257:114265.

Burt S (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. Int J Food Microbiol 94:223-253.

Büyükkılıç Beyzi S, Konca Y, Kaliber M, Sarıözkan S, Kocaoğlu Güçlü B, Aktuğ E & Şentürk M (2020) Effects of thyme essential oil and A, C, and E vitamin combinations to diets on performance, egg quality, MDA, and 8-OHdG of laying hens under heat stress. J Appl Ani Res 48:126-132

Campolina JP, Coelhor SG, Belli AL, Machado FS, Pereira LG, Tomich TR, Carvalho WA, Silva RO, Voorsluys AL, Jacob DV & Campos MM (2020) Effects of a blend of essential oils in milk replacer on performance, rumen fermentation, blood parameters and health scores of dairy heifers. bioRxiv.

Cardinali R, Cullere M, Dal Bosco A, Mugnai C, Ruggeri S, Mattioli S, Castellini C, Trabalza Marinucci M & Dalle Zotte A (2015). Oregano, rosemary and vitamin E dietary supplementation in growing rabbits: Effect on growth performance, carcass traits, bone development and meat chemical composition. Livest Sci 175:83-89.

Cardozo PW, Calsamiglia S, Ferret A, Kamel C (2004). Effects of natural plant extracts on ruminal protein degradation and fermentation profiles in continuous culture. J Anim Sci 82:3230-3236.

Cázares-Gallegos R, Silva-Vázquez R, Hernández-Martínez CA, Gutiérrez-Soto JG, Kawas-Garza JR, Hume ME & Méndez-Zamora GM (2019) Performance, Carcass Variables, and Meat Quality of Broilers Supplemented with Dietary Mexican Oregano Oil. Brazilian J Poul Sci: 21.

Cerioli MF, Moliva MV, Cariddi LN & Reinoso EB (2018) Effect of the Essential Oil of Minthostachys verticillata (Griseb.) Epling and Limonene on Biofilm Production in Pathogens Causing Bovine Mastitis. Front Vet Sci 5:146.

Cheshmehgachi SM, Moeini MM, Hozhabri F & Soror MEN (2019) Effect of different levels of fennel essential oil on in vitro gas production parameters and protozoa population of goat rumen. Ani Prod Res 8.

Cho JH, Kim HJ & Kim IH (2014) Effects of phytogenic feed additive on growth performance, digestibility, blood metabolites, intestinal microbiota, meat color and relative organ weight after oral challenge with Clostridium perfringens in broilers. Livest Sci 160:82-88.

Cho Y, Kim H, Beuchat LR & Ryu JH (2020) Synergistic activities of gaseous oregano and thyme thymol essential oils against Listeria monocytogenes on surfaces of a laboratory medium and radish sprouts. Food Microbiol 86:103357.

Christaki E, Giannenas I, Bonos E & Florou-Paneri P (2020) Innovative uses of aromatic plants as natural supplements in nutrition. In: Feed Additives, Academic Press: pp. 19-34.

Cobellis G, Petrozzi A, Forte C, Acuti G, Orrù M, Marcotullio MC, Aquino A, Nicolini A, Mazza V & Trabalza-Marinucci M (2015) Evaluation of the Effects of Mitigation on Methane and Ammonia Production by Using Origanum vulgare L. and Rosmarinus officinalis L. Essential Oils on in Vitro Rumen Fermentation Systems. Sustainability 7:12856-12869.

Cobellis G, Trabalza-Marinucci M, Marcotullio MC & Yu Z (2016) Evaluation of different essential oils in modulating methane and ammonia production, rumen fermentation, and rumen bacteria in vitro. Anim Feed Sci Technol 215:25-36.

Coma V (2012) Antimicrobial and antioxidant active packaging for meat and poultry. In: Advances in Meat, Poultry and Seafood Packaging, Woodhead Publishing Series in Food Science, Technology and Nutrition. Woodhead Publishing: pp. 477-503.

Coneglian SM, Serrano RDC, Cruz OTB & Branco AF (2019) Effects of essential oils of Cashew and Castor on intake, digestibility, ruminal fermentation and purine derivatives in beef cattle fed high grain diets. Semina: Ciências Agrárias 40:2057-2070.

Cufadar Y (2018) Effects of Dietary Oregano Essential Oil Supplementation on Performance and Eggshell Quality in Laying Hens. Selçuk Tarım ve Gıda Bilimleri Dergisi 32:158-161.

Davoodi SM, Mesgaran MD, Vakili AR, Valizadeh R & Pirbalouti AG (2019) In vitro Effect of Essential Oils on Rumen Fermentation and Microbial Nitrogen Yield of High Concentrate Dairy Cow Diet. Biosci Biotech Res Asia 16:333-341.

de Oliveira Monteschio J, de Souza KA, Vital AC, Guerrero A, Valero MV, Kempinski EM, Barcelos VC, Nascimento KF & do Prado IN (2017) Clove and rosemary essential oils and encapsuled active principles (eugenol, thymol and vanillin blend) on meat quality of feedlot-finished heifers. Meat Sci 130:50-57.

Dehghani N, Afsharmanesh M, Salarmoini M, Ebrahimnejad H & Bitaraf A (2018) Effect of pennyroyal, savory and thyme essential oils on Japanese quail physiology. Heliyon 4(10): e00881

Diler O, Gormez O, Diler I & Metin S (2017) Effect of oregano (Origanum onites L.) essential oil on growth, lysozyme and antioxidant activity and resistance against Lactococcus garvieae in rainbow trout, Oncorhynchus mykiss (Walbaum). Aqua Nut 23:844-851.

Diniz-Silva HT, Brandao LR, de Sousa Galvão M, Madruga MS, Maciel JF, de Souza EL & Magnani (2020) M Survival of Lactobacillus acidophilus LA-5 and Escherichia coli O157:H7 in Minas Frescal cheese made with oregano and rosemary essential oils. Food Microbiol 86:103348.

Dudareva N, Pichersky E & Gershenzon J (2004). Biochemistry of Plant Volatiles. Plant Physiol 135:1893-1902.

Ebani VV, Nardoni S, Bertelloni F, Pistelli L & Mancianti F (2018) Antimicrobial Activity of Five Essential Oils against Bacteria and Fungi Responsible for Urinary Tract Infections. Molecules 23:1668.

Elcocks ER, Spencer-Phillips PTN & Adukwu EC (2020). Rapid bactericidal effect of cinnamon bark essential oil against Pseudomonas aeruginosa. J App Microbiol 128:1025-1037.

Elcoso G, Zweifel B & Bach A (2019) Effects of a blend of essential oils on milk yield and feed efficiency of lactating dairy cows. Appl Ani Sci 35:304-311.

Eler G, Gomes AV, Trindade BS, Almeida LS, Dilelis F, Cardoso VS & Lima CA (2019) Oregano essential oil in the diet of broilers: performance, carcass characteristics, and blood parameters. South African J Ani Sci 49:753-762.

El-Essawy AM, Abdou AR, Khattab IM & Abdel-Wahed AM (2019) Effect Of Addition Of Anise, Clove And Thyme Essential Oils On Barki Lambs Performance, Digestibility, Rumen Fermentation, Carcass Characteristics And Intramuscular Fatty Acids. Egyptian J Nut Feeds 22:465-477.

El-Gogary MR (2020) Ecofriendly Synthesis Of Calcium Nanoparticles With Biocompatible Rosmarinus Officinalis Extract On Physiological And Immunological Effects In Broiler Chickens. Egyptian Poult Sci J 40:81-102.

El-Katcha MI, Soltan MA & Essi MS (2016) Effect of Garlic Extract Supplementation on Growth Performance, Nutrient Digestibility and Some Blood Serum Biochemical Changes of Fattening Lambs. AJVS

48:24-133.
El-Shall NA, Nahed A, Shewita RS, Abd El-Hack ME, AlKahtane A, Alarifi S, Alkahtani S, Abdel-Daim MM & Sedeik ME (2020) Effect of essential oils on the immune response to some viral vaccines in broiler chickens, with special reference to Newcastle disease virus. Poult Sci.
Ezzeldeen NA, Amin AA, Khyralla HA & Abdelmonem MA (2015) Effect of some essential oils on verotoxin-producing Escherichia coli isolated from minced meat. Adv Env Biol 9:33-42.
Falowo AB, Mukumbo FE, Idamokoro EM, Afolayan AJ & Muchenje V (2019) Phytochemical Constituents and Antioxidant Activity of Sweet Basil (Ocimum basilicum L.) Essential Oil on Ground Beef from Boran and Nguni Cattle. Int J Food Sci. doi:https://doi.org/10.1155/2019/2628747
Fathi MM, Al-Homidan I, Ebeid TA, Abou-Emera OK & Mostafa MM (2020) Dietary supplementation of Eucalyptus leaves enhances eggshell quality and immune response in two varieties of Japanese quails under tropical condition. Poult Sci 99:879-885.
Florou-Paneri P, Christaki E, Giannenas I (2019). Feed Additives: Aromatic Plants and Herbs in Animal Nutrition and Health. In: Feed Additives, Academic Press.
Fratini F, Casella S, Leonardi M, Pisseri F, Ebani VV, Pistelli L & Pistelli L (2014) Antibacterial activity of essential oils, their blends and mixtures of their main constituents against some strains supporting livestock mastitis. Fitoterapia 96:1-7.
Giannenas I, Bonos E, Christaki E & Florou-Paneri P (2018) Oregano: A Feed Additive with Functional Properties. Therapeutic Foods, Academic Press: pp. 179-208.
Giannenas I, Sidiropoulou E, Bonos E, Christaki E & Florou-Paneri P (2020) The history of herbs, medicinal and aromatic plants, and their extracts: Past, current situation and future perspectives. In: Feed Additives. Academic Press: pp. 1-18.
Giller K, Rilko T, Manzocchi E, Hug S, Bolt R & Kreuzer M (2020) Effects of mixed essential oils from eucalyptus, thyme and anise on composition, coagulation properties and antioxidant capacity of the milk of dairy cows. J Anim Feed Sci 29:3-10.
Govindarajan M, Rajeswary M, Hoti SL, Bhattacharyya A & Benelli G (2016). Eugenol, α-pinene and β-caryophyllene from Plectranthus barbatus essential oil as eco-friendly larvicides against malaria, dengue and Japanese encephalitis mosquito vectors. Parasitol Res 115:807-815.
Gumus R, Ercan N, Imik H, Gumus R, Ercan N & Imik H (2017) The Effect of Thyme Essential Oil (Thymus Vulgaris) Added to Quail Diets on Performance, Some Blood Parameters, and the Antioxidative Metabolism of the Serum and Liver Tissues. Brazilian J Poult Sci 19:297-304.
Guo M, Zhang L, He Q, Arabi SA, Zhao H, Chen W, Ye X & Liu D (2020) Synergistic Antibacterial Effects of Ultrasound and Thyme Essential Oils Nanoemulsion against Escherichia coli O157: H7. Ultrason Sonochem 25:104988.
Hamdani H, Chami N, Oukhouia M, Jabeur I, Sennouni C & Remmal A (2019) Effect of a thymol-based additive on rumen fermentation, on methane emissions in eructed gas and on milk production in Holstein cows. Livest Res Rural Develop 31.
Hart KJ, Jones HG, Waddams KE, Worgan HJ, Zweifel B & Newbold CJ (2019) An Essential Oil Blend Decreases Methane Emissions and Increases Milk Yield in Dairy Cows. Open J Ani Sci 09:259.
Hassan HA (2019) Effects of Thyme Oil, Peppermint Oil and their Combination on Productive Performance, Carcass Criteria and Blood Profile of Broiler Chickens. J Ani Poult Prod 10:105-108.
Huang Y, Lin M, Jia M, Hu J & Zhu L (2020) Chemical composition and larvicidal activity against Aedes mosquitoes of essential oils from Arisaema fargesii. Pest Manage Sci 76:534-542.
Ismail FSA, El-Gogary MR & El-Morsy MN (2019) Impact Of Dietary Supplementation Of Different Levels Of Thyme And Its Essential Oils On Performance, Blood Parameters, Metabolic And Immune Response Of Broiler Chickens. Egyptian Poult Sci J 39:365-379.
Jalal H, Akram MZ, Doğan SC, Fırıncıoğlu SY, Irshad N & Khan M (2019) Role of Aloe Vera as A Natural Feed Additive in Broiler Production. TURJAF 10(sp1):163-166.
Janacua-Vidales H, Peña-González E, Alarcon-Rojo AD, Ortega-Gutiérrez J & Aguilar-Palma N (2019) Determination of carcase yield, sensory and acceptance of meat from male and female pigs with dietary supplementation of oregano essential oils. Italian J Ani Sci 18:668-678.
Jayasena DD & Jo C (2014). Potential Application of Essential Oils as Natural Antioxidants in Meat and Meat Products: A Review Food Rev Int 30:71-90.
Kareem MH, Khalaf JM, Hasan MS, Saleh EN & D Salih NO (2020) Effects of Eucalyptus Alcoholic Extracts on Pathogenic E. coli, Invitro Study. Inter J Pharma Res 12:1033-1034.
Khalili ST, Mohsenifar A, Beyki M, Zhaveh S, Rahmani-Cherati T, Abdollahi A, Bayat M & Tabatabaei M (2015) Encapsulation of Thyme essential oils in chitosan-benzoic acid nanogel with enhanced antimicrobial activity against Aspergillus flavus. LWT-Food Sci Technol 60:502-508.
Khateri N, Azizi O & Jahani-Azizabadi H (2017) Effects of a specific blend of essential oils on apparent nutrient digestion, rumen fermentation and rumen microbial populations in sheep fed a 50:50 alfalfa hay:concentrate diet. Asian-Australas J Anim Sci 30:370-378.
Kiarsi Z, Hojjati M, Behbahani BA & Noshad M (2020) In vitro antimicrobial effects of Myristica fragrans essential oil on foodborne pathogens and its influence on beef quality during refrigerated storage. J Food Safety: e12782.
Knezevic P, Aleksic V, Simin N, Svircev E, Petrovic A & Mimica-Dukic N (2016) Antimicrobial activity of Eucalyptus camaldulensis essential oils and their interactions with conventional antimicrobial agents against multi-drug resistant Acinetobacter baumannii. J Ethnopharm 178:125-136.
Kosari F, Taheri M, Moradi A, Hakimi Alni R & Alikhani MY (2020) Evaluation of cinnamon extract effects on clbB gene expression and biofilm formation in Escherichia coli strains isolated from colon cancer patients. BMC Cancer 20:267.
Kotsampasi B, Tsiplakou E, Christodoulou C, Mavrommatis A, Mitsiopoulou C, Karaiskou C, Sossidou E, Fragioudakis N, Kapsomenos I, Bampidis VA, Christodoulou V & Zervas G (2018). Effects of dietary orange peel essential oil supplementation on milk yield and composition, and blood and milk antioxidant status of dairy ewes. Anim Feed Sci Technol 245:20-31.
Kulma M, Bubová T, Kopeckỳ O & Rettich F (2017) Lavender, eucalyptus, and orange essential oils as repellents against Ixodes ricinus females. Sci Agri Boh 48:76-81.
Kumar S, Mendiratta SK, Agrawal RK, Sharma H & Singh BP (2018) Anti-oxidant and anti-microbial properties of mutton nuggets incorporated with blends of essential oils. J Food Sci Technol 55:821-832.
Kurniawati A, Wigati DN, Hasanah C & Yusiati LM (2020) Improvement of ruminal feed fermentation by addition of eucalyptus based mix essential oil. IOP Conf Ser: Earth Environ Sci 425:012086.
Le TB, Beaufay C, Bonneau N, Mingeot-Leclercq M-P & Quetin-Leclercq J (2018) Anti-protozoal activity of essential oils and their constituents against Leishmania, Plasmodium and Trypanosoma. Phytochimie 18:1.
Lee JE, Jung M, Lee SC, Huh MJ, Seo SM & Park IK (2020) Antibacterial mode of action of trans-cinnamaldehyde derived from cinnamon bark (Cinnamomum verum) essential oil against Agrobacterium tumefaciens. Pest Biochem Physiol 2:104546.
Lee JW, Kim DH, Kim YB, Jeong SB, Oh ST, Cho SY & Lee KW (2020) Dietary Encapsulated Essential Oils Improve Production Performance of Coccidiosis-Vaccine-Challenged Broiler Chickens. Animals 10:481.
Lei Z, Zhang K, Li C, Jiao T, Wu J, Wei Y, Tian K, Li C, Tang D, Davis DI & Casper DP (2019) Ruminal metagenomic analyses of goat data reveals potential functional microbiota by supplementation with essential oil-cobalt complexes. BMC Microbiol 19:30.
Liu T, Chen H, Bai Y, Wu J, Cheng S, He B & Casper DP (2020) Calf starter containing a blend of essential oils and prebiotics affects the growth performance of Holstein calves. J Dairy Sci 103:2315-2323.
Maqbul MS, Bokhari YA, Gumaan S, Basalib SN, Omar BM, Khan AA, Iqubal SS & Mohammed T (2020) A Comparative Study of Different Types of Thyme Essential oils Against Streptococcus pyogenes to Determine their Biochemical and Antimicrobial Properties. Orient J Chem 36(2).

Masood A, Qureshi AS, Shahid RU & Jamil H (2020) Effects of Oral Administration of Essential Oil (Mix Oil®) on Growth Performance and Intestinal Morphometry of Japanese Quails (Coturnix coturnix japonica). Pak Vet Journal.

Matloup OH, Abd El Tawab AM, Hassan AA, Hadhoud FI, Khattab MSA, Khalel MS, Sallam SMA & Kholif AE (2017). Performance of lactating Friesian cows fed a diet supplemented with coriander oil: Feed intake, nutrient digestibility, ruminal fermentation, blood chemistry, and milk production. Anim Feed Sci Technol 226:88-97.

Maty HN & Hassan AA (2020) Effect of supplementation of encapsulated organic acid and essential oil Gallant+® on some physiological parameters of Japanese quails. Iraqi J Vet Sci 34:181-188.

McIntosh FM, Williams P, Losa R, Wallace RJ, Beever DA & Newbold CJ (2003). Effects of Essential Oils on Ruminal Microorganisms and Their Protein Metabolism. Appl Env Microbiol 69:5011-5014.

Mekuiko HW, Tendonkeng F, Ngoula F, Miegoue E, Lemoufouet J, Zogang BF, Muhindo ZK, Chounna A, Mouchili M & Tedonkeng EP (2018) Effect of Added Quantity of the Callistemon viminalis Essential Oil on the in Vivo Digestibility of Pennisetum clandestinum Hay and Some Biochemical Parameters on the West African Dwarf Goat. J Agric Chem Environ 07:81.

Mercati F, Dall'Aglio C, Acuti G, Faeti V, Tardella FM, Pirino C, De Felice E & Scocco P (2020) Oregano Feed Supplementation Affects Glycoconjugates Production in Swine Gut. Animals 10(1):149.

Michailoff AA, Silveira MF, Maeda EM, Sordi ACB, Francisco LF & Farenzena R (2020) Effect of including functional oils in ovine diets on ruminal fermentation and performance. Small Rum Res 2:106084.

Migliorini MJ, Boiago MM, Roza LF, Barreta M, Arno A, Robazza WS, Galvao AC, Galli GM, Machado G, Baldissera MD & Wagner R (2019) Oregano essential oil (Origanum vulgare) to feed laying hens and its effects on animal health. Anais da Academia Brasileira de Ciências 91: e20170901.

Mohammadi A, Ghazanfari S & Sharifi SD (2019) Comparative effects of dietary organic, inorganic, and Nano-selenium complexes and rosemary essential oil on performance, meat quality and selenium deposition in muscles of broiler chickens. Livest Sci 226:21-30.

Mohammadi A, Ghazanfari S & Sharifi SD. Comparative effects of dietary organic, inorganic, and Nano-selenium complexes and rosemary essential oil on performance, meat quality and selenium deposition in muscles of broiler chickens. Livest Sci 226:21-30.

Mohammed RK, Musa FH, Mehdi BY & Al-Rawe AM (2020). Impacts of the Alcoholic Extract and Essential Oil of Thymus vulgaris L. against the Causative Agent of Acne Formation (Staphylococcus aureus). Syst Rev Pharm 11:495-498.

Mohan A & Purohit AS (2020) Anti-Salmonella activity of pyruvic and succinic acid in combination with oregano essential oil. Food Control 110:106960.

Mountzouris KC, Paraskeuas V, Griela E, Papadomichelakis G & Fegeros K (2020) Effects of phytogenic inclusion level on broiler carcass yield, meat antioxidant capacity, availability of dietary energy, and expression of intestinal genes relevant for nutrient absorptive and cell growth-protein synthesis metabolic functions. Anim Prod Sci 60:242-253.

Muir WI, Bryden WL & Husband AJ (2000). Immunity, vaccination and the avian intestinal tract. Dev Comp Immunol 24:325-342.

Olaimat AN, Al-Holy MA, Ghoush MHA, Al-Nabulsi AA, Osaili TM & Holley RA (2019) Inhibitory effects of cinnamon and thyme essential oils against Salmonella spp. in hummus (chickpea dip). J Food Process Preserv 43:e13925.

Oliva A, Costantini S, De Angelis M, Garzoli S, Božović M, Mascellino MT, Vullo V & Ragno R (2018) High potency of melaleuca alternifolia essential oil against multi-drug resistant gram-negative bacteria and methicillin-resistant Staphylococcus aureus. Molecules 23:2584.

Onel SE & Aksu T (2019) The Effect of Thyme (Thymbra spicata L. var. spicata) Essential Oil on the Antioxidant Potential and Meat Quality of Japanese Quail Fed in Various Stocking Densities. Atatürk Üniversitesi Veteriner Bilimleri Dergisi 14:129-136.

Ortuño J, Serrano R, Jordán MJ & Bañón S (2014) Shelf life of meat from lambs given essential oil-free rosemary extract containing carnosic acid plus carnosol at 200 or 400mgkg−1. Meat Sci 96:1452-1459.

Paiano RB, Bonilla J, Sousa RLM de, Moreno AM & Baruselli PS (2020). Chemical composition and antibacterial activity of essential oils against pathogens often related to cattle endometritis. J Infec Develop Count 14:177-183.

Park JH & Kim IH (2018) Effects of a protease and essential oils on growth performance, blood cell profiles, nutrient retention, ileal microbiota, excreta gas emission, and breast meat quality in broiler chicks. Poult Sci 97:2854-2860.

Parvar R, Ghoorchi T, Kashfi H & Parvar K (2018) Effect of Ferulago angulata (Chavil) essential oil supplementation on lamb growth performance and meat quality characteristics. Small Rum Res 167:48-54.

Parvizi O, Taherkhani R & Abouzari M (2020) Evaluation the effect of using thyme and oregano powder in comparison to the antibiotic and probiotic supplementation on growth, some immune responses and intestinal morphology of broiler chicks. Ukrainian J Vet Agri Sci 3:3-8.

Pathak M, Mandal GP, Patra AK, Samanta I, Pradhan S & Haldar S (2017) Effects of dietary supplementation of cinnamaldehyde and formic acid on growth performance, intestinal microbiota and immune response in broiler chickens. Anim Prod Sci 57:821-827.

Patra AK & Saxena J (2010). A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 71:1198-1222.

Patra AK & Yu Z (2012). Effects of Essential Oils on Methane Production and Fermentation by, and Abundance and Diversity of, Rumen Microbial Populations. Appl Env Microbiol 78:4271-4280.

Pawar MM, Kamra DN, Chaudhary LC, Agarwal N & Chaturvedi VB (2019) Nutrients utilization, methane emission, immune function, blood metabolites and performance of buffalo calves fed Trachyspermum copticum seed oil. Indian J Ani Sci 89:63-67.

Peñalver P, Huerta B, Borge C, Astorga R, Romero R & Perea A (2005). Antimicrobial activity of five essential oils against origin strains of the Enterobacteriaceae family. APMIS 113:1-6.

Peng QY, Li JD, Li Z, Duan ZY & Wu YP (2016). Effects of dietary supplementation with oregano essential oil on growth performance, carcass traits and jejunal morphology in broiler chickens. Anim Feed Sci Technol 214:148-153.

Placha I, Ocelova V, Chizzola R, Battelli G, Gai F, Bacova K & Faix S (2019) Effect of thymol on the broiler chicken antioxidative defence system after sustained dietary thyme oil application. British poult sci 60:589-596.

Pourghanbari G, Nili H, Moattari A, Mohammadi A & Iraji A (2016) Antiviral activity of the oseltamivir and Melissa officinalis L. essential oil against avian influenza A virus (H9N2). VirusDis 27:170-178.

Pournazari M, AA-Qotbi A, Seidavi A & Corazzin M (2017) Prebiotics, probiotics and thyme (Thymus vulgaris) for broilers: performance, carcass traits and blood variables. Revista Colombiana de Ciencias Pecuarias 30:3-10.

Pu J, Chen D, Tian G, He J, Zheng P, Mao X, Yu J, Huang Z, Luo J, Luo Y & Yu B (2020) Effects of benzoic acid, Bacillus coagulans and oregano oil combined supplementation on growth performance, immune status and intestinal barrier integrity of weaned piglets. Ani Nut.

Pukrop JR, Campbell BT & Schoonmaker JP (2019) Effect of essential oils on performance, liver abscesses, carcass characteristics and meat quality in feedlot steers. Ani Feed Sci Technol 257:114296.

Puvača N, Petrović A, Tomić DH, Tatham EK, Čabarkapa I, Lević J & Sparagano O (2019). Influence of essential oils as natural poultry red mite (Dermanyssus gallinae) repellents. JATEM 2:168-77.

Puvača, N, Lika E, Cocoli S, Shtylla Kika T, Bursić V, Vuković G, Tomaš Simin M, Petrović A & Cara M (2020) Use of Tea Tree Essential Oil (Melaleuca alternifolia) in Laying Hen's Nutrition on Performance and Egg Fatty Acid Profile as a Promising Sustainable Organic Agricultural Tool. Sustainability 12: 3420.

Rafat Khafar K, Mojtahedin A, Rastegar N, Kalvani Neytali M & Olfati A (2019) Dietary Inclusion of Thyme Essential Oil Alleviative Effects of Heat Stress on Growth Performance and Immune System of Broiler Chicks. Iranian J Appl Ani Sci 9:509-517.

Rewatkar HN, Agashe JL & Jadhao GM (2019) The effect of supplementation of oregano oil and probiotic on blood biochemicals and immune response of broiler chicken. J Pharmacog Phytochem 8:1894-1897.

Ri CS, Jiang XR, Kim MH, Wang J, Zhang HJ, Wu SG, Bontempo V & Qi GH (2017) Effects of dietary oregano powder supplementation on the

growth performance, antioxidant status and meat quality of broiler chicks. Italian J Ani Sci 16:246-252.

Ribeiro AD, Ferraz Junior MV, Polizel DM, Miszura AA, Gobato LG, Barroso JP, Susin I & Pires AV (2019) Thyme essential oil for sheep: effect on rumen fermentation, nutrient digestibility, nitrogen metabolism, and growth. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 71:2065-2074.

Rinttilä T, Apajalahti J (2013). Intestinal microbiota and metabolites—Implications for broiler chicken health and performance1. J Appl Poult Res 22:647-658.

Rivaroli DC, del Mar Campo M, Sañudo C, Guerrero A, Jorge AM, Vital AC, Valero MV, do Prado RM & do Prado IN (2020) Effect of an essential oils blend on meat characteristics of crossbred heifers finished on a high-grain diet in a feedlot. Anim Prod Sci 60:595-602.

Rivaroli DC, Guerrero A, Valero MV, Zawadzki F, Eiras CE, del Mar Campo M, Sañudo C, Jorge AM & do Prado IN (2016) Effect of essential oils on meat and fat qualities of crossbred young bulls finished in feedlots. Meat Sci 121:278-284.

Rotondi P, Colonna MA, Marsico G, Giannico F, Ragni M & Facciolongo AM (2018) Dietary Supplementation with Oregano and Linseed in Garganica Suckling Kids: Effects on Growth Performances and Meat Quality. Pak J Zoo 50:1421.

Roy S, Chaurvedi P & Chowdhary A (2015) Evaluation of antiviral activity of essential oil of Trachyspermum Ammi against Japanese encephalitis virus. Pharma Res. 7:263-267.

Ruben NT, Raphaël KJ, Désiré TT, Hervé MK, Doriane YM, Alexis T & Amir M (2018) Effects of dietary thyme and oregano essential oils entrapped in chitosan and Canarium charcoal stable matrix on growth performances in broiler chickens. Int J Inn Appl Stud; Rabat 22:114-122.

Salem A, El-Awady H, EL-Dein MT & Eisa D (2019) Effect Of Supplementation Of Aromatic Plants Oils On Immunity, Udder Health And Milk Production Of Friesian Cows. Slovenian Vet Res 56: 523-530.

Sarengaowa, Hu W, Feng K, Xiu Z, Jiang A & Lao Y (2019) Tandem mass tag-based quantitative proteomic analysis reveal the inhibition mechanism of thyme essential oil against flagellum of Listeria monocytogenes. Food Res Inter 125:108508.

Sariözkan S, Güçlü BK, Konca Y, Aktuğ E, Kaliber M, Beyzi SB & Şentürk M (2020) The effects of essential thyme oil and vitamin combinations on performance, carcass quality and oxidation parameters in broilers exposed to heat stress. Ankara Üniversitesi Veteriner Fakültesi Dergisi.

Schären M, Drong C, Kiri K, Riede S, Gardener M, Meyer U, Hummel J, Urich T, Breves G & Dänicke S (2017) Differential effects of monensin and a blend of essential oils on rumen microbiota composition of transition dairy cows. J Dairy Sci 100:2765-2783.

Sharafati Chaleshtori R, Rokni N, Rafieian-Kopaei M, Deris F & Salehi E (2015) Antioxidant and antibacterial activity of basil (Ocimum basilicum L.) essential oil in beef burger. J Agri Sci Technol 17:817-826.

Sharma H, Mendiratta SK, Agrawal RK, Gurunathan K, Kumar S & Singh TP (2017) Use of various essential oils as bio preservatives and their effect on the quality of vacuum packaged fresh chicken sausages under frozen conditions. LWT - Food Sci Technol 81:118-127.

Shokrollahi B, Amini F, Fakour S & Andi MA (2015) Effect of rosemary (Rosmarinus officinalis) extract on weight, hematology and cell-mediated immune response of newborn goat kids. JARTS 116: 91-97.

Silva RB da, Pereira MN, Araujo RC de, Silva W de R & Pereira RAN (2020) A blend of essential oils improved feed efficiency and affected ruminal and systemic variables of dairy cows. Trans Anim Sci 4:182-193.

Simitzis PE (2017). Enrichment of Animal Diets with Essential Oils—A Great Perspective on Improving Animal Performance and Quality Characteristics of the Derived Products. Medicines 4:35.

Singh RK, Dey A, Paul SS, Singh M, Dahiya SS & Punia BS (2019) Associative effects of plant secondary metabolites in modulating in vitro methanogenesis, volatile fatty acids production and fermentation of feed in buffalo (Bubalus bubalis). Agroforest Sys 17:1-2.

Smeti S, Hajji H, Bouzid K, Abdelmoula J, Muñoz F, Mahouachi M & Atti N (2015) Effects of Rosmarinus officinalis L. as essential oils or in form of leaves supplementation on goat's production and metabolic statute. Trop Anim Health Prod 47:451-457.

Smeti S, Hajji H, Mekki I, Mahouachi M & Atti N (2018) Effects of dose and administration form of rosemary essential oils on meat quality and fatty acid profile of lamb. Small Rum Res 158:62-68.

Šojić B, Pavlić B, Zeković Z, Tomović V, Ikonić P, Kocić-Tanackov S & Džinić N (2018) The effect of essential oil and extract from sage (Salvia officinalis L.) herbal dust (food industry by-product) on the oxidative and microbiological stability of fresh pork sausages. LWT 89:749-755.

Souza VGL, Pires JR, Vieira ÉT, Coelhoso IM, Duarte MP & Fernando AL (2019) Activity of chitosan-montmorillonite bionanocomposites incorporated with rosemary essential oil: From in vitro assays to application in fresh poultry meat. Food Hydrocolloids 89:241-252.

Su G, Zhou X, Wang Y, Chen D, Chen G, Li Y & He J (2018) Effects of plant essential oil supplementation on growth performance, immune function and antioxidant activities in weaned pigs. Lipids in Healt Dis 17:139.

Su G, Zhou X, Wang Y, Chen D, Chen G, Li Y, He J (2020) Dietary supplementation of plant essential oil improves growth performance, intestinal morphology and health in weaned pigs. J Ani Phys Ani Nut 104:579-589.

Tapki I, Ozalpaydin HB, Tapki N, Aslan M & Selvi MH (2020) Effects of Oregano Essential Oil on Reduction of Weaning Age and Increasing Economic Efficiency in Holstein Friesian Calves. Pak J Zoo 52:745.

Tayeb IT, Artoshi NH & Sögüt B (2019) Performance of broiler chicken fed different levels thyme, adiantum, rosemary and their combination. Iraqi J Agri Sci 50:1522-1532.

Tekce E, Çınar K, Bayraktar B, Takma Ç & Gül M (2020) Effects of an Essential Oil Mixture Added to Drinking Water for Temperature-Stressed Broilers: Performance, Meat Quality, and Thiobarbituric Acid-Reactive Substances. J Appl Poult Res 29:77-84.

Teobaldo RW, De Paula NF, Zervoudakis JT, Fonseca MA, Cabral LS, Martello HF, Rocha JK, Ribeiro IJ & Mundim AT (2020) Inclusion of a blend of copaiba, cashew nut shell and castor oil in the protein-energy supplement for grazing beef cattle improves rumen fermentation, nutrient intake and fibre digestibility. Anim Prod Sci.

Thao NT, Wanapat M, Cherdthong A & Kang S (2014) Effects of Eucalyptus Crude Oils Supplementation on Rumen Fermentation, Microorganism and Nutrient Digestibility in Swamp Buffaloes. Asian-Australas J Anim Sci 27:46-54.

Thao NT, Wanapat M, Kang S & Cherdthong A (2015) Effects of Supplementation of Eucalyptus (E. Camaldulensis) Leaf Meal on Feed Intake and Rumen Fermentation Efficiency in Swamp Buffaloes. Asian-Australas J Anim Sci 28:951-957.

Tian Q & Piao X (2019) Essential Oil Blend Could Decrease Diarrhea Prevalence by Improving Antioxidative Capability for Weaned Pigs. Animals 10:847.

Tomkins NW, Denman SE, Pilajun R, Wanapat M, McSweeney CS, Elliott R (2015) Manipulating rumen fermentation and methanogenesis using an essential oil and monensin in beef cattle fed a tropical grass hay. Anim Feed Sci Technol 200:25-34.

Tongnuanchan P & Benjakul S (2014) Essential Oils: Extraction, Bioactivities, and Their Uses for Food Preservation. J Food Sci 79:1231-1249.

Ultee A, Bennik MHJ & Moezelaar R (2002) The Phenolic Hydroxyl Group of Carvacrol Is Essential for Action against the Food-Borne Pathogen Bacillus cereus. Appl Env Microbiol 68:1561-1568.

Vendramini THA, Takiya CS, Silva TH, Zanferari F, Rentas MF, Bertoni JC, Consentini CEC, Gardinal R, Acedo TS & Rennó FP (2016) Effects of a blend of essential oils, chitosan or monensin on nutrient intake and digestibility of lactating dairy cows. Anim Feed Sci Technol 214:12-21.

Wade M, Manwar S, Kuralkar S, Waghmare S & Ingle V (2018) Effect of thyme essential oil on performance of broiler chicken. J Ent Zoo Stud 6:25-28.

Wadhwa M & Bakshi MPS (2019) Effect of supplementing total mixed ration with ajwain (Trachyspermum ammi) oil on the performance of buffalo calves. Indian J Ani Sci 89:424-430.

Wallace RJ (2004). Antimicrobial properties of plant secondary metabolites. Proc Nutr Soc 63:621-629.

Wang LM, Mandell IB & Bohrer BM (2020) Effects of feeding essential oils and benzoic acid to replace antibiotics on finishing beef cattle

growth, carcass characteristics, and sensory attributes. Appl Ani Sci 36:145-156.

Wells JE, Berry ED, Guerini MN & Varel VH (2015) Evaluation of essential oils in beef cattle manure slurries and applications of select compounds to beef feedlot surfaces to control zoonotic pathogens. J Appl Microbiol 118:295-304.

Wells JE, Berry ED, Guerini MN & Varel VH (2015). Evaluation of essential oils in beef cattle manure slurries and applications of select compounds to beef feedlot surfaces to control zoonotic pathogens. J Appl Microbiol 118:295-304.

Witkowska D, Sowińska J, Murawska D, Matusevičius P, Kwiatkowska-Stenzel A, Mituniewicz T & Wójcik A (2019) Effect of peppermint and thyme essential oil mist on performance and physiological parameters in broiler chickens. South African J Ani Sci 49:29-39.

Xu YT, Liu Li, Long SF, Pan L & Piao XS (2018) Effect of organic acids and essential oils on performance, intestinal health and digestive enzyme activities of weaned pigs. Ani Feed Sci Technol 235:110-119.

Yalçin S, Eser H, Onbaşilar İ & Yalçin S (2020) Effects of dried thyme (Thymus vulgaris L.) leaves on performance, some egg quality traits and immunity in laying hens. Ankara Üniversitesi Veteriner Fakültesi Dergisi.

Yang SY, Cao L, Kim H, Beak SE & Song KB (2018) Utilization of Foxtail Millet Starch Film Incorporated with Clove Leaf Oil for the Packaging of Queso Blanco Cheese as a Model Food. Starch - Stärke 70:1700171.

Yang WZ, Ametaj BN, Benchaar C & Beauchemin KA (2010). Dose response to cinnamaldehyde supplementation in growing beef heifers: Ruminal and intestinal digestion. J Anim Sci 88:680-688.

Yang X, Xin H, Yang C & Yang X (2018) Impact of essential oils and organic acids on the growth performance, digestive functions and immunity of broiler chickens. Ani Nut 4:388-393.

Yang YF, Zhao LL, Shao YX, Liao XD, Zhang LY, Lin LU & Luo XG (2019) Effects of dietary graded levels of cinnamon essential oil and its combination with bamboo leaf flavonoid on immune function, antioxidative ability and intestinal microbiota of broilers. J Integ Agri 18:2123-2132.

Yesilbag D, Biricik H, Cetin I, Kara C, Meral Y, Cengiz SS, Orman A & Udum D (2017) Effects of juniper essential oil on growth performance, some rumen protozoa, rumen fermentation and antioxidant blood enzyme parameters of growing Saanen kids. J Ani Phys Ani Nut 101:e67-e76.

Zengin H & Baysal AH (2015) Antioxidant and Antimicrobial Activities of Thyme and Clove Essential Oils and Application in Minced Beef. J Food Proc Preserv 39:1261-1271.

Zhang Y, Liu X, Wang Y, Jiang P & Quek S (2016) Antibacterial activity and mechanism of cinnamon essential oil against Escherichia coli and Staphylococcus aureus. Food Control 59:282-289.

Zhou R, Wu J, Lang X, Liu L, Casper DP, Wang C, Zhang L & Wei S (2020) Effects of oregano essential oil on in vitro ruminal fermentation, methane production, and ruminal microbial community. J Dairy Sci 103:2303-2314.

Zhou R, Wu J, Zhang L, Liu L, Casper DP, Jiao T, Liu T, Wang J, Lang X, Song S & Gong X (2019) Effects of oregano essential oil on the ruminal pH and microbial population of sheep. PloS one 14:e0217054.

Zhu H, Du M, Fox L & Zhu MJ (2016) Bactericidal effects of Cinnamon cassia oil against bovine mastitis bacterial pathogens. Food Control. 66:291-299.

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Review article
Ανασκόπηση

Effects of L-carnitine supplementation in diets of broiler chickens

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ABSTRACT: L-carnitine is a nutritional supplement having fat-burning property and plays an important role in lipid metabolism, energy release, and also improve the production yield, immunity and blood constitute. This paper reviews the effects of L-carnitine on quantitative and qualitative characteristics of broilers. According to the reviewed literature, the application of L-carnitine (50-200 mg/kg) has no significant effect on the growth performance, however, using L-carnitine as much as 300-800 mg/kg resulted to an improvement in the body weight (2226.00-2575.00 g) compared to the control chicks (1998.40-2338.75 g). The feed conversion ratios of the chickens fed the same amount of L-carnitine were 1.66-1.86 kg/kg, which was improved in comparison with the control chicks (1.87-2.09 kg/kg). Abdominal fat of the broiler chickens fed 50-900 mg/kg L-carnitine was 0.98-1.75%, which is lower than the control chicks (1.79-2.16%). For immunity, the antibody titers against the Newcastle virus in the chickens fed 250 mg/kg L-carnitine was between 4.6- 5.5 which is more than control chicks (4.3-5.2). The antibody titer against the influenza virus in the chickens fed the same amount of L-carnitine was between 5.6-6.3, which was more than the control chicks (4.3-5.8). The use of 100-600 mg/kg L-carnitine could reduce triglyceride (90-104.4 mg/dL) compared to the control chicks (125-104.7 mg/dL) and also reduced the cholesterol (109-115 mg/dL) compared to the control chicks (129.25-131 mg/dL). The application of 100-600 mg/kg L-carnitine also could reduce low-density lipoprotein (LDL) from 19.1-72.2 mg/dL to 16.5-49.0 mg/dL. However, the application of 100-900 mg/kg L-carnitine had no significant effect on the sensory characteristics of broiler chicken meat. In general, it can be concluded that L-carnitine can be used as a dietary supplement on the health of broiler chickens without any negative effect on growth performance.

***Keywords*:** Dietary supplements; fat burning; triglycerides, cholesterol, L-carnitine

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INTRODUCTION

With the spread of various nutrition-caused diseases, producing food and meat consistent with human health has become increasingly important and this has limited the consumption of fatty-rich food such as high-fat chicken meat. Besides, increased fat accumulation in chickens is considered as waste in slaughterhouse operations, and manufacturers always seek fat removal strategies to reduce the cost of production and to produce healthy chickens (Golzae Adabi et al., 2011). In this regard, changes in the rate and nutrient level of the diet and the use of fat burning supplements, such as medicinal plants were found to be effective in reducing fat. Fatty chemicals used to control fat in humans have been introduced to address this problem.

Today, the use of L-carnitine, as a component of the essential substances in body to increase energy efficiency and dietary fat, as well as reduce the accumulation of abdominal fat and plasma, is increasing in the food industry.

L-carnitine is an anti-fatty drug for controlling fat in humans and its use in poultry feed including broilers has been reported to be effective in controlling lipid and ventricular fat)Golzae Adabi et al., 2011; Hrncar et al., 2015; Khatibjoo et al., 2016(

L-carnitine is made in the liver of humans and animals and then transferred to muscle tissue. Most L-carnitine is stored in skeletal and heart muscle, and is a mixture of L-carnitine synthesized in the body and L-carnitine absorbed from the diet (Taklimi et al., 2015). The use of L-carnitine in poultry feed helps increase energy efficiency so that poultry can more quickly and easily obtain the energy they need from dietary lipids. Positive effect of L-carnitine on reducing feed intake (Khatibjoo et al., 2016; Mirzapor Sarab et al., 2016), live weight gain (Kidd et al., 2009), increased final weight, improved feed conversion ratio, improved carcass characteristics (Hrncar et al., 2015) and reduced abdominal fat (Babazadeh Aghdam et al., 2015) have been reported for different poultry. L-carnitine also increases the antioxidant and immune capacity of poultry and prevents cardiovascular disease, pulmonary hypertension and ascites in poultry by affecting nitric oxide production and recovery of myocardial energy reserve (Buyse et al., 2007; Xue et al., 2007; Khajali et al., 2011). The effect of L-carnitine on broiler chickens will be the subject of this review.

L-CARNITINE

L-carnitine (L-Trimethyl-3-hydroxyamino-butanoate) is a pseudo-vitamin and deformed amino acid, first isolated in 1905 from muscle tissues, and its structure was identified in 1927.

Synthesis of carnitine is endogenous and requires two amino acids lysine and methionine. Lysine is the supplier of the carbon chain and the hydrogen atom and methionine also play a role as a methyl donor in this process (Flanagan et al., 2010). Carnitine has two L and D isomers, the L isomer being important for humans and animals. The synthesis of L-carnitine in the body is a multi-stage process and is performed by adding a hydroxyl group to a third carbon lysine in various organs such as mitochondria, kidneys, liver, brain and muscles. The complete process for the synthesis of L-carnitine is presented in Figure 1.

L-carnitine has two main roles in the body, including facilitating the entry of long-chain fatty acids into mitochondria, as well as the release of short and medium-chain fatty acids from the mitochondria. In addition to the roles such as eliminating the toxic effects of acyl groups from cells, adjusting the ratio of coenzyme A to the acyl-coenzyme A in cytosol and mitochondria, gluconeogenesis, are also reported for L-carnitine (Corduk et al., 2007; Parsaeimehr et al., 2014a).

L-carnitine is a drug for lowering plasma lipids that reduces cholesterol, triglycerides, free fatty acids, phospholipids, and low-density lipoproteins and increases high-density lipoprotein. In addition to the role of L-carnitine in the oxidation of fatty acids, its role in carbohydrate metabolism has also been reported (Rajasekar and Anuradha 2007). Today, the use of L-carnitine supplementation is increasing as part of the body's essential nutrients to increase the energy and fat intake of food and to reduce the accumulation of ventricular and plasma fat.

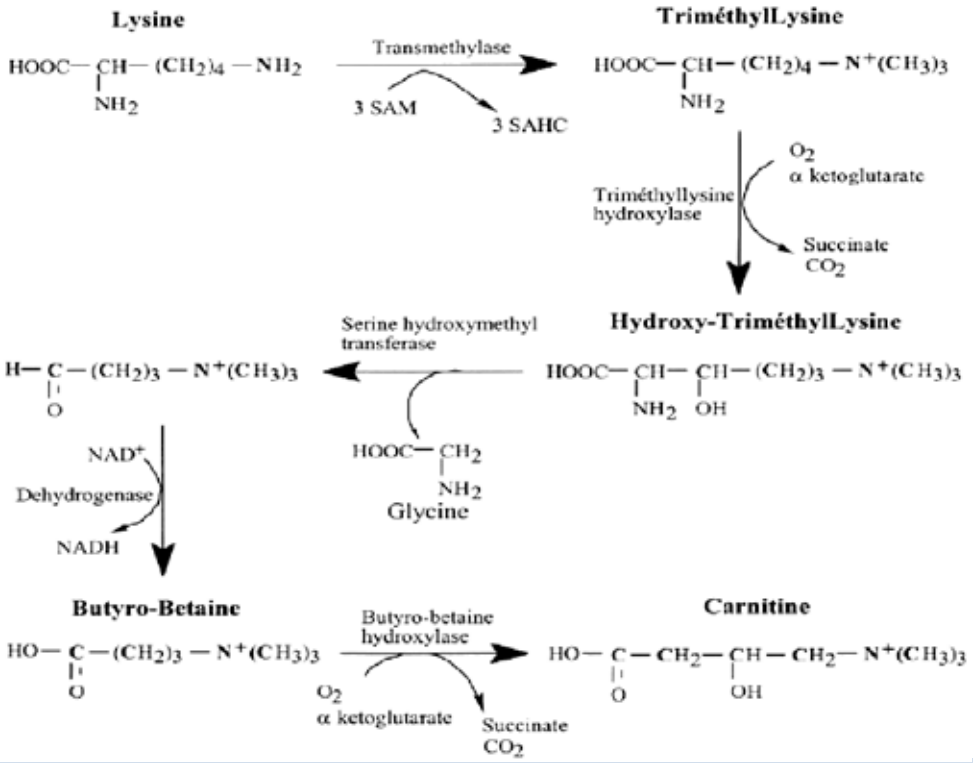

Figure 1. Biosynthesis of L-carnitine (Arslan, 2006).

THE NECESSITY OF USING L-CARNITINE IN POULTRY DIET

L-carnitine is an essential and water-soluble substance naturally existing in plants, animals and microorganisms, however, the amount of L-carnitine in herbs and plants is lower than that of the animals, and since the poultry diet is mostly formed of feed of plant origin, using the supplementation of L-carnitine in a diet or drinking water is necessary to achieve optimal performance (Taklimi et al. 2015). It should be noted that the normal amount of L-carnitine per kilogram of poultry feed is 2 to 5 milligrams and an average of 25 to 50 milligrams per day (Mirzapor Sarab et al., 2016).

L-carnitine is involved in many metabolic processes, such as lipid metabolism, energy liberation, improved production efficiency, immune enhancement, etc. (Khatibjoo et al., 2016; Mirzapor Sarab et al., 2016). This substance prevents lipogenesis and increases the burning rate of fat. Burning of fatty acids happens in body cells and energy production occurs in the mitochondria; the mitochondrial membrane is infused; therefore, it is required to have a carrier to transport fatty acids to the oxidation place mitochondria, that L-carnitine acts as a carrier and by the transport of long-chain fatty acids into mitochondria increases the oxidation of fatty acids and facilitates the production of energy (Kheirkhah et al., 2009).

The use of L-carnitine in poultry feed helps increase the energy efficiency of poultry so that poultry can quickly and easily absorb the energy needed from the dietary lipids. Besides, the use of L-carnitine causes a significant reduction in feed intake in poultry (Khatibjoo et al., 2016; Mirzapor Sarab et al., 2016). Several studies have shown that sufficient amounts of L-carnitine in poultry diets with different levels and sources of fat, can be appropriate to optimal yield, increased feed efficiency and increased economic returns (Parsaeimehr et al. 2014b; Abedpour et al. 2017; Swarna et al. 2018).

Effect of L-carnitine cofactors in broiler diet

Carnitine is synthesized in different parts of the body in living creatures. For example, the synthesis of carnitine in the liver of all mammals, muscles of sheep, brain and kidneys of humans and all the cats occurs through methioninolysin. The synthesis of carnitine in the body of different living organisms requires enzymes as well as cofactors such as ascorbic acid, niacin, pyridoxine and iron oxide. Researchers

believe that poultry and mammals can produce carnitine if they are sufficiently present in the diet (Ghoreyshi et al., 2019).

The use of lysine and methionine amino acids in the diet was effective in supplying carnitine in poultry. Celik and Ozturkcan (2003) examined the effect of ascorbic acid, a precursor of carnitine synthesis in the body, on broiler chickens under thermal stress. In this study, the use of carnitine and ascorbic acid in normal conditions significantly reduced the growth of broiler chickens. In another study, the use of carnitine alone and in combination with niacin, which is a cofactor of carnitine production in the body, improved the performance of broiler chicks in the early stages of growth (Celik et al., 2003).

EFFECT OF L-CARNITINE ON THE PERFORMANCE OF BROILER CHICKENS

Kidd et al. (2009) reported that supplementation of L-carnitine supplementation in broiler diets does not have a significant effect on yield, but thigh and body weight gain is increased with 40 mg/kg of L-carnitine. Hrncar et al. (2015) used L-carnitine (1 ml/1.2 l) in drinking water in Ross 308 broiler chickens. In that study, the application of L-carnitine increased final weight and improved feed conversion ratio. Kamal et al. (2019) stated that consumption of L-carnitine in the diet of broilers increases live weight, final weight and reduces feed intake and feed conversion ratio. Ghoreyshi et al. (2019) stated that the use of 15% L-carnitine + 30% methionine + 30% lysine throughout the whole period reduces feed intake compared to the control, but the highest weight gain was achieved with the use of 15% of the above mentioned compounds.

Tufarelli et al. (2020) found that L-carnitine consumption did not have a significant effect on live weight of broiler chickens in the starter, grower, and finisher periods, but feed intake increased with the use of L-carnitine during the starter period. The feed conversion ratio increased with the use of L-carnitine in the starter period and decreased in the final period by consuming 50 mg/kg; feed intake was also improved with the application of different levels of L-carnitine in broiler chickens in different experiments (Celik et al., 2003).

It is stated that consumption of L-carnitine increases the weight of the chickens during the first three weeks of growth, but did not affect the weight of the chickens in the final three weeks (Celik et al., 2003). Researchers have found that body weight gain in broiler chickens by the use of L-carnitine in the diet is due to the role of L-carnitine in increasing the oxidation of long-chain fatty acids and increasing the level of acetyl coenzyme A in mitochondria, which increases the use by chickens of the diet protein (Mirzapor Sarab et al., 2016).

Improvement of the nutritional conversion ratio in broiler chickens and laying hens with the combined use of L-carnitine and vitamin C was reported (Celik and Ozturkcan, 2003; Hassan et al., 2011). Xu et al. (2003) reported that L-carnitine had no significant effect on functional traits (live weight, feed intake and feed conversion ratio) at 0, 25, 50, 75 and 100 mg/kg levels. Khajali and Khajali (2014) used 200 mg/kg of L-carnitine in broiler diets; the results showed that the application of L-carnitine had no significant effect on body weight, feed intake and feed conversion ratio.

Wang et al. (2013) investigated the effect of L-carnitine supplemented diet in low-temperature conditions on broiler chickens Ross 308 strains. The results showed that the performance of chicken was not affected by L-carnitine, but the susceptibility to ascites was greatly reduced. Parsaeimehr et al. (2013) used a diet containing plant and animal fat with L-carnitine. The results of that study showed that the addition of L-carnitine to a diet containing animal fat increases the weight of the Ross 308 chickens during the growth and the whole period. Also, feed intake significantly decreased with this treatment. Adding L-carnitine to the diet reduced the feed conversion rate at the finisher period.

In a study, 0, 150, 300, 450 and 600 mg/kg of L-carnitine was used in the base diet of Ross 308 male broiler chickens; the results showed that adding L-carnitine up to 600 mg/kg had no effect on weight gain of the initial period (1-21 days), but, it was effective on the weight of chicken during the growth period and the whole period. There was no significant difference between the treatments in feed intake during the growth period and the whole period. Also, the addition of L-carnitine in the diet did not have a significant effect on the feed conversion ratio in three periods of starter, grower, and finisher (Parsaeimehr et al., 2014b).

Shirali et al. (2015) examined the effects of different levels of vitamin E and L-carnitine in the diet of chickens under thermal stress and reported that the

interaction of vitamin E and L-carnitine had a significant effect on yield, feed intake, feed conversion ratio of chickens during the breeding season. Treatment of 100 mg/kg vitamin E with 100 mg/kg L-carnitine had the highest live weight during the starter period (1-21 days). However, during the starter period and the whole period, the treatments did not significantly affect the weight gain of chickens.

Farrokhyan et al. (2014) examined the effect of L-carnitine (0, 150 and 300 mg/kg) and gemfibrozil (0, 1 and 2 g/kg) on broiler diets. The results showed that application of the treatment without gemfibrozil and with 300 mg/kg of l-carnitine, yielded the highest live weight. Also, the highest feed intake during the 1-6 week period was achieved with 300 mg/kg of L-carnitine without gemfibrozil whereas the lowest was achieved with a diet supplemented with 1 g/kg of gemfibrozil with 300 mg/kg of L- carnitine. The lowest feed conversion ratio was observed in diets supplemented with 1 g/kg gemfibrozil with 300 mg/kg of L-carnitine and a diet supplemented with 300 mg/kg of L-carnitine with no gemfibrozil added.

Rajabzadeh-Nesvan et al. (2013) reported the effect of L-carnitine (0 and 125 mg/kg) on the diets containing different sources of fat (soybean oil, animal fat, and their mixture) on Ross 308 broiler chickens. The results showed that different sources of fat and L-carnitine had no significant effect on chicken yield. The effect of L-carnitine on the performance of chickens was not significant, but the use of L-carnitine reduced the feed conversion ratio, increased feed intake and increased weight of chickens compared to the control.

Arslan and Tufan (2018) investigated the effect of a diet supplemented with oligosaccharide chitosan and L-carnitine on broiler chickens and showed that there was no significant difference between diets on body weight, feed intake and feed conversion ratio. Rezaei et al. (2007) indicated that the application of fat (1, 3 and 5%) and 2 levels of L-carnitine (0 and 250 mg/kg) in the diet of broiler chickens reduced feed conversion ratio and increased feed intake.

Taraz and Dastar (2008) showed that the application of 125 mg/kg of L-carnitine in a diet containing various protein levels would increase body weight and carcass performance of cookable broiler chickens. Kheirkhah et al. (2009) reported that L-carnitine had no effect on feed conversion ratio, but reduced feed intake and increased weight of broiler chickens. Darsi Arani et al. (2010) reported that application of 50 mg/kg of L-carnitine in a diet containing three levels of crude protein (21, 19.5 and 18%) did not affect body weight, feed intake or feed conversion ratio.

Akbari Azad et al. (2010) reported that the application of 375 mg/kg of L-carnitine in the diet significantly reduced consumption and weight gain and thus increasing European production efficiency. Research results showed that broiler chickens fed diets supplemented with L-carnitine and butyric acid were not significantly different in terms of feed intake, weight gain and feed conversion ratio (Norreh et al., 2015).

Jalali et al. (2015) investigated the effects of L-carnitine in a diet of soybean oil and sunflower oil on broiler chickens, the results showed that soybean oil with 120 mg/kg of L-carnitine significantly improved live weight and feed conversion ratio in the grower and finisher stages. Fallah et al. (2016) showed that adding terbutaline and L-carnitine to the diet increased weight gain compared to the control group. Feed intake and feed conversion ratio also significantly improved with the use of these substances in the diet.

Application of L-carnitine in drinking water in broiler chickens improved performance (Nouboukpo et al., 2010). Mehdizadeh Taklimi et al. (2015) evaluated the effect of L-carnitine (0, 400, 600 and 800 mg/kg) as liquid and powder on the performance of broiler chickens and reported that chickens from the diet containing 600 mg L-carnitine per kg of the diet were more likely to grow faster in grower period than in the other experimental groups. The highest live weight of the entire course was also 800 mg/kg of L-carnitine. The researchers stated that the L-carnitine type (powder and liquid) did not affect body weight. Feed intakes were significantly higher in chickens fed the 400 mg/kg of the L-carnitine diet. The most suitable feed conversion ratio was observed in the growth stage (1- 21 days) as well as the entire experimental period (1-42 days) in a diet containing 800 mg/kg of L-carnitine.

Rehman et al. (2017) used vitamin E (250 mg/kg), ginger (2 g/kg) and l-carnitine (500 mg/kg) in the diet of Hubbard and Cobb broiler chickens exposed to heat stress. The results of this study showed that feed intake and feed conversion ratio increased with the application of L-carnitine compared to other treatments. Murali et al. (2013) used 2 levels of L-carnitine (0 and 900 mg/kg) in the base diet of Van Cobb

chickens. The results of this study showed that growth performance did not change under the influence of L-carnitine.

Khatibjoo et al. (2016) investigated the effects of L-carnitine and butyric acid on 192 broiler chickens of Ross 308 and reported that the chickens fed by a diet containing 125 mg/kg of L-carnitine and 2 g/kg of butyric acid had a lower feed intake and a better feed conversion ratio relative to the control group. In the study by Murali et al. (2015), the application of 900 mg/kg of L-carnitine in the diet of broiler chickens strain Cobb did not have any effect on yield. Celik and Ozturkcan (2003) used two levels of L-carnitine (0 and 50 mg/kg) and 2 levels of ascorbic acid (0 and 50 mg/kg) in drinking water of broiler chickens under different thermal regimes. The results showed that live chicken weight significantly improved with the use of L-carnitine, ascorbic acid and L-carnitine plus ascorbic acid under high-temperature conditions. The effect of L-carnitine on feed conversion ratio and water and food consumption was not significant.

Application of 300 mg/kg of L-carnitine to broiler diets caused weight gain in the final course and the whole period. Feed conversion ratio in chicken fed 200 and 300 mg/kg L-carnitine was the lowest in the finisher period (Babazadeh Aghdam et al., 2015). Parsaeimehr et al. (2014a) reported that a complementary diet of L-carnitine and 5% animal fat improved body weight and feed conversion ratio in broiler chickens.

The conclusion that can be drawn from this review is that the application of L-carnitine in base diets and complemented with different levels of fat did not have a negative effect on the performance of broiler chickens. Although in some studies, the effect of L-carnitine on body weight gain, feed intake and feed conversion ratio were positive; but in many cases, L-carnitine did not have a significant effect compared to control.

In general, it can be concluded that the use of L-carnitine supplementation in diets with different levels of plant and/or animal fat was found to be beneficial for achieving optimal performance, however, it is not recommended if the purpose is to improve performance only.

Table 1. Effect of L-carnitine on the performance of broiler chickens

Reference	Applied treatment	Effects
Kidd et al., 2009	40 mg/kg	Live weight increase
Hrncar et al., 2015	1 ml /1.2 L	Live weight increase and improved feed conversion ratio
Celik et al., 2003	50 mg/kg	Improved feed intake and increased weight in the first three grower weeks
Xu et al., 2003	25, 50, 75, 100 mg/kg	No significant effect on performance traits
Khajali and Khajali, 2014	200 mg/kg	No significant effect on performance traits
Wang et al., 2013	100 mg/kg	No significant effect on performance traits and positive effect on ascites reduction
Parsaeimehr et al., 2013	150, 300, 450 , 600 mg/kg	Weight increase in grower period and whole period decreased feed intake and decreased feed conversion ratio in the finisher period
Shirali et al., 2015	100 mg/kg	No significant effect on performance traits
Rajabzadeh-Nesvan et al., 2013	125 mg/kg	Increased feed intake, weight gain increase, and reduced feed conversion ratio
Arslan and Tufan, 2018	100 mg/kg	No significant effect on performance traits
Rezaei et al., 2007	250 mg/kg	Increased feed intake and reduced feed conversion ratio
Taraz and Dastar, 2008	125 mg/kg	Bodyweight increase and carcass cooking performance
Kheirkhah et al., 2009	100 , 200 mg/kg	Weight increase, feed intake decrease and no significant effect on the feed conversion ratio
DarsiArani et al., 2010	50 mg/kg	No significant effect on performance traits
Akbari Azad et al., 2010	375 mg/kg	Increasing weight and improving European production efficiency, decreasing feed consumption
Norreh et al., 2015	125, 250 mg/kg	No significant effect on performance traits

Jalali et al., 2015	120 mg/kg	Improving live weight and feed conversion ratio in the grower and finisher periods
Fallah et al., 2016	100 mg/kg	Live weight increase, improved feed consumption and feed conversion ratio
Nouboukpo et al., 2010	500, 1000 mg/kg	Improving performance
MehdizadehTaklimi et al., 2015	400, 600, 800 mg/kg	Increased live weight, feed intake and improved feed conversion ratio
Rehman et al.,2017	500 mg/kg	Increased feed intake and improved feed conversion ratio
Murali et al.,2013	900 mg/kg	No significant effect on performance traits
Khatibjoo et al., 2016	125 mg/kg+2 g Butyric acid	Decreased feed intake and improved feed conversion ratio
Murali et al., 2015	900 mg/kg	No significant effect on performance traits
Celik and Ozturkcan, 2003	500 mg/kg	Improved live weight, no significant effect on feed conversion ratio and water and feed consumption
BabazadehAghdam et al., 2015	200, 300 mg/kg	Increased live weight and decreased feed conversion ratio
Parsaeimehr et al., 2014a	300 mg/kg	Improved live weight and feed conversion ratio
Corduk et al., 2007	100 mg/kg	No effect on weight increase
Parsaeimehr et al., 2014b	150, 300,450,600 mg/kg	No effect on performance, feed intake and conversion ratio

Effect of L-carnitine on carcass characteristics of broiler chickens

Ghoreyshi et al. (2019) stated that the use of L-carnitine, lysine and methionine had no significant effect on body weight, breast, heart, liver, gizzard, pancreas and abdominal fat, but significantly reduced thigh, neck, duodenum, jejunum and ileum weight. Islam and Ouda (2020) stated that consuming L-carnitine increases carcass weight but does not have a significant effect on the weight of heart, gizzard and abdominal fat of broilers. Fujimoto et al. (2020) showed that L-carnitine consumption has no significant effect on the carcass characteristics of broilers.

Murali et al. (2015) examined the effect of dietary supplement with L-carnitine (900 mg/kg) and animal fat (5%) on the carcass characteristics of broiler chickens. The results showed that the least abdominal fat was achieved in chicken treated with L-carnitine supplement. In that study, it was found that L-carnitine had no effect on body weight, full carcass weight, empty carcass weight, carcass yield, meat characteristics and internal organs weight.

Tufarelli et al. (2020) reported that the use of 100 and 50 mg/kg L-carnitine in the diet of broilers increased breast weight but had no significant effect on heart weight, pancreas, gizzard and abdominal fat. Xu et al. (2003) stated that the effect of 25 mg/L L-carnitine was useful in reducing the abdominal fat of broilers. However, there are also some reports stating that L-carnitine has no effect on reducing the abdominal fat of broilers (Tufarelli et al., 2020).

Babazadeh Aghdam et al. (2015) reported that consuming 300 mg/kg of L-carnitine in the diet reduced the fat content of the abdominal area of the broiler chickens with ROSS strain. However, different levels of L-carnitine (100, 200 and 300 mg/kg) had no significant effect on carcass characteristics. In the study of Xu et al. (2003), L-carnitine had a positive effect on foot muscle function, breast muscles and abdominal fat loss. Hrncar et al. (2015) reported that the addition of L-carnitine to broiler chicken diet had a positive but non-significant effect on abdominal fat, heart, liver and gizzard. Rezaei et al. (2007) stated that the addition of 250 mg/kg of L-carnitine to the diet of broiler chickens ROSS strain does not affect carcass characteristics.

In a study by Celik et al., (2003), it was found that supplementation of the diet of broiler chickens with L-carnitine had no significant effect on carcass weight and yield and ventricular fat. In the study of Daskiran and Teeter (2001), the use of L-carnitine had no significant effect on carcass yield and ventricular fat content, but Xu et al. (2003) reported increased breast muscles in male broiler chickens using 50 and 75 mg/kg of L-carnitine in the diet.

The effect of 200 mg/kg of L-carnitine in broil-

er chicken diet did not affect carcass, but the amount of ventricular fat significantly decreased compared to the control (Khajali and Khajali, 2014). The results showed that using supplementation of L-carnitine in the base diet of Ross 308 broiler chickens increases the relative weight of the breast and thighs (Parsaeimehr et al., 2014b). Also, the use of L-carnitine caused a significant decrease in abdominal cavity fat at 42 days of age (Parsaeimehr et al., 2014b). The use of L-carnitine did not have a significant effect on the relative weight of the bursa, spleen, heart and liver of broiler chickens (Parsaeimehr et al., 2014b).

In a study, vitamin E (0, 100 and 200 mg/kg) and L-carnitine (0, 50 and 100 mg/kg) were used in broiler diets. Carcass analysis showed that the effect of treatments on the percentage of breast, thigh, liver, gizzard and pancreas was not significant. However, the use of L-carnitine significantly reduced ventricular fat. The interaction of vitamin E and L-carnitine was also effective in reducing the ventricular fat in broiler chickens (Shirali et al., 2015). Evaluation of carcass characteristics of broiler chickens fed diets supplemented with different levels of L-carnitine and gemfibrozil showed that with the use of L-carnitine alone, the weight of carcasses was reduced compared to the control. The heaviest weights of full and empty carcasses were obtained using 300 and 150 mg/kg of L-carnitine plus 2 grams per kilogram of gemfibrozil, respectively. The interaction of L-carnitine and gemfibrozil increased weights of breast, wings, heart, liver and decreased ventricular fat. But there was no effect on the thigh weight (Farrokhyan et al., 2014).

Study of different levels of L-carnitine supplementation and fat source on the percentage of carcass components in broiler chickens at the age of 42 days showed that using L-carnitine had no significant effect on heart, liver, breast and thigh weights. But significantly reduced abdominal fat. The lowest abdominal fat was obtained in the treatment in which soy oil and 125 mg/kg L-carnitine were combined. L-carnitine was also effective in reducing fat in thigh, breast and full carcass (Rajabzadeh-Nesvan et al., 2013).

The study of the effect of butyric acid and L-carnitine on carcass characteristics of broiler chickens showed that the interaction of treatments on abdominal fat percentage and carcass fat percentage was not significant. Application of 250 mg/kg L-carnitine in the diet improved the percentage of thebreast in chickens (Khatibjoo et al., 2016). The use of chitosan oligosaccharide and L-carnitine in broiler diets had no significant effect on carcass weight, nor on breast, wing and leg to carcass weight ratios. The percentage of ventricular fat in diets with chitosan oligosaccharide, L-carnitine and L-carnitine plus chitosan oligo-saccharide was less than in a control diet. The use of L-carnitine and L-carnitine + chitosan oligosaccharide reduced the weight of the liver relative to the control and chitosan oligosaccharide diets (Arslan and Tufan, 2018).

Application of different levels of fat and L-carnitine in broiler chicken diets increased breast meat and liver weight but decreased ventricular and fetal fat (Rezaei et al., 2007). Research results showed that application of 50 mg/kg L-carnitine in a diet containing various levels of crude protein significantly reduces fat in thigh muscles, the full carcass as well as abdominal fat (Darsi Arani et al., 2010). The addition of terbutaline and L-carnitine in broiler diet significantly increased the weights of heart, gizzards, spleen, and the bursa of fabricius, and reduced the amount of abdominal fat (Fallah et al., 2016).

Mehdizadeh Taklimi et al. (2015)reported that mean crude protein and carcass fat content was not affected by different levels of L-carnitine in the diet, but with the use of L-carnitine powder in the diet, the amount of carcass ash significantly decreased. Using L-carnitine, broiler chicken carcass fat decreased compared to control and treatment of vitamin E and ginger (Rehman et al., 2017). Khadem et al. (2006) reported that the use of L-carnitine induced a significant reduction in chicken abdominal fat. A similar reduction of abdominal fat by the use of L-carnitine in broiler diets was also reported by Parsaeimehr et al. (2014a).

Jafari Golrokh et al. (2016) showed that the use of L-carnitine and atorvastatin in the diet of broiler chickens improved the quality of carcasses by affecting the amount and distribution of muscle fats, carcass traits and blood parameters. In that study, L-carnitine at both levels of 150 and 300 mg/kg increased the weights of empty body and full carcass, breast, thigh, gizzard, liver and heart as compared to the control.

According to the above review, it can be concluded that L-carnitine is effective in reducing ventricular fat and fat in different carcass parts (thigh and breast) organs and significantly decreases ventricular fat in broiler chickens. But in most cases, there was no significant effect on weight and yield of internal organs and carcasses.

Table 2. Effect of L-carnitine on carcass characteristics of broiler chickens

Reference	Applied treatment	Effects
Murali et al., 2015	900 mg/kg	Positive effect on reducing abdominal fat and no effect on carcass weight and internal organs
Xu et al., 2003	25, 50 mg/kg	Reducing abdominal fat, improving the performance of leg and breast muscles
BabazadehAghdam et al., 2015	300 mg/kg	Reduced abdominal fat and no effect on carcass characteristics
Hrncar et al., 2015	1 mL/1.2 L	Positive effect on abdominal fat, weights of heart, liver and gizzard
Rezaei et al., 2007	250 mg/kg	No significant effect on carcass characteristics
Celik et al., 2003	50 mg/kg	No significant effect on carcass weight and weight of abdominal fat
Daskiran and Teeter, 2001	150 mg/kg	No significant effect on carcass traits and content of abdominal fat
Khajali and Khajali, 2014	200 mg/kg	No significant effect on carcass and breast, positive effect on abdominal fat
Parsaeimehr et al., 2014b	300 mg/kg	Increasing the relative weight of carcass, thigh and significant decrease of abdominal cavity fat, no effect on the weight of bursa of Fabricius, spleen, heart and liver
Shirali et al., 2015	50 , 100 mg/kg	No effect on the percentage of breast, thigh, liver, gizzard, pancreas and significant decrease of abdominal fat
Farrokhyan et al., 2011	150, 300 mg/kg L-carnitine + 2 g / kgGemfibrozil	Increased weights of carcass and internal organs
Rajabzadeh-Nesvan et al., 2013	125 mg/kg	No significant effect on the weight of internal organs and a positive effect on reducing carcass fat
Khatibjoo et al., 2016	250 mg/kg	Improving breast percentage and no significant effect on abdominal fat
Arslan and Tufan, 2018	100 mg/kg L-carnitine+ 100 mg/kgChitosan Oligosaccharide	Positive effect on carcass weight and reducing abdominal fat
Rezaei et al., 2007	250 mg/kg	Increasedbreast meat and liver weight and reduced abdominal fat and fat of breast meat
DarsiArani et al., 2010	50 mg/kg	Reduced-fat in thigh and carcass and also abdominal fat
Fallah et al., 2016	10 mg/kg + 100 mg/ kgterbutaline	Positive effect on increasing weights of heart, gizzard, spleen, bursa of Fabricius and reducing abdominal fat
MehdizadehTaklimi et al., 2015	400, 600, 800 mg/kg	Reduced level of carcass ash, no significant effect on mean crude protein orcarcass fat content
Rehman et al., 2017	500 mg/kg	Reduced abdominal fat
Khadem et al., 2006	50 mg/kg	Reduced abdominal fat
Parsaeimehr et al., 2014a	300 mg/kg	Reduced abdominal fat
JafariGolrokh et al., 2016	150, 200 mg/kg	Positive effect on improving carcass quality
Miah et al., 2004	50 mg/kg	Reduced abdominal fat
Kheirkhah et al., 2009	100, 200 mg/kg	No effect on carcass traits
Xu et al., 2003	50 , 75 mg/kg	Increased weight of breast muscle and reducedabdominal fat
Daskiran andTeeter, 2001	150 mg/kg	No effect on carcass yield and abdominal fat
Oladele et al., 2011	60 mg/kg	Increased carcass yield and reduced abdominal fat
Zhang et al., 2010	300, 600, 900 mg/kg	Reduced abdominal fat
Kheiri et al., 2011	60, 120 mg/kg	Reduced abdominal fat
Ardekani et al., 2012	50 mg/kg	No significant effect on abdominal fat
Celik and Ozturkcan, 2003	500 mg/kg	No effect on the weight of internal carcass organs

Effect of L-carnitine on the immunity of broiler chickens

The effect of L-carnitine on enzymatic activity in subcutaneous fat in male broiler chickens showed that hormone-sensitive lipase (HSL)-activity was higher at 50 and 75 mg/kg L-carnitine levels than at 0, 25 and 100 mg/kg L-Carnitine levels. The activity of lipoprotein lipase (LPL), glucose-6-phosphate dehydrogenase, malic dehydrogenase and iso-citrate dehydrogenase decreased with the use of L-carnitine. The activity of carnitine-palmityl transferase-I by increasing the level of carnitine in the diet decreased significantly (Xu et al., 2003).

The use of L-carnitine in broiler diets affects the function of the immune system. Mirzapor Sarab et al. (2016) found that the use of L-carnitine in broiler chicken diets does not affect antibody production against Newcastle and Sheep red blood cells (SRBC). Research results showed that heterophil to lymphocyte ratio in broiler chickens fed diets supplemented with L-carnitine had no significant difference compared to the control (Khajali and Khajali, 2014).

In a study, it was stated that the effect of different levels of vitamin E and L-carnitine was not significant on the relative weight of the immune organs (thymus, spleen, and bursa of fabricius). The application of 50 mg/kg of L-carnitine increased the antibody titer against SRBC during the initial response (Shirali et al., 2015). Akbari Azad et al. (2010) reported that the application of 375 mg/kg of L-carnitine increased the antibody titer against Newcastle disease and influenza. Also, this treatment had a positive effect on the improvement of the population of white and red blood cells, hemoglobin, hematocrit, and triglyceride in broiler chickens.

Research results of Khatibjoo et al. (2016) showed that the application of 0.025% L-carnitine and 1.5% garlic powder to broiler chicken diet did not affect cellular and humoral immunity but increased the immunity through blood cells and improved the body defense system in broiler chickens. Norreh et al. (2015) reported that the use of L-carnitine and butyric acid in the diet increased lymphocyte percentages. Also, the initial IgG titre (31 days) in response to the sheep's red blood cell was higher in the chicken fed a diet containing 125 mg/kg of L-carnitine compared to the control.

Jalali et al. (2015) found that the use of supplementation of L-carnitine in a diet of broiler chickens containing soybean oil would increase antibody titer against Newcastle virus. Researchers reported an increase of flu and Newcastle antibody titersin the 18th and 28th day of life using Terbutaline and L-carnitine in thediet of Ross 308 broiler chickens (Fallah et al., 2016). Rehman et al. (2017) investigated the effect of vitamin E (250 mg/kg), ginger (2 g/kg) and L-carnitine (500 mg/kg) on the antibody titer against infectious bursa IBD and Geometric mean titer(GMT) of broiler chickens under thermal stress. The results showed that all treatments increased titers on days 21, 28, 35, and 42. But vitamin E was more effective in these traits.

Parsaeimehr et al. (2014a) reported that the use of L-carnitine had a significant effect on Newcastle antibody titer at 32 days of age but had no effect on Newcastle-antibody titer at 42 days of age. Researchers investigating the effects of L-carnitine (0 and 200 mg/kg), coenzyme Q10 (0 and 40 mg/kg), and ractopamine (0 and 10 mg/kg) in a factorial experiment on chicken Ross 308 male broiler showed that the addition of coenzyme Q10 and L-carnitine in the diet of broiler chickens has a positive effect on immune response (Asadi et al., 2016).

The results of a research showed that using the mixture of L-carnitine, lysine and methionine in the basic diet of broilers did not have a significant effect on antibody titers against sheep red blood cells, bronchitis and weight of the bursa of fabricius and spleen, but increased antibody titers against Newcastle (Ghoreyshi et al., 2019).

In most studies, the use of L-carnitine in the diets of broiler chickens led to improvements in the immune system. In some studies, the effect of L-carnitine on control of the immune system in broiler chickens was not statistically significant. Overall, L-carnitine can be effective in improving immunity, increasing resistance to diseases and decreasing ascites in broiler chickens.

Table 3. Effect of L-carnitine on the immunity of broiler chickens

Reference	Applied treatment	Effects
Xu et al., 2003	25, 50, 75, 100 mg/kg	Increased activity of hormone-sensitive lipase (HSL), decreased Lipoprotein lipase (LPL) activity, glucose-6-phosphate dehydrogenase, malic hydrogenase, iso-citrate dehydrogenase and carnitine palmitoyltransferase I
MirzaporSarab et al., 2016	50, 100 mg/kg	No significant effect on the production of antibody against SRBC
Khajali and Khajali, 2014	200 mg/kg	No significant effect on heterophil to lymphocyte ratio
Shirali et al., 2015	50 mg/kg	Increased antibody titeragainst SRBC, no significant effect on the relative weight of immune organs (thymus, spleen and bursa of Fabricius).
Akbari Azad et al., 2010	375 mg/kg	Increasing antibody titers against Newcastle disease and influenza and positive effect on white and red blood cells, hemoglobin, hematocrit and triglyceride
Khatibjoo et al., 2016	0.025 %	No effect on cellular and humoral immunity and increased immunity through blood cells and improvement of the immune system.
Norreh et al., 2015	125 mg/kg	Increased percentage of lymphocytes and primary IgG titers
Jalali et al., 2015	120 mg/kg	Increased antibody titeragainst Newcastle virus
Fallah et al., 2016	100 mg/kg l-carnitine + 100 mg/kgterbutaline	Increase in antibody titers against Newcastle and Influenza viruses during the 18th and 28th day of life
Rehman et al., 2017	500 mg/kg	Increased antibody titers against IBD and GMT
Parsaeimehr et al., 2014a	300 mg/kg	Significant effect on Newcastle disease antibody titer at 32 days of age but no effect at 42 days
Asadi et al., 2016	200 mg/kg	Positive effect on the immune system
Azadmanesh and Jahanian, 2014	100 mg/kg	No effect on antibody titers against Newcastle disease and bronchitis
Kheirkhah et al., 2009	100, 200 mg/kg	No effect on the production of antibodies against SRBC
Golzaradabi et al., 2011	100 mg/kg	Increase in antibody titers against SRBC and Newcastle disease. Weight gain of thymus, spleen, and bursa of Fabricius
Deng et al., 2006	1000 mg/kg	Increased thymus weight, increased antibody level against SRBC

Effect of L-carnitine on blood parameters of broiler chickens

Xu et al. (2003) showed that the application of (0, 25, 50, 75 and 100 mg/kg) L-carnitine, especially 50 mg/kg, reduced serum triglyceride in broiler chickens. Levels of serum-free fatty acids increased significantly with the use of L-carnitine compared to the control. L-carnitine supplementation in the diet of broiler chickens containing 1, 3 and 5% fat significantly decreased triglyceride, cholesterol and VLDL in blood serum (Rezaei et al., 2007). In the study of Hassan et al. (2011), they found that elevated levels of L-carnitine had a greater effect on cholesterol levels. It is showed that triglyceride levels of chicken fed L-carnitine decreased compared to the control, but the concentration of cholesterol, phospholipids and serum lipoprotein was not affected by L-carnitine. The reduction in triglyceride content of blood serum with the use of L-carnitine was reported by Zhang et al. (2010) and the reason was increased catabolism of fatty acids by L-carnitine.

Tufarelli et al. (2020) stated that the use of L-carnitine along with 20% lysine-methionine reduced uric acid and increased total cholesterol in broilers. The effect of L-carnitine consumption had no significant effect on glucose and triglyceride levels.

The reduction of serum triglyceride and very low-density lipoproteins (VLDL) was reported by Xu et al. (2003).

Kamal et al. (2019) reported that L-carnitine does not have a significant effect on blood parameters and liver enzymes. The results of Islam and Ouda (2020) showed that by consuming L-carnitine in the diet of broilers, the total protein content increases and the amount of cholesterol, triglycerides, LDL and blood glucose decreases. Abouzed et al. (2019) stated that the consumption of L-carnitine in the drinking water of broilers does not have a significant effect on blood parameters and digestive enzymes.

Application of 200 mg/kg of L-carnitine in the approved Cobb 500 strain reduced the concentration of malondialdehyde in plasma and hematocrit (PCV) and increased plasma nitric oxide (Khajali and Khajali, 2014). The results showed that the application of 100 mg/kg L-carnitine reduced plasma malondialdehyde (MDA) levels in broiler chickens (Tan et al., 2008). In Yousefi et al. (2013), plasma MDA concentration also decreased significantly with the use of a diet containing 50 and 150 mg/kg of L-carnitine. Research results showed that chickens fed with L-carnitine had significantly lower red blood cells (RBC), hemoglobin (HGB) and hematocrit (HCT) at 42 days of age. L-carnitine also significantly reduced MDA levels from 21 to 35 days of age, and increased superoxide dismutase (SOD) and Gsh-Px activity at 21 to 42 days of age. The use of L-carnitine significantly reduced serum triglyceride, glucose, uric acid and a significant increase in total serum protein and globulin content was observed (Wang et al., 2013).

Zhang et al. (2010) also reported a significant decrease in serum cholesterol and triglyceride levels with increasing levels of L-carnitine in the diet. The use of L-carnitine reduced the level of triglyceride and increased the level of serum cholesterol (Kheiri et al., 2011). Parsaeimehr et al. (2013) investigated the use of L-carnitine and a diet containing animal and plant fats for broiler chickens Ross strain. The results showed that the use of L-carnitine had no effect on blood glucose and HDL however, it lowers cholesterol, triglycerides, LDL, and VLDL levels.

Parsaeimehr et al. (2014b) completed the base diet of Ross broiler chickens with 0, 150, 300, 450 and 600 mg/kg of L-carnitine and showed that use of L-carnitine in the diet reduced levels of triglycerides, cholesterol, LDL, VLDL, and elevated blood albumin and globulin. However, the effect of L-carnitine on the level of glucose, protein, and HDL was not significant. The use of L-carnitine on blood parameters other than the concentration of triglyceride was not significant in broiler chickens under thermal stress at 42 days of age (Shirali et al., 2015). Corduk et al. (2007) reported that the use of L-carnitine did not affect total serum cholesterol and triglyceride levels. In the study of Azadmanesh and Jahanian (2014), the use of carnitine increased triglyceride levels throughout the rearing period.

When gemfibrozil and L-carnitine were used in diets of broiler chickens, total cholesterol and serum triglyceride declined, although the differences were not statistically significant (Farrokhyan et al., 2014). Arsan and Tufan (2018) reported that the use of L-carnitine and chitosan oligo-saccharide had no significant effect on total cholesterol, triglyceride, HDL, LDL, VLDL, total protein and albumin concentration in serum of broiler chickens. The use of 300 mg/kg L-carnitine increased blood glucose levels in broiler chickens under thermal stress. However, L-carnitine supplementation at levels 0, 100, 200 and 300 mg/kg had no significant effect on cholesterol, albumin, protein and HDL in broiler chickens (Babazadeh Aghdam et al., 2015).

Research results of Taraz and Dastar (2008) showed that the use of L-carnitine supplementation in broiler diets had no significant effect on blood parameters such as cholesterol, total protein, albumin, triglyceride, glucose and blood uric acid. Usage of L-carnitine in herbal oil diet increased total protein, globulin, cholesterol, HDL, and LDL in blood serum of broiler chicken (Jalali et al., 2015). Fallah et al. (2016) revealed that broiler chickens Ross 308, fed with diet containing L-carnitine and terbutaline, had lower total cholesterol, HDL, and LDL than the control.

Corduk et al. (2007) reported that the use of complementary L-carnitine in broiler diets increased β-oxidation of fatty acids to produce adenosine triphosphate (ATP) energy and improved energy efficiency. Mehdizadeh Taklimi et al. (2015) showed that calcium and phosphorus levels of blood in broiler chickens were not affected by a diet containing L-carnitine.

The effect of a diet containing L-carnitine, ginger and vitamin E on serum paraoxonase activity and oxidative status indicated that all treatments increased serum paroxonizations and decreased total oxidant status on 21, 28, 35 and 42 days of age, in comparison with the control group. Reducing glucose and increasing total protein was also reported (Rehman et al., 2017). Murali et al. (2013) fed chickens 900 mg/kg of L-carnitine and found that the treatment significantly reduced cholesterol and serum LDL-cholesterol levels compared to the control. The effect of L-carnitine on the levels of triglycerides, HDL, and VLDL was not significant.

Abd El-Wahab et al. (2015) showed that the lowest levels of cholesterol were obtained in chickens fed lysine (15.5 g/kg) and methionine (5.8 g/kg) plus 350 mg/kg L-carnitine. In the study of Parsaeimehr et al. (2014b), the L-carnitine diet had no significant effect on blood protein.

Hosseintabar et al. (2015) examined the effects of different levels of L-carnitine, lysine and methionine on blood parameters of broiler chickens Ross 308. In that 3×3 factorial design study, three levels of L-carnitine (0, 75 and 150 mg/kg) and 3 levels of lysine and methionine (0, 15 and 30%) were used. The results showed that the diets supplemented with L-carnitine had a significant effect on uric acid, HDL, LDL, and total cholesterol. L-carnitine-, lysine- and methionine-fed chickens had the highest plasma uric acid and the lowest total cholesterol and LDL. Also, L-carnitine significantly reduced total cholesterol compared to control and lysine and methionine-fed chickens. Generally, the diet with 150 mg/L of carnitine and 15% of lysine and methionine was the best treatment to maintain the concentration of TC, LDL, and HDL. Jafari Golrokh et al. (2016) reported a decrease of cholesterol, triglycerides, LDL, alkaline phosphate (ALP) in chickens treated with L-carnitine.

From the above researches, it is concluded that positive and significant effects of L-carnitine on blood parameters of broiler chickens were evident. L-carnitine, as an edible anti-fat substance, reduced cholesterol, triglycerides, LDL, and VLDL, in most studies, and had a positive effect on blood parameters. In a few studies, the effect of L-carnitine on the improvement of blood parameters was not significantly different compared with the control. But in general, the effect of this supplement was positive in improving the blood parameters of broiler chickens.

Table 4. Effect of L-carnitine on blood parameters of broiler chickens

Reference	Applied treatment	Effects
Xu et al., 2003	25,50, 75, 100 mg/kg	Reduced serum triglyceride and increased free fatty acid levels
Rezaie et al., 2007	250 mg/kg	Reduced blood triglycerides, cholesterol, serum VLDL
Zhang et al., 2010	300, 600,900 mg/kg	Increased fatty acid catabolism and reduced serum triglyceride levels
Khajali and Khajali, 2014	200 mg/kg	Reduced malondialdehyde in plasma, reduced hematocrit and increased plasma nitric oxide
Tan et al., 2008	100 mg/kg	Reduced levels of plasma malondialdehyde
Yosefi et al., 2013	50, 150 mg/kg	Reduced levels of plasma malondialdehyde
Wang et al., 2013	100 mg/kg	Reduction of red blood cells, hemoglobin and hematocrit, malondialdehyde, serum triglyceride, glucose and uric acid, increase of activity of superoxide dismutase and GSH-PX and increased serum total protein
Zhang et al., 2010	300, 600, 900 mg/kg	Reduced triglycerides and cholesterol
Kheiri et al., 2011	60, 120 mg/kg	Reduced triglycerides and increased serum cholesterol levels
Parsaeimehr et al., 2013	150, 300, 450, 600 mg/kg	No significant effect on blood glucose, HDL, and a significant reduction in cholesterol, triglyceride, LDL and VLDL levels.
Parsaeimehr et al., 2014b	300 mg/kg	Reduced triglyceride, cholesterol, LDL and VLDL, increased albumin and globulin levels

Shirali et al., 2015	150, 300, 450, 600mg/kg	Significant and positive effects on blood triglyceride concentration
Corduk et al., 2007	100 mg/kg	No significant effect on total serum cholesterol and triglyceride levels
Azadmanesh and Jahanian, 2014	100 mg/kg	Increased triglyceride levels throughout the course
Farrokhyan et al., 2014	150, 300 mg/kgL- carnitine + 1, 2 mg/kg gemfibrozil	Reduced total serum cholesterol and triglyceride
Arslan and Tufan, 2018	100 mg/kgL- carnitine	No significant effect on total cholesterol, triglycerides, HDL, LDL, VLDL, total protein and albumin concentration in serum
BabazadehAghdam et al., 2015	100, 200, 300 mg/kg	Increased blood glucose but no significant effect on cholesterol, albumin, protein and HDL
Jalali et al., 2015	120 mg/kg	Increased total serum protein, globulin, cholesterol, HDL and LDL
Fallah et al., 2016	100 mg/kg L- carnitine plus 10 mg/kg terbutaline	Reduced total cholesterol, HDL, and LDL
Corduk et al., 2007	100 mg/kg	Increased oxidation of fatty acids and increased energy production
MehdizadehTaklimi et al., 2015	400, 600, 800 mg/kg	No significant effect on calcium and phosphorus levels
Rehman et al., 2017	500 mg/kg	Increased serum and total protein paroxonization activity, decreased total oxidant and glucose status
Murali et al., 2013	900 mg/kg	Decreased cholesterol, LDL levels and no significant effect on triglyceride, HDL and VLDL levels
Abd El Wahab et al., 2015	350 mg/kg	Reduce cholesterol levels
Parsaeimehr et al., 2014a	300 mg/kg	No significant effect on blood protein
Hosseintabar et al., 2015	75, 150 mg/kg	Positive effects on uric acid, HDL, LDL, and total cholesterol
JafariGolrokh et al., 2016	150, 300 mg/kg	Reduced cholesterol, triglycerides, LDL, and alkaline phosphate
Kheirkhah et al., 2009	100 , 200 mg/kg	No effect on the concentration of cholesterol or blood triglycerides
Taraz and Dastar, 2008	125, 250 mg/kg	No effect on blood parameters
Murali et al., 2015	900 mg/kg	Increased metabolism and facilitatedthe oxidation of fatty acids

Effect of L-carnitine on the microbial flora of broiler chickens

Hosseintabar et al. (2013) investigated the effect of L-carnitine, methionine-lysine on themicrobial flora of broiler cecum. In that study, L-carnitine (0, 75 and 150 mg/kg), methionine-lysine (0, 15 and 30%) were used at three levels. The results showed that there is a significant difference between the levels of L-carnitine and methionine-lysine in the total population of aerobic bacteria, producing lactic acid bacteria, *Escherichia coli* and lactobacilli at a probability level of 5%.

Table 5. Effect of L-carnitine on the microbial flora of broiler chickens

Reference	Applied treatment	Effects
Hosseintabar et al., 2013	75 , 150 mg/kg	Positive effect in reducing the total population of aerobic bacteria, producing lactic acid bacteria, Escherichia coli and lactobacillus

Effect of L-carnitine on the fatty acid profile and meat characteristics of broiler chickens

Abd El-Wahab et al. (2015) used two levels of L-carnitine (175 and 350 mg/kg) in the diet of broiler chickens containing methionine (6.5 and 8.5 g/kg) and lysine (13.5 and 15.5g/kg). The results of that study showed that L-carnitine significantly reduced lipid profiles in all 2 levels.

The effect of butyric acid (0 and 2 g) and L-carnitine (0, 125 and 250 mg) on broiler chicken meat color showed that statistically there was no significant difference between the treatments. However, L-carnitine at 125 milligrams level had the highest brightness and redness of breast meat among treatments. The effect of L-carnitine and butyric acid on the characteristics of thigh meat (yellowness, redness and brightness) was not significant. However, the chickens fed a diet containing 250 mg of L-carnitine had darker thigh meat compared to the other treatments. In that study, the chickens fed a diet containing 125 mg/kg of L-carnitine had thigh meat with higher pH of. The maximum pH of the breast meat was 250 mg/kg of L-carnitine plus 2 g/kg of butyric acid. The researchers linked this study of the effect of L-carnitine on the brightness of meat color as a consequence of that material in preventing the oxidation of muscle myoglobulin (Khatibjoo et al., 2016).

The results of Zhang et al. (2010) showed that the effect of L-carnitine (0, 300, 600 and 900 mg/kg) on pH of breast meat, colour brightness of breast and thigh meat, was not statistically significant. However, the effect of treatments on pH of thigh meat showed that using) L-carnitine, increased pH of the thigh, redness of breast and thigh meat. The yellowness of thigh and breast meat decreased with L-carnitine usage compared to the control. Breast and thigh shear forces declined by using high levels of L-carnitine (600 and 900 milligrams). Corduk et al. (2007) showed that the use of L-carnitine (0 and 100 mg/kg) did not affect meat quality in broiler chickens.

Table 6. Effect of L-carnitine on the fatty acid profile of broiler chickens

Reference	Applied treatment	Effects
Abd El Wahab et al., 2015	175, 350 mg/kg	Reduced lipid profile

Table 7. Effect of L-carnitine on the characteristics of broiler chicken meat

Reference	Applied treatment	Effects
Khatibjoo et al., 2016	250 mg/kg	More darkness of thigh meat
Khatibjoo et al., 2016	125 mg/kg	Increased pH of thigh meat and brightness and redness of breast meat
Khatibjoo et al., 2016	250 mg/kg+ 2 g/ kg butyric acid	Increased pH of breast meat
Zhang et al., 2010	300, 600, 900 mg/kg	Increased pH of the thigh, redness of breast and thigh meat
Corduk et al., 2007	100 mg/kg	No significant effect on meat quality

CONCLUSION

L-carnitine, as a nutritional substance was found effective in reducing fat content in the body of broiler chickens leading to resulting in the production of low-fat and healthier chickens without affecting the performance and qualitative characteristics of broiler chickens. Therefore, L-carnitine can be used at levels of 125-300 mg/L as an effective nutritional supplement to resolve the problem of fat accumulation as well as maintaining the health of chickens. Although higher levels of L-carnitine were also found effective, economic considerations, and cost relative to benefit balance, the dose 300 mg/L is the one to be recommended.

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CONFLICT OF INTEREST

None declared by the authors.

REFERENCES

Abd El-Wahab A, Aziza A, El-Adl M (2015) Impact of dietary excess methionine and lysine with or without addition of L-carnitine on performance, blood lipid profile and litter quality in broilers. Journal of Animal and Veterinary Advances. 10 (5): 191-202.

Abedpour A, Jalali SMA, Kheiri F (2017). Effect of vegetable oil source and L-carnitine supplements on growth performance, carcass characteristics and blood biochemical parameters of Japanese quails (*Coturnix japonica*). Iranian Journal of Applied Animal Science. 7(1): 147-153.

Abouzed TK, Dorghamm DA, Kahilo KA, Elkattawy AM, Nassef E, El-Sawy HB (2019) Impact of l-carnitine supplementation on growth of broiler chicken through determination of changes in the expression of CAT2, MYOD and MYF5 genes. Veterinary Research. 56 (Suppl 22): 665-672.

Akbariazad G, Haghighi-Khoshkhoo P, Ila N, Moayer F, Dehghan- Nayeri H (2010) The effects of dietary L - carnitine supplementation on overall performance, carcass traits, blood components and immune response in broiler chickens. Journal of Veterinary Clinical Research. 1(1): 7-17. (Persian).

Ardekani HM, Shevazad M, Chamani M, Aminafshar M, Arani ED(2012) The effect of L-carnitine and low crude protein supplemented with crystalline essential amino acids diets on broiler chickens. Annals of Biological Research. 3(2): 1085-1093.

Arslan C, Tufan T (2018) Effects of chitosan oligosaccharides and L-carnitine individually or concurrent supplementation for diets on growth performance, carcass traits and serum composition of broiler chickens. Revue de Médecine Vétérinaire. 169: 130-137.

Asadi H, Sadeghi AA, Eila N, Aminafshar M (2016) Carcass traits and immune response of broiler chickens fed dietary L-carnitine, coenzyme Q10 and ractopamine. Brazilian Journal of Poultry Science. 18 (4): 677-682.

Azadmanesh V, Jahanian R (2014) Effect of supplemental lipotropic factors on performance, immune responses, serum metabolites and liver health inbroiler chicks fed on high-energy diets. Animal Feed Science and Technology. 195(1):92-100.

BabazadehAghdam A, Ghazi HarsiniSh, Daneshyar M (2015) The effect of different levels of L-carnitine on performance, blood parameters and carcass characteristics of broiler chickens fed with high fat diets under heat stress condition. Journal of Veterinary Research. 70(3): 341-348. (Persian).

Buyse J, Quirine Swennen TA, Niewold KC, Klasing GPJ, Janssens M, Baumgartner BMG (2007) Dietary L-carnitine supplementation enhances thelipopolysaccharide-induced acute phase protein response in broiler chickens. Veterinary Immunology and Immunopathology. 118: 154-159.

Cakir S, Yalcin S (2007) Effects of L-carnitine supplementation in diets with low energy level on growth performance and carcass traits in broilers. Review of Veterinary Medicine. 158: 291-296.

Celik L, Ozturkcan O (2003) Effects of dietary supplemental L-carnitine and ascorbic acid on performance, carcass composition and plasma L-carnitine concentration of broiler chicks reared under different temperature. Archives of Animal Nutrition. 57:27-38.

Celik L, Ozturkcan O, Inal TC, Canacankatan N, Kayrin L (2003) Effects of L-carnitine and niacin supplied by drinking water on fattening performance, carcass quality and plasma L-carnitine concentration of broiler chicks. Archives of Animal Nutrition. 57: 127-136.

Corduk M, Ceylan N, Ildiz F (2007) Effects of dietary energy density and L-carnitine supplementation on growth performance, carcass traits and blood parameters of broiler chickens. South African Journal of Animal Science. 37(2): 65-73.

DarsiArani E, Shivazad M, Zaghari M, Namroiud NF (2010) Effect of L-carnitine on growth performance andcarcass composition of broiler chicks fed low crude protein diet. Iran. Iranian Journal of Animal Science. 41(2): 153-161. (Persian).

Daskiran M, Tetter RG (2001) Effect of dietary L-carnitine (carnicking) supplementation on overall performance and carcass characteristics of seven week-old broiler chickens. Animal Science Research. 1:1-5.

Deng K, Wong CW, Nolan JV (2006) Long-term effects of early-life dietary L-carnitine on lymphoid organs and immune responses in Leghorn-type chickens. Journal of Animal Physiology and Animal Nutrition. 90: 81-86.

Fallah R, Nazari P, Mirzaei E (2016) Effect of different levels of terbutaline and L-carnitine on the growth performance, carcass traits, blood parameters and immune system of broiler chickens. Malaysian Society of Animal Production. 19(2): 57-69.

Farrokhyan P, Bouyeh M, Lartey FM, Seidavi A (2014) The effects of dietary L-carnitine and gemfibrozil on performance, carcass characteristics, cholesterol and triglycerides in broiler chicks. Avian Biology Research. 7(3): 160-166.

Flanagan JL, Simmons PA, Vehige J, Willcox MD, Garrett Q (2010) Role of carnitine in disease. Nutrition and Metabolism. 7: 30-37.

Fujimoto H, Matsumoto K, Koseki M, Yamashiro H, Yamada T, Takada R (2020) Effects of rice feeding and carnitine addition on growth performance and mRNA expression of protein metabolism related genes in broiler grower chicks. Animal Science Journal. 91(1): e13390

Ghoreyshi SM, OmriB, Chalghoumi R, Bouyeh M, Seidavi A, Dadashbeiki M, Lucarini M, Durazzo A, Hoven RVD, Santini A (2019) Effects of dietary supplementation of l-carnitine and excess lysine-methionine on growth performance, carcass characteristics, and immunity markers of broiler chicken. Animals. 9(6): 362.

Golzar Adabi S, Cooper RG, Ceylan N, Corduk M (2011) L-carnitine and its functional effects in poultry nutrition. World's Poultry Science Journal. 67: 277-296.

Hassan MSH, Youssef SF, El-Bahy NM (2011) Effects of L-carnitine and ascorbic acid supplementation on productive, reproductive, physiological and immunological performance of golden montazah laying hens. Egyptian Poultry Science. 31(2) :557-578.

Hosseintabar B, Dadashbeiki M, Bouyeh M, Seidavi A (2013) Is the amount of L-carnitine and methionine-lysine effect on the microbial flora of broiler cecum? Journal of Pure and Applied Microbiology. 8(1): 353-360.

Hosseintabar B, Dadashbeiki M, Bouyeh M, Seidavi A, Van Den Hoven R, Gamboa S (2015) Effect of different levels of L-carnitine and lysine-methionine on broiler blood parameters. Revista MVZ Córdoba. 20 (3): 4698-4708.

Hrncar C, Verguliaková S, Svorad P, Weis J, Arpášová H, Mindek S, Fik M, Bujko J (2015) Effect of L-carnitine supplementation on fattening and carcass parameters of broiler chickens. Acta Fytotechn Zootechn. 18 (1): 15-19.

Iben C, Meinhart S, Leibetseder J (1991) Carnitine in broilers. Part 1 Effects of carnitine precursors. Wiener Tierarztliche Monatsschrift. 78: 403-407.

Ismail EI, Ouda MM (2020) Impact of dietary L-carnitine as a feed additive on performance, carcass characteristics and blood biochemical measurements of broiler. Journal of Animal and Poultry Production. 11(2): 21-25.

Jafari Golrokh AR, Bouyeh M, Seidavi AR, Van Den Hoven R, Laudadio V, Tufarelli V (2016) Effect of different dietary levels of atorvastatin and L-carnitine on performance, carcass characteristics and plasma constitutes of broiler chickens. Journal of Poultry Science 53(3): 201-207.

Jalali SMA, Rabiei R, Kheiri F (2015) Effects of dietary soybean and sunflower oils with and without L-carnitine supplementation on growth performance and blood biochemical parameters of broiler

chicks. Archives Animal Breeding. 58: 387-394.

Kamal T, Dorghamm D, Kahilo K, ElkattawyA, Nassef E, El-sawyH (2019) Impact of l-carnitine supplementation on growth of broiler chicken through determination of changes in the expression of CAT2, MYOD and MYF5 genes. Slovenian Veterinary Research. 56: 665-672.

Khadem AA, Nouri-Emamzadeh A, Sami A (2006) The effects of quantitative feed restriction and the protein and L-carnitine density of diets on the performance of broiler chickens. Pakistan Journal of Biological Sciences. 9(8): 1509-1515.

Khajali F, Khajali Z (2014) L-carnitine supplementation decreases lipid peroxidation and improves cardiopulmonary function in broiler chickens reared at high altitude. Acta Veterinaria Hungarica. 62(4): 489-499.

Khajali F, Liyanage R, Wideman RF (2011) Methylglyoxal and pulmonaryhypertension in broiler chickens. Poultry Science. 90: 1284-1296.

Khatibjoo A, Poormalekshahi AA, Fattahnia F, Jaefai H, Aelaei M (2016) Effects of supplementation time of L-carnitine and garlic powder on performance and carcass characteristics of broiler chickens. Iranian Journal of Animal Science Research. 8(1): 132-140. (Persian).

Kheiri F, Pourreza J, Ebrahimnezhad Y, Nazeradl K, Jalali Haji-Abadi SMA (2011) Effects of supplemental ractopamine and L-carnitine on growth performance, blood biochemical parameters and carcass traits of male broiler chicks. African Journal of Biotechnology. 10(68): 15450-15455.

Kheirkhah AR, Rahimi Sh, Karimi Torshizi MA, Malekmohamadi H (2009) Effect of different levels of L-carnitine supplementation in broiler breeders and their progeny's diets on performance, blood factors, carcass characteristics and immune system of broilers. Journal of Veterinary Research. 64(4): 283-289. (Persian).

Kidd MT, Gilbert J, Corzo A, Page C, Virden WS, Woodworth JC (2009) Dietary L-carnitine influences broiler thigh yield. Asian-Australasian Journal of Animal Sciences. 22(5): 681-685.

Mc Dowell LR (1989) Vitamins in animal nutrition. Ed. Tony J. Cunha, Chapter 16, pp: 393-394, Academic Press, Inc., 1250 Sixth Avenue, San Diego, California.

Mcdowell LR (2000)Vitamins in animal and human nutrition. Second edition, Iowa State University Press, Ames, Iowa, 794p.

MckeeJs, Harrison PC (1995) Effects of supplemental ascorbic acid on the performance of broiler chickens exposed to multiple concurrent stressors. Poultry Science. 74(11): 1772-1785.

MehdizadehTaklimi SM, Ghazvinian K, Ahmadi Kasgari MR (2015) Effect of L-carnitine on performance and carcass quality of broiler chickens. Academia Journal of Scientific Research. 3(3): 050-054.

Miah MY, Rahman MS, Islam MK, Monir MM (2004) Effects of saponin and L-carnitine on the performance and reproductive fitness of male broiler. International Journal of Poultry Science. 3(8): 530-533.

Mingrone G, Greco AV, Capristo E, Benedetti G, Giancaterini A, Gaetano A, De Gasbarrini G (1999) L-carnitine improves glucose disposal in type 2 diabetic patients. Journal of the American College of Nutrition. 18(1): 77-82.

Mirzapor Sarab S, Salari S, MirzadehKh, Aghaei A (2016) Effect of different levels of vitamin C and L-carnitine on performance and some blood and immune parameters of broilers under heat stress. Iranian Journal of Animal Science Research. 8(1):141-153. (Persian).

Murali P, George Sherin K, Mercy AD, Dipu MT, Narayanankutty K (2013) Effects of L-carnitine supplementation on serum lipid profile of broiler chicken fed with animal fat. International Journal of Agriculture and Food Science Technology. 4(8): 773-774.

Murali P, George SK, Ally K, Dipu MT, Dominic G (2015) Effect of dietary L-carnitine supplementation with animal fat on carcass characteristics of broiler chicken. Journal of Animal Research. 5(4): 713-717.

Norreh Z, Khatibjoo A, Fattahnia F, Akbari-Gharaei M (2015) Investigation of performance and immune response of broiler chickens fed diet containing butyric acid and L-carnitine supplement. Journal of Animal Production. 17(2): 269-279. (Persian).

Nouboukpo KE, Tona K, Kamers B, Everaert N, Willemsen H, Onagbesan OM, Gbeassor M, Decuypere E (2010) Effects of in ovo administration of L-carnitine on hatching events and juvenile performance of layer-type chick. International Journal of Poultry Science. 9: 980-983.

Novotny BJ (1998) L-carnitine. What difference does it make? Symposium Proceedings, Leuven, Belgium 15th December 1998.

Oladele OA, Adeboye F, Richard S, Zainob H (2011) Growth response, carcass yield and serum biochemistry of broiler chicken feds with supplemental L-carnitne in feed or drinking water. Poultry Science. 48: 223-228.

Parsaeimehr K, Afrouziyeh M, Hoseinzadeh S (2014a). The effects of L-carnitine and different levels of animal fat on performance, carcass characteristics, some blood parameters and immune response in broiler chicks. Iranian Journal of Applied Animal Science. 4(3): 561-566.

Parsaeimehr K, Farhoomand P, Afrouziyeh M, Cheraghi H, Hoseinzadeh S (2014b) The effects of different levels of L-carnitine on performance, carcass characteristics and some blood parameters of broiler chickens. Animal Science Researches. 24(3): 43-51. (Persian).

Parsaeimehr Kh, Farhoomand P, Afrouziyeh M, Najafi R, Ahmadie Naghdehi AA (2013) Effects of L-carnitine with different dietary fat sources on performance and some blood metabolites of broiler chicken. Animal Production Research. 1(4): 27- 34. (Persian).

Rajabzadeh Nesvan M, Rezaee M, Ansari Pirsaraee Z (2013) Effect of L-carnitine supplementation to finisher diets with different sources of fat on the performance, carcass characteristics and body composition in broiler chickens. Journal of Management System. 92(2): 51-68. (Persian).

Rajasekar P, Anuradha CV (2007) Fructose-induced hepatic gluconeogenesis: Effect of L-carnitine. Life Sciences. 80(13): 1176-1183.

Rebouche CJ (1992) Carnitine function and requirements during the life cycle. The Faseb Journa. l6(15): 3379-3386.

Rehman Z, Chand N, Khan RU (2017) The effect of vitamin E, L-carnitine, and ginger on production traits, immune response, and antioxidant status in two broiler strains exposed to chronic heat stress. Environmental Science and Pollution Research. 24: 26851-26857.

Rezaei M, Attar A, Ghodratnama A, Kermanshahi H (2007) Study the effects of different levels of fat and L-carnitine on performance and carcass characteristics of broiler chicks. Pakistan Journal of Biological Sciences 10(12):1970-1976.

Sayed AN, Shoeib HK, Abdel-Raheem HA (2001) Effect of dietary L-carnitine on the performance of broiler chickens fed on different levels of fat. Assiut Veterinary Medical Journal. 45: 37-47.

Shirali MA, Salari S, TabatabayiVakili S, Sari M, Jahanian R (2015) Effect of vitamin E and L-carnitine on growth performance, blood parameters and immune response of broiler chickens under heat stress. Animal Science Journal of Pajouhesh va Sazandegi. 110:115-128. (Persian).

Swarna CL, Rao SV, Srinivas G, Reddy VR (2018). Effect of Unsaturated to Saturated Fatty Acids Ratio of Supplemental Fat in the Diet with or without L-Carnitine on Performance of Broiler Chicken. Indian Journal of Animal Nutrition. 35(1): 90-96.

Tan X, Hu SH, Wang XL (2008) The effect of dietary L-carnitine supplementation on pulmonary hypertension syndrome mortality in broilers exposed to low temperatures. Animal Physiology and Animal Nutrition. 92(2): 203-210.

Taraz Z, Dastar B (2008) Effects of L-carnitine supplementation in diets with different levels of protein on performance and blood parameters in broiler chickens. Journal of Agricultural Sciences and Natural Resources15(5): 114-122. (Persian).

Tufarelli V, Mehrzad-Gilmalek H, Bouyeh M, Qotbi A, Amouei H, SeidaviA, Paz E, Laudadio V (2020) Effect of different levels of L-carnitine and excess, lysine-methionine on broiler performance, carcass characteristics, blood constituents, immunity and triiodothyronine hormone. Agriculture 10(4): 138-146.

Wang YW, Ning D, Peng YZ, Guo YM (2013) Effects of dietary L-carnitine supplementation on growth performance, organ weight, biochemical parameters and ascites susceptibility in broilers reared under low-temperature environment. Asian-Australasian Journal of Animal Sciences. 26(2): 233-240.

Xu ZR, Wang MQ, Mao HX, Zhan XA, Hu CH (2003) Effect of L-carnitine on growth performance, carcass composition and metabolism of lipids in male broiler. Poultry Science. 82: 408-413.

Xue YZ, Wang LX, Liu HZ, Qi XW, Wang XH, Ren HZ (2007) L-carnitineas an adjunct therapy to percutaneous coronary intervention for non-STelevation myocardial infarction. Cardiovasc. Drugs Therapy. 21: 445-448.

Yousefi A, Khajali F, Hassanpour H, Andkhajali Z (2013) Dietary L-carnitine improves pulmonary hypertensive response in broiler chickens subjected to hypobaric hypoxia. Asian-Australasian Journal of Animal Sciences. 50: 143-149.

Zhang YQ, Bai MX, Zhao L, Wang Q, Andji C (2010) Effects of dietary acetyl- L-carnitine on meat quality and lipid metabolism in arbor acres broilers. Asian-Australian Journal of Animal Science. 23:1639-1644.

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Review article
Ανασκόπηση

Update on Tail biting in pigs: An undesirable damaging behavior

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ABSTRACT: Tail-biting is an abnormal behavior of multifactorial origin, that consists a major problem in modern pig industry. It has a serious impact on both welfare and health status of the pigs involved, as well as on economic profitability of the farm. It is considered to be a problem of pig adaptation in poor environment triggered by a plethora of external and internal risk factors interacting with each other. A great variation exists on prevalence of tail biting between different studies across the world. Tail docking is the common practice applied by farmers to prevent this behavior, while treatments are based on enrichment material provision. The aim of this review is to explore the most recent literature on risk factors and impacts of tail biting and to discuss promising areas on early prediction and treatment of the topic.

Keywords: tail biting, pig health, pig welfare, pig behaviour

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INTRODUCTION

Tail biting is an abnormal behavior frequently occurring in pigs compromising welfare, health and economic gain in pig industry (Harley et al., 2014; Larsen et al., 2019; Niemi et al., 2012). It had been reported in pig farms since the 1950's (Schrøder-Petersen and Simonsen, 2001). However, it is generally accepted that this abnormality became more severe with the development of more intensive production systems (Edwards, 2006). Although this type of behavior is reported in conventional and organic free-range production systems (Kongsted and Sørensen, 2017), surprisingly, it is not documented in other non-domesticated Suids or Peccaries kept in captivity or found in wild life (Taylor et al., 2010).

The prevalence of severe tail biting can be as high as 3.1% (Van Staaveren et al., 2017b) while it may reach up to 72.0% for mild lesions (Teixeira et al., 2016). In general, abattoir data for tail biting cannot easily help to identify its prevalence, as a standard tail damage scoring system is lacking (Harley et al., 2014; Keeling et al., 2012) and particularly a system associating meat inspection with welfare (Harley et al., 2012a; Vom Brocke et al., 2019).

This pattern of behavior may cause acute pain in bitten pigs but also results in long-term consequences such as secondary infections (Valros and Heinonen, 2015) leading to carcass abscesses (Heinonen et al., 2010), respiratory organ inflammation (Munsterhjelm et al., 2013) and lung pathologies (Kritas and Morrison, 2007). Tail biting consists not only a welfare problem for pigs receiving this behavior, due to the pain caused, but is also an indication of decreased welfare in pigs performing it, since the inadequacies in their environment make them feel discomfort and bite (Thodberg et al., 2018; Valros et al., 2015, 2004).

Although poor health status is considered a high-risk factor for tail biting (European Food Security Authority (EFSA), 2007), suboptimal health has not received adequate attention so far (D'Eath et al., 2014; Valros and Heinonen, 2015). More research is needed on the possible correlations between pig health and the expression of tail biting behavior (Munsterhjelm et al., 2016; van Staaveren et al., 2016; Naya et al., 2019a). Moreover, due to secondary infections, carcasses from tail bitten pigs are trimmed or condemned in abattoirs (Kritas and Morrison, 2007) reflecting a direct economic loss of 1.10 Euro per pig (Harley et al., 2014) while this amount is even higher if we consider the indirect losses due to lower average daily gain (ADG) that can come up to 3% (Sinisalo et al., 2012).

Tail biting is known to be a multifactorial syndrome which is related to internal risk factors influenced by a great variety of external factors (Schrøder-Petersen and Simonsen, 2001). Although a lot of risk factors, especially environmental, have been identified the exact cause remains unknown (Sutherland and Tucker, 2011). As a consequence, tail biting outbreaks are difficult to be predicted and even more challenging to understand their cause as several environmental and husbandry factors associated with this behavior are likely to be unknown at their exact magnitude. Even under one roof where the same managerial practices are implemented, tail biting is sporadic (Edwards, 2006; Sutherland and Tucker, 2011). Furthermore, due to its multifactorial origin, this behavior is rare not to be present in commercial farms (Thodberg et al., 2018) leading farmers to routinely tail dock under fear of economic losses and fear of losing control of the situation (D'Eath et al., 2016). The existing percentages of tail docking over 95% in Europe (EFSA, 2007) indicates that 25 years after the first EU legislation forbidding the procedure on routine basis, there is an information gap in alternative steps that farmers could adopt (D'Eath et al., 2016) while the insufficiencies of current control methods is proven by high prevalence's of tail-docking (Harley et al., 2012b). European Commission audits for tail docking in Germany and Denmark, both being the biggest producers of pork in EU, report over 95% of pigs in Germany and over 98.5% in Denmark are tail docked (EU Commission, 2018). As no experimental curative treatment has stopped 100% of tail biting outbreak (Chou et al., 2019b; Zonderland et al., 2008), farmers could benefit from research in the field of automated monitoring of pigs' behavior in order to predict such outbreak (Niemi et al., 2012; Zonderland et al., 2016).

On the other hand, animal welfare is related to ethics and also a point of concern for the public and a part of EU values (European Commission, 2012). Consequently, this is an important political issue both at National and European level (Harley et al., 2014). Moreover, successful implementation is conditioned by knowing farmers perceptions (Kakanis et al., 2019) and consumer willingness to pay a price premium for better animal welfare (Niemi et al., 2012; D'Eath et al., 2016).

As a lot of research on tail biting is conducted throughout Europe, this review aims at presenting the

main outcomes based on current literature, to pinpoint the possible research gaps and highlight the potential pathways for new research needed.

PREVALENCE

The percentage of tail bitten pigs in a given population is used as the prevalence of tail biting in most studies although time should be also incorporated (Taylor et al., 2010). There seems to be an underestimation of tail biting lesion prevalence in the official statistics as the Official Veterinarians (OVs) record only the severe cases (Keeling et al., 2012; Wallgren et al., 2019c) and data should be used cautiously in order to evaluate the tail biting incidence in farm (Lahrmann et al., 2017). Underestimation could also be attributed to the high-speed lines of abattoirs (Correia-Gomes et al., 2017). On the other side, Wallgren et al. (2019c) support that in countries where farmers keep undocked pigs there could be an overestimation of tail damage in abattoirs due to other reasons, like tail necrosis due to toxins in straw. Tail appearance is not always the best way to quantify tail biting activity as under intact tails severe histopathological reactions may be found (Simonsen et al., 1991; Munsterhjelm et al., 2013). Moreover, the standing point of the observer in the abattoir plays a role in this discrepancy between studies, as some do the scoring before scalding and dehairing (Keeling et al., 2012) while others do it after them for better assessment of the minor lesions (Harley et al., 2014) or of every score of severity (Carroll et al., 2015). The variation in abattoir studies is further amplified by different scoring systems (van Staaveren et al., 2016), ways of record keeping (Harley et al., 2012b) and differences in estimation between individuals (Keeling et al., 2012).

Tail biting prevalence based on abattoir data, can't give an exact representation of the problem on farm as it misses out those tail bitten pigs that die due to severe lesions or euthanasia and lesions that get healed (Marques et al., 2012; Harley et al., 2012b; Lahrmann et al., 2017). Moreover, docking practices could differentiate the prevalence, as pigs raised under welfare schemes have more odds than conventional farmed pigs for tail lesions, probably due to being undocked (Kongsted and Sørensen, 2017; Alban et al., 2015). Also, increase of on farm prevalence could be expected in countries where enrichment material is not a prerequisite by legislation, as in USA (Li et. al., 2017). In a registered based study performed by Fertner et al. (2017), it was pinpointed that relatively low prevalence in tail biting lesions could be attributed to measures taken for the prevention of tail biting. In addition, variation of prevalence between farms (Becskei et al., 2018) is attributed to some producers being more capable of keeping pigs under optimum conditions thus avoiding tail biting outbreaks (Van Staaveren et al., 2016). This variation indicates the importance of farm related risk factors (Valros et al., 2004) and emphasize the different conditions that exist in pig farms around Europe (Wallgren et al., 2019b).

Overall, tail lesion prevalence may vary greatly between farms (Fertner et al., 2017; Van Staaveren et al., 2017a) abattoirs (Keeling et al., 2012) and regions (Harley et al., 2012b). As the prevalence of this behavior is expected to increase if the trend towards less docking continues in E.U. (Keeling et al., 2012; D 'Eath et al., 2016), it is important to have a clear definition and standard record practices in order to have reliable data (Harley et al., 2012b). A summary of the most recent studies data is shown in Table 1.

It is evident that the lack of a clear definition of tail biting scoring system makes difficult the comparison of the studies run among different countries. Tail biting research could benefit from incorporating scoring in official veterinary duties, and thus an increased volume of data to become available. This will also allow correlations between different welfare indicators in slaughterhouse (e.g. hind-limb bursitis) to be made, resulting in the establishment of a welfare indicator system applied among all countries.

Table 1. Tail biting prevalence between different studies

COUNTRY	DATE	Number of pigs	PLACE	DOCKED		UNDOCKED		Reference
				MILD*	SEVERE**	MILD*	SEVERE**	
FINLAND	2000	10852	ABBATOIR			34.60%	1.30%	Valros et al., 2004
USA	2002	1895	FARM	16.30%	9.70%			Kritas & Morrison, 2004
SWEDEN	2003	15068	ABBATOIR			7.00%	1.5%-1.9%	Keeling et al., 2012
IRELAND	2010	35288	ABBATOIR	58.10%	1.03%			Harley et al. 2012b
IRELAND	2012	3422	ABBATOIR	72.50%	2.50%			Harley et al. 2014
IRELAND	2014	3889	ABBATOIR	30.80%	1.60%			Carrol et al. 2015
ITALY	2014	128/320	FARM				3.60%	Martino et al., 2015
DENMARK	2015	472	FARM	2.20%	0.00%	3.30%	2.40%	Paoli et al.,2016
IRELAND	2012	3143	ABBATOIR	72.00%	2.30%			Teixeira et al., 2016
IRELAND	2015	4491	ABBATOIR	7.30%	2.40%			Staaveren et al., 2016
IRELAND	2014	13133	ABBATOIR	25.20%	3.10%			Staaveren et al., 2017
DENMARK	2016	1624	FARM	49.00%				Larsen et al., 2017
DENMARK	2015	962/960	FARM	0.00%	0.00%	23.00%		Larhmann et al., 2017
USA	2016	120/120	FARM	42.50%	5.00%	59.20%	30.00%	Li et al., 2017
GREECE	2017	461	ABBATOIR	46.42%***				Becskei Zolt, 2017

5 scale climax: 0=No evidence of tail biting 1= Healed of mild lesions 2=Evidence of chewing or puncture wounds, no swelling 3= Evidence of chewing or puncture wounds with swelling and signs of infection 4=Partial or total loss of the tail

3 scale climax : 0= No visible tail lesion. Earlier lesion is healed 1= Tail appears red and/or has minor scratches 2= Visible wound with obvious tissue damage

*Mild: Tail biting lesions of score 1 and 2 in a 5-scale climax or 1 in a three-scale climax.

**Severe: Tail biting lesions of score 3 and 4 in a 5-scale climax or 2 in a three-scale climax.

*** Mild & Severe tail biting lesions

TAIL BITING IMPACT ON PIG HEALTH AND WELFARE

Pigs that are tail bitten feel acute pain meaning lower welfare but also later they might develop health problems through infection of the biting wound (Valros, 2017). Wallenbeck and Keeling (2013) found that pigs that are mostly tail bitten (victims) had lower daily feed consumption up to 2 weeks after a tail biting outbreak while Munsterhjelm et al. (2015) reports reduced feed intake for even 20 days before becoming a victim and additionally suggest that feed intake on day 0 of tail biting could be used as predictor of recovery. Li et al. (2017) didn't observe any differences in body weight (BW) between tail biters and victims although they recorded reduced ADG in the period of production when most of the tail biting outbreaks occurred.

Victim pigs have a lower ADG (Marques et al., 2012; van Staaveren et al., 2017b) up to 3% although the latter is not correlated with feed conversion ratio (Sinisalo et al., 2012). Marques et al. (2012) explained this reduction by means of stress, secondary effects or lower feed intake. A correlation between high percentage of tail lesions and lower ADG has been found also at farm level (Pandolfi et al., 2018).

The infection from tail wound due to tail biting can spread through the body and lead to secondary infection to different organs (Schrøder-Petersen and Simonsen, 2001). Pigs with severe tail lesions present more often locomotion disorders and a higher mortality rate (Marques et al., 2012). In their epidemiological study, Pandolfi et al. (2018) reported a correlation between tail bitten pigs and hepatic scaring. Tail bitten pigs have more abscesses in lungs and at vertebral column (Marques et al., 2012). Table 2 presents the major outcome of tail biting on health of pigs as reported by previous research efforts.

The lack of available data on the topic, reveals the need for further research to investigate the link between tail biting and health status of the animal on a cause and effect basis. Moreover, the overall mortality on farm due to tail biting should be quantified to complete the record of tail biting effect.

Table 2. Major outcomes of tail biting on health of pigs

Main outcomes	Reference
Tail lesions increases the risk of carcass condemnation mainly due to abscesses and arthritis.	Valros et al. (2004)
Healed tail damage also increases the risk significantly.	
The severity of tail biting is associated with the presence of pleuritis and lung abscesses.	Kritas and Morrison. (2007)
There are significant association between the severity of tail-biting and external carcass abscesses.	
Along with suboptimal production systems, tail biting wounds can increase the risk of disease and injury lesions in pigs sent for slaughter.	Harley et al. (2012b)
In addition to an increased mortality rate, tail biting is associated with locomotion disorders and with the presence of abscesses at vertebral column, inguinal area and lungs.	Marques et al. (2012)
The average daily gain of tail biting victims is reduced by 1 to 3%.	Sinisalo et al. (2012)
Pigs with tail injuries due to tail biting consume decreased amounts of feed.	Wallenbeck and Keeling. (2013)
Prevalence of severe tail lesions is associated with the prevalence of enzootic pneumonia-like lesions and pyaemia.	Pandolfi et al. (2018)
Pigs with severe tail lesions tend to have more severe pleurisy where the lungs remained attached to the chest wall than pigs with moderate tail lesions.	Staaveren et al. (2016)
Severe tail lesions are correlated with a lower average daily gain	Staaveren et al. (2017)

TAIL BITING IMPACT ON FARM ECONOMY

Financial losses due to tail biting are significant and a constant threat for a pig farm (Harley et al., 2014). The cost of tail biting for a finishing farm of 4,000 pigs is estimated to be at 2,383 Euro per year (Zonderland et al., 2011c). In their study, Harley et al. (2014) estimated the profit loss per pig at 0.59 Euro considering only carcass reduced weight, while this amount goes up to 1.69 Euro when carcass condemnations, trimmings and smaller carcass weight are considered.

The risk of carcass condemnation has been positively correlated with the severity of tail lesion (Marques et al., 2012; Harley et al., 2014) while even healed lesions raise the above risk significantly (Valros et al., 2004). This positive association exists also between tail lesions severity and carcass trimmings (Harley et al., 2014; Kritas and Morrison, 2007). Moreover, tail lesions have a negative correlation with carcass weight (Harley et al. 2014; Carroll et al., 2015) while Valros et al. (2013) suggest that due to lower carcass weight they produce less lean meat.

Indirect economic losses have also to be estimated due to possible morbidity and mortality such as drug use for treatment of tail bitten pigs and increased labor costs (Marques et al., 2012). In a simulation study, the cost per tail bitten pig in a pen was estimated at 18.96 Euro due to increased medicine, veterinary, labor and material costs, increased mortality, carcass disposal and carcass condemnations, reduced daily gain and extra feed consumption (D'Eath et al., 2016).

On the economic impact of tail biting, it seems that farmers will treat tail biting in time if they are aware of the impact on their income. Thus, there is a need for more studies in the correlation between tail biting and production loss. Studies should quantify and take into account all the possible factors tail biting lesions affect the farmers' revenue.

BEHAVIORAL BASIS OF PIG TAIL BITING

Due to its sporadic and unpredictable presentation tail biting behavior is hard to be studied. The associations between tail damage and other lesions could be explained by shared risk factors (Teixeira et al., 2016), let alone the weakness of experimental studies to provoke it (Edwards, 2006; D 'Eath et al., 2014) and the fact that treatments that work once maybe proven useless to another outbreak (Hunter et al., 2001).

Three distinct types of tail biting have been proposed (Taylor et al., 2010): a) Two stage: a pre-injury one followed by an injurious one, b) sudden-forceful, and c) obsessive, based on a

different motivation basis. Later studies seem to support the different motivation (Ursinus et al., 2014a,2014c). The three types could be prevented by the identification of the different factors that are more usually involved to each type. Not all tail bites result in a tail biting outbreak (Holling et al., 2017; Lahrmann et al., 2018b). In most cases however, finding the pathway of actions that each risk factor triggers the processes that control tail biting expression is very difficult (Brunberg et al., 2016; D'Eath et al., 2016). Some studies are categorizing pigs as 'tail-biters' that do most of the biting, 'victims' who receive most of the bites and 'neutrals' that neither perform nor receive (Zonderland et al., 2011b; Brunberg et al., 2011) in order to find distinctive characteristics of pig behavior. In addition, Ursinus et al. (2014a) suggest that while there is an inconsistency in tail biting behavior by tail biters through rearing phases, victims seem to stay victims throughout their lives.

According to Valros et al. (2015) tail biters have differences in certain neurotransmitter's metabolism, dopamine and serotonin, in different brain areas than victims, indicating different ways of coping with stress between these two behavioral phenotypes. In addition, Brunberg et al., 2013 who reported differences in gene expression in the hypothalamus and prefrontal cortex among tail biters, receivers and neutral pigs suggested that pigs perform more pig-directed abnormal behavior due to selection for better production traits. Ursinus et al. (2014c) support that tail biters in enriched pens may be motivated by unsatisfied high nutrition demands while in barren pens (pens without enrichment material) this behavior could be associated with boredom. Brunberg et al. (2016) suggested a possible mechanism for tail biting behavior that includes gut microbiota, the immune system, hypothalamic-pituitary-adrenal (HPA)-axis reactivity, and the ability to cope with challenges. More specifically, insufficient ingredients in feed provoke a response from the stress-related HPA-axis and consequently the immune system, altering this way the individual's behavior. Preweaning behavior seems to correlate with tail biting behavior later in life (Ursinus et al., 2014a).

On the behavioral basis of tail biting, studies focus on three possible phenotypes in pig level: the biter, the victim and the neutral. Molecular studies should also be performed to help the early diagnosis of the biter. Comparison in genes expressions of these three phenotypes could reveal new characteristics of the different phenotypes.

RISK FACTORS FOR PIG TAIL BITING

Most of the studies on risk factors focused on the use of environmental enrichment are experimental while studies focused on farm conditions, health status and feeding are based mainly on epidemiological data (Valros et al., 2016). Table 3 presents the main results of studies on main risk factors for pig's tail biting.

On a breed basis, Breuer et al., (2005) found tail biting to be heritable in Landrace breed but not in Large White breed. Moreover, above-mentioned authors concluded that in the Landrace population, tail-biting was unfavorably genetically correlated with leanness (lean tissue growth rate) and back fat thickness at 90 kg. A link between production traits (fat content) and both performing and receiving tail biting, as well as other pig-directed abnormal behaviors, was also confirmed by Brunberg et al. (2013) meaning that the genetic background has to be taken into account when dealing with tail biting (Bulens et al., 2018). Furthermore, (Sinisalo et al., 2012) indicated that Yorkshire breed is more susceptible to become victim than Landrace breed. Taylor et al. (2010) suggests it is better to look in strains or lines inside a breed for tail biting correlations while D 'Eath et al. (2014) support that the evidence for a breed predisposal to be a victim or a biter is quite weak.

The relationship between health and biting behavior is complex and thus difficult to be established in a cause and effect way (Munsterhjelm et al., 2017; Van Der Meer et al., 2017) and there is no clear proof of evidence until now (Munsterhjelm et al., 2019). Tail biting has been positively associated with respiratory diseases and greater mortality on farm (Moinard et al., 2003). Pleuritis and lung abscesses are correlated with severe tail biting but not enzootic pneumonia (EP) (Kritas and Morrison, 2007) although an association with EP-like lesions has been reported in an epidemiological study (Pandolfi et al., 2018). Moreover, pigs from batches with higher tail lesions had high

prevalence of pleurisy, pneumonia and pleuropneumonia supporting the association between poor health and poor welfare on farm (Teixeira et al., 2016). On the contrary, van Staaveren et al., (2016) established this association only for severe pleurisy where lungs are attached to chest wall. A connection between infected tail lesions and respiratory organ inflammation has also been shown by (Munsterhjelm et al., 2013) who also suggested that tail biting behavior can be induced by a combination of individual factors and disease.

Additionally, it has been shown that tail biting victims have higher concentration of Acute phase proteins (APPs) triggering this way an acute phase response and the creation of abscesses (Heinonen et al., 2010) while Ursinus et al. (2014b), who found lower blood serotonin in tail biters, suggest that fluctuations of serotonergic measures and tail biting behavior over rearing phases should be taking into account before characterizing individual pigs. In addition, Li et al. (2017) observed higher concentrations of total serum protein and IgG in victim pigs 5 days after tail biting outbreak suggesting inflammation while the lower IgG and serum proteins concentrations of tail biters could indicate a possible compromised immune function due to chronic stress. In addition, it is suggested that living in a tail biting pen affects some physiological parameters of pigs (Palander et al., 2013). Furthermore, Munsterhjelm et al. (2019) suggested that pens with sick pigs are more in danger to show tail biting after the sick pigs have recovered than in the acute phase while there is an indication of correlation of specific cytokines with behaviors characterizing tail biters (Munsterhjelm et al., 2017).

Table 3. Studies on main risk factors for pig's tail biting and their results.

Risk Factor	Materials	Age	D/I	Sex	Results	Reference
ENRICHMENT	Easyfix® floor toy for weaners and a Piglyx® lick block for finishers- Floor toy-Wood post-Hanging wood-Loose material in long rack-Fabric-Hanging chew toy-Loose material in container	W-G-F	I	Mx	The types of enrichment provided over time doesn't significantly reduce tail damage or tail directed behavior (TDB). Pigs have preferences for certain enrichment materials, it is important to consider enrichment characteristics, presentation, location, and maintenance when providing enrichment.	Chou et al (2019a)
	Straw (7 g/pig/day) Bite-Rite Rope	W	I	Mxc	Providing additional straw on the floor during a tail biting outbreak reduced the risk of an escalation in tail damage more effectively than providing a Bite-Rite, Bite-Rite cannot keep pigs interested in very long and it should be combined or rotated with other materials to successfully stop tail biting.	Lahrmann et al (2019)
	Straw (7 g/pig/day) Haylage (22 g/pig/day) Rope	W	I	Mxc	Tail biting outbreaks can in many cases be prevented by giving the pigs access to extra enrichment material, when the first minor tail damage is noticed Not every case of tail biting behavior escalate into a tail biting outbreak	Lahrmann et al (2018)

	Hanging toy Straw blocks Hiding wall Pigs predisposed to better carcass traits vs. pigs predisposed to better growth	G-F	I	Mxc	Pigs with intact tails have higher daily weight gains in enriched pens when hiding walls and straw dispensers are provided. Genetic background should be considered when investigating the cause of tail-biting outbreaks and when evaluating the effect of enrichment on tail biting.	Bulens et al. (2018)
	Wood	F	***	F	A wooden stick close to the feeder is associated with more exploratory behavior in growing female pigs compared with a similar stick placed opposite to the feeder, Novelty of enrichment material plays a significant role.	Dalmau et al. (2018)
	Tail docking at ½ Straw (150gr/pig/day) Stocking density (S.D.) 0,73m^2/pig -1,21 m^2 /pig	G-F	D/I	Mxc	Incidences of first tail damage mainly in week 1 and in the first half of the finisher period. Tail docking is more successful preventive measure than provision of straw. Combination of straw provision and lower stocking density is as preventive as tail docking	Larsen et al. (2017)
	Jute sack	W-G	I	F	The provision of a jute sack can reduce tail-biting behavior of gilts directed to pen mates by up to half as much compared to gilts kept in barren pens. For tail biting boredom rather than a metabolic motivation plays the largest role in pigs kept in barren pens. Displaying high levels of tail-biting behavior is generally related to displaying higher levels of all kinds of biting behavior, a relatively high (phenotypic and possibly genotypic) growth, and originating from a large litter.	Ursinus et al. (2014c)
	Straw	G	D/I	Mc/F	Straw is an important tool in both increasing explorative behavior and preventing biting and lesions, particularly in the early stage of fattening. Tail biting represents an issue for heavy pigs as for standard rearing weights.	Scollo et al. (2013)
HEALTH		F	I	Mc/F	Tail biting induces inflammation in the tail end leading to a strong systemic acute phase response and formation of abscesses in the carcass	Heinonen et al. (2010)

		G	***	F	Recovered animals from sickness may have an increased propensity to become tail biters. Increased attention towards a sick animal by penmates, may increase the risk for the sick individual to become a victim of tail biting. The pen-level risk for tail biting may be higher after a bout of illness in the group than during the acute stage.	Munsterheim et al. (2019)
		P-W-G-F	I	Mxc	Within specific phases of life, tail biters and to a lesser extent also victims have lower levels of blood serotonin compared to non-performers/receivers. Tail biters also seems to have higher blood platelet uptake velocities.	Ursinus et al. (2014b)
		G-F	I	Mc/F	No associations between health status and tail-biting activity. Being a victim of tail biting is associated with severe inflammatory lesions in the respiratory organs. Deep infections may exist under healthy skin in bitten tails	Munsterheim et al. (2013)
		G-F	I	Mc/F	Free access feeding with restricted feeding space, compared with feeding twice a day with unrestricted feeding space was associated with an overall reduction in EAA levels in blood and deepened crypts in the jejunum. The observed differences differ according to the behavioral role of pigs (Biter, victim, neutral) in a tail-biting pen during the outbreak.	Palander et al. (2013)
GENDER		W	I	Mu/F Mx	Female piglets are more likely to tail bite than male piglets	Zonderland et al. (2010)
		F	D	Mxc	Barrows have 2.6-fold higher odds to be bitten compared to gilts. Victim pigs are smaller in size.	Kritas & Morrison (2004)
GROUP HOUSING		W-G	I	Mxc	No clear results of group housing before weaning and a prolongation of the suckling period from four to five weeks on tail biting.	Naya et al. (2019a)
STOCKING DENSITY	Solid floor area:1/3-2/3 S.D.:0.7m^2/p-0.89m^2/p Group Size:15p-12p Straw: Floor-Rack	G-F	I	Mxc	Increased space allowance + increased area of solid flooring+ straw allocated onto the floor + reduced group size= lower tail damage +tendency for more tail-directed behavior.	Brandt et al. (2019)
	Solid floor area:1/3-2/3 S.D.:0.77m^2/p-1.00m^2/p Group Size:15p-12p Straw: Floor-Rack	G-F	I	Mxc	No difference in tail directed behavior in pens with fewer pigs, a higher space allowance per pig and a larger area of solid floor.	Klaborg et al. (2019)

	W	D/I	***	Higher prevalence of tail lesions in undocked weaner pigs Higher prevalence of tail lesions when <7.5 litters are mixed at weaning In docked pigs lower prevalence of tail lesions in pens with lower stocking density	Grumpel et al. (2018)
MIXING	W	I	Mx	No direct effect of mixing animals at weaning on tail-biting at rearing.	Veit et al. (2017)
TAIL DOCKING	W	D/I	Mxc	Tail docking may be more effective because pigs are able to perform more damaging bites to intact tails because they are longer, so pigs are able to hold them across the mouth and crush them with the premolar teeth, which is not possible for the shorter docked tails.	Paoli et al. (2016)
	W-G-F	D/I	Mc/F	In a rearing cycle prolonged up to 40 weeks of age, the presence of intact tails causes higher levels of tail lesions but not a generalized welfare endangerment	Martino et al. (2015)
	W-G-F	D/I	Mxc	Tail lesions are more prevalent among 30 to 60 kg pigs than in the late finishing period (60 to 90 kg). Recordings from abattoir routine meat inspection when used to evaluate the level of tail biting in a herd, highly underestimates the number of bitten pigs.	Lahrmann et al. (2017)
	G-F	D/I	Mxc	Pigs with short docking length manipulates pen mates' tail less compared with pigs with longest docked tails Pigs with short docking length have a lower risk of a tail biting outbreak compared with undocked pigs .	Thodberg et al. (2018)
GENETICS	W-G-F	I	Mu/F	Landrace tends to show a higher prevalence of tail-biting than Large White under the same farm conditions. The heritability of tail biting in Landrace is correlated to two key performance parameters, lean tissue growth rate and back fat thickness.	Breuer et al. (2005)
	G-F	I	Mx	Selection on production traits, especially those related to meat/fat ratios, has contributed to the development of pigs that are more vulnerable to become performers and victims of tail biting behaviors. The development of abnormal behaviors may be influenced by the dopaminergic system.	Brunberg et. al. (2013a)

	G-F	I	Mx	Genes differently expressed in neutral pigs are associated with the cause, rather than the consequence, of them not performing and receiving tail biting. Given similar physical environmental conditions, whether an individual becomes a tail biter, has its tail bitten or remains neutral to a tail biting outbreak, is related to how much its behavior is targeted towards pen mates. Neutral pigs are less pig-directed in their behavior	Brunberg et. al. (2013b)
FEED	P-W-G-F	I	Mx	No distinct effect of a higher content of fibre in the piglet diet on tail biting in growing pigs.	Naya et al. (2019b)
	P-W-G-F	I	Mx	Provision of raw material through life reduces occurrence of tail biting but doesn't prevent it.	Veit et al. (2016)

Production Stage: P=Piglets (0-4weeks),W=Weaners (4-9 weeks), G=Growers (10-17 weeks),F=Finishers(17-Abbatoir)
I=Intact tail (Not docked), D=Docked tail (at first week of life)
Sex: Mx (Mixed group of uncastrated males and females) Mxc (Mixed group of castrated males and females) Mc/F(Single sex group MaleCastrated/Female), Mu/F(Single sex group MaleUncastrated/Female)
*** Not Reported

Tail biting is increasing with time starting mainly at two weeks after weaning (Veit et al., 2017) independent of the weaning age (Naya et al., 2019b), although Ursinus et al. (2014a) reported tail damage even at preweaning phase. Severe tail lesions are recorded in pigs aged 10 weeks (Carroll et al., 2018) or more (van Staaveren et al., 2018; Scollo et al., 2016) probably as damage is accumulated over time (Haigh et al., 2019; Chou et al., 2019a). On the other side, some authors report a decrease in tail lesions during the fattening cycle (Vermeer et al., 2017) even in pigs with a prolonged fattening cycle reared in intensive production (Scollo et al., 2013).

Gender is considered to be a risk factor for tail damage as castrated male pigs tend to have more tail biting lesions than gilts (Valros et al., 2004; Kritas and Morrison, 2004, 2007) and these lesions are more likely to be severe in mixed groups (Keeling et al., 2012). Similar findings regarding frequency and severity of tail lesions apply also to entire male pigs (Harley et al., 2014; van Staaveren et al., 2016; Calderon Diaz et al., 2017). In single sex groups, a higher frequency of biting behavior in females compared to males has been documented in weaners (Zonderland et al., 2010) and later in finishers (Haigh et al., 2019) while the same goes for castrated males and females (Kritas and Morrisson, 2004; Li et al., 2017).

Climate in the barn (temperature, gas, dust) keeping constantly out of the comfort zone could act as stressor that create discomfort and chronic stress to the animal leading to tail biting outbreaks (Taylor et al., 2010; Schrøder-Petersen and Simonsen, 2001). The same applies for great fluctuations in temperature during the day at certain times of the year or intense draughts as the capacity of the ventilation/heating/cooling systems is not limitless (D'Eath et al., 2014). In an epidemiological study in intensive pig production from weaning to 170 Kg live weight (Scollo et al., 2016) found poor air quality (as perceived by the author) to be a significant factor for tail biting.

Slatted floors are considered to be an important risk factor for tail biting outbreaks both in weaning and fattening phase of production (Schrøder-Petersen and Simonsen, 2001; Moinard et al., 2003; Van De Weerd et al., 2005). An increase in internal biosecurity has been positively associated with a reduction in tail biting (Pandolfi et al., 2018).

Up to some extend bigger herd size farms have lower risk of moderate tail lesions (van Staaveren et al., 2016) while tail biting sequelae (osteomyelitis and hind abscesses) have also low occurrence (Fertner

et al., 2017). On the contrary, Harley et al. (2012b) suggested that risk of injury and illness in animals in large herds is bigger than small herds based on an association of batch size and carcass condemnations at slaughterhouse while Scollo et al. (2017) are indicating that middle size farms seem to be more in danger of tail biting as big farms have good level of automation and small ones good stockman per pig analogy.

High stocking density is associated with the risk of tail biting as it interrupts normal social interactions (Moinard et al., 2003). Surprisingly, stocking density and group size as risk factors are not well documented in experimental studies (D'Eath et al., 2014) and available studies are not conclusive for the effect of group size in negative social behavior (Averós et al., 2010; Sutherland and Tucker, 2011). Tail lesions have been associated with high stocking density (Grümpel et al., 2018) and pens with more than 30 pigs (Pandolfi et al., 2018). However, Klaaborg et al. (2019) found no effect of bigger space allowance on pen mate directed behavior and Meyer-Hamme et al., (2016) didn't consider group size as a risk factor in tail docked pigs. We have to consider that in most studies is difficult to differentiate space allowance and group size as they are confounded (Klaaborg et al., 2019) while there are interactions with other factors. According to Averos et al. (2010) the positive effect of increased space allowance is conditioned to the provision of enrichment material.

Although EFSA (2007) considers mixing of piglets to be a risk factor, Veit et al. (2017) indicated that there is no direct effect between mixing and tail biting at rearing period while Grümpel et al. (2018) found more tail lesions in farms that mixed less than 7.5 litters. In addition, Li et al. (2018) suggested that pigs from same litter could be predisposed to tail biting as they are less socially connected.

Enrichment material in pig's pen play a vital role in controlling tail biting (Schrøder-Petersen and Simonsen, 2001) although they cannot eliminate it (Ursinus et al., 2014c) as the genetic predisposition to tail bite remains (Camerlink et al., 2014). This material has to be economic, labor not intensive and durable in order for farmers to adopt it (van de Weerd and Day., 2009; Chou et al., 2018). According to Commission recommendation EU 2016/336, the optimum enrichment material is described as: edible, chewable, investigable and manipulable (EU Commission, 2016).

Straw as a bedding material allows pigs to express their species-specific behavior (van de Weerd and Day., 2009) reduces tail biting prevalence (van de Weerd et al., 2005; Wallgren et al., 2019b) and keeps pig more occupied than plastic objects (Scott et al., 2009). The most serious problem about enrichment material given as a bedding is the possible blockage of the slurry system (Zonderland et al., 2008; D 'Eath et al., 2014; Lahrmann et al., 2018b) but also problems with availability (Wallgren et al., 2019b) mycotoxins ingestion (Nordkvist and Häggblom, 2014) and biosecurity in times of African Swine Fever have to be considered (Wallgren et al., 2019c). Straw effectiveness as environmental enrichment is decreased when given in other ways (Zonderland et al., 2008) or forms (Haigh et al., 2019) and when provided through dispensers doesn't totally prevent tail biting (Holling et al., 2017) but still remains better than toys (Bulens et al., 2018).

Although chains are considered of marginal interest and must be accompanied by an optimal or suboptimal enrichment material (Commission Recommendation (EU) 2016/336) still are the main enrichment provided (Bracke et al., 2013; Valros et al., 2016), especially in countries that don't use straw (Bracke and Koene, 2019). The value of chains as enrichment material is the lowest comparing to hard wood or plastic (Boyle et al., 2019) or freshly cut wood (Telkänranta et al., 2014) and doesn't seem to have an effect on tail biting prevalence (Buijs and Muns, 2019). However, it could benefit from a new design of branched ends (Bracke, 2017). Dry wood keeps the interest of pigs more than plastic "toys" (Beaudoin et al., 2019) although type of wood seems to have no differences in efficacy (Chou et al., 2018).

Object manipulation is affected by space, other enrichment materials offered to pigs, (Larsen et al., 2019; van de Weerd et al., 2009) and the position of it (Scott et al., 2009; Larsen et al., 2019; Dalmau et al., 2019). Object manipulation diminishes by time as pigs become habituated to them and as aging changes their choices (Trickett et al., 2009; van de Weerd et al., 2009; Dalmau et al., 2019) while the variation of enrichment materials does not seem to have a significant effect in reducing tail biting severity (Chou et al., 2019a). Cleanliness plays a role (Averos et al., 2010) but everyday cleaning or replacement doesn't seem to have an effect on attractiveness for short periods (Beaudoin et al., 2019).

Although a lot of research is ongoing in enrichment material and its efficacy on preventing tail bit-

ing there is no consensus between experts on what is acceptable enrichment (Scott et al., 2009; Bracke and Koene, 2019; Briyne et al., 2018) nor does legislation helps competent authorities assess the degree that this need is fulfilled (Weerd et al., 2009; Wallgren et al., 2019c). Except materials used as beddings, only a combination of other enrichment could possibly comply with EU criteria (Buis and Muns, 2019) whereas the kind of environmental enrichment a farmer uses as preventive measure, could indicate the efficiency of management (Boyle et al., 2019)

Variations and delays in feeding time pose a risk for tail-biting (Scollo et al., 2017). Also feed type plays a role as there is higher prevalence of tail biting when pigs are fed liquid (Pandolfi et al., 2017) or pellets (Hunter et al., 2001) than meal. Moreover, artificially fed piglets show more tail lesions than piglets that are fed by a sow (Schmitt et al., 2019) while restricted feeding space is suggested to be associated with a reduction of amino acids in blood of victims and control pigs due to stressful environment or because they can't access as successfully as tail biters the feeder (Palander et al., 2013). The external factors that affect pig behavior have been widely investigated, however, the research is still in need of studies for identification of internal factors, including health and genetics, and their impact on pig behavior.

PREVENTION AND TREATMENT

It's been over 25 years since the first EU legislation (EU Council Directive, 91/630/EEC) prohibited tail docking on a routine basis (Nalon and De Briyne, 2019). However, even though a new directive came in force in 2008 (EU Council Directive, 2008/120/EC), this practice is still the most widely preventive measure against tail biting used by up to 95% of farms in Europe (EFSA, 2007; EU Commission, 2018). The Directive itself leaves enough place for misinterpretation by Competent Authorities in Member States (D'Eath et al., 2016). The high prevalence of tail docking indicates a serious and chronic problem of tail biting in pig farming (Harley et al., 2012b) as it is used to conceal other welfare problems (Zonderland et al., 2008; D'Eath et al., 2016).

The equipment used for tail docking in pigs includes teeth clippers, cutting pliers, scissors, a scalpel blade, gas or electrical cautery iron while the length of the remaining part of the tail varies among different countries (Sutherland and Tucker, 2011). Moreover, Thodberg et al. (2018) demonstrated that leaving a very short tail lowers the risk of tail biting outbreak in comparison not only with undocked tails but also to tails with a longer remnant.

Pigs that are tail docked have lower incidence and severity of tail lesions compared to intact ones in weaner (Fu et al., 2018; Grümpel et al. 2018) and finisher phase of production (Di Martino et al., 2015; Li et al. 2017; Lahrmann et al. 2017). However, there is no clear explanation why tail docking reduces tail biting (Paoli et al., 2016; Valros, 2017; Grümpel et al., 2018). Simonsen et al. (1991) suggested that formation of neuromas in tail tip could lead to hypersensitivity or another explanation could be that the hairy intact tail remains more attractive to bite. However, there is the idea that the shorter the tail the less time will a pig be bitten (Harley et al., 2012b). Paoli et al. (2016) suggest that the longer the tail the more powerful the grasp of the tail by the biter as he demonstrates that pigs with intact tails don't show more tail directed behavior than the docked ones. This finding is inconsistent with the results of Thodberg et al. (2018) who support that pigs with short docked tails manipulate other pigs' tails less in comparisons with undocked, while tail docking doesn't seem to alter the social functions of finisher pigs.

Tail docking has an acute impact on the welfare of the pig due to pain demonstrated by histopathological studies (Simonsen et al., 1991) and by behavioral studies (Sutherland and Tucker, 2011). At the long term, in the site of amputation, there is possible neuroma development (Herskin et al., 2015) that still goes on even 16 weeks after (Sandercock et al., 2016) and is accompanied with significant changes in gene expression linked with possible chronic pain in the tail stump (Sandercock et al., 2019). It is generally accepted that tail docking reduces the incidents of tail biting (Kakanis et al., 2019; Bracke et al., 2013; Valros et al., 2016), but it cannot eliminate the problem (Li et al., 2017; Larsen 2018a) especially when environmental conditions remain the same (EFSA, 2007; Thodberg et al. 2018).

On the other hand, there are not so many studies to offer effective treatments of tail biting (Zonderland et al., 2008; Chou et al., 2019b) although straw provision is the most successful (Lahrmann et al., 2019). Allocating extra manipulable material early can prevent a tail biting outbreak (Zonderland et al., 2008; Lahrmann et al., 2018b). The biters/victim's ratio seems to play more important role to the success of a treatment than the method selected but no real dif-

ferences were found between removing the biter or the victim for an outbreak stop (Chou et al., 2019b) although Zonderland et al. (2011a) suggested is better to remove the biter than the victim. Experimental curative treatment of removing pigs (biters or victims) or adding enrichment material (straw or ropes) hasn't stopped 100% of tail biting (Zonderland et al., 2008; Chou et al., 2019b).

Tail docking is still perceived by many farmers to be the only way to deal with the problem even though it is not permitted by legislation. Research should be driven to identify successful prevention and treatment schemes. Moreover, as strategies are implemented on a farm basis approach, those schemes should be diversified between different countries and farming system to be effectively adopted by farmers.

PREDICTION OF PIG TAIL BITING

Today the EU regulation 2008/120 EC requires that staff do inspect the pigs at least once a day. Tail biting behavior is difficult to follow due to the sporadic nature of outbreaks (Statham et al., 2009), the diurnal pattern of behaviors (Domun et al., 2019; Chou et al., 2019b) and small changes in early predictors that are difficult to be detected by caretakers daily (Wedin et al., 2018). The ability to automatically detect and track individual pigs without the need for human observation could help in early detection of potential welfare problems (Zhang et al., 2019) and could help develop on farm strategies to reduce tail biting outbreaks (Zonderland et al., 2011b). Precision livestock farming techniques, besides the disadvantages of cost and technical challenges, gives the opportunity to farmers to go from group level to individual level welfare (Benjamin et al., 2019).

Back to 1969, van Putten et al. concluded that tail is an indicator of unpleasant surroundings. Tail posture seems to be correlated with tail biting (Schrøder-Petersen and Simonsen 2001). Statham et al. (2009) found evidence that before an outbreak occurs there are more pigs with tails tucked under. Zonderland et al. (2009) also found that piglets who had their tail between their legs had significantly higher possibility to have tail damage 2-3 days afterwards even though at that moment they didn't have any, while the number of pigs with lowered tails is increasing in pens towards day 0 of the outbreak (Lahrmann et al., 2018a; Wedin et al., 2018). D'Eath et al. (2018) using 3D cameras also found lowered tail posture in pen level as far as 2 weeks prior to a tail biting outbreak and interestingly the same effect even 2 weeks post outbreak although they comment that each pen has its own baseline of low posture tails. At pig level, a hanging tail is correlated positively with having a lesion (Larsen et al., 2018b; D 'Eath et al., 2018; Wallgren et al., 2019a) although we must take into account tail posture in relation to different activities and emotions expressed by pigs (Wallgren et al., 2019a) or the diurnal pattern of pig behavior (Larsen et al., 2019) in order to use it as an early predictor.

Except tail posture, a higher level of pen activity days prior to a tail biting outbreak (Statham et al., 2009; Zonderland et al., 2011b; Ursinus et al., 2014a; Larsen et al., 2019) object manipulation (Ursinus et al., 2014a; Larsen et al., 2019) as well as a lower feeding frequency at pen level (Wallenbeck and Keeling, 2013) could be used as early behavioral indicators of tail biting outbreaks. There are indications that these differences in activity could be attributed to different phenotypes of pigs categorized by their tail in mouth behavior as performers, receivers or neutral but also that a pen level threshold exists (Munsterhjelm et al., 2016).

The advances in technology have helped to better understand the behavioral patterns of pigs. Precision livestock farming studies are already being performed for a number of production characteristics and behaviors, including tail biting. This kind of studies should be focused on early diagnosis of tail biting outbreaks and interestingly on a pig level of recognizing the possible biter.

POLICY MEASURES TO ADDRESS PIG TAIL BITING

EU as a major pig exporter has set high standards of welfare driving the trends in pig welfare worldwide while they do not seem to have a significant effect on competitiveness (Nalon and Briyne, 2019). More research is needed in order to get away of amputation methods towards a sustainable pig production in welfare aspects (Larsen et al., 2018a). As welfare policy is mainly considered to be driven by views of the non-producers public, farmers' concerns should be taken into account (Spooner et al., 2014) even though it seems to differ across countries (Valros et al., 2016). This is especially important for choosing public policy measures that are more efficient in improving animal welfare (Niemi et al., 2012). Additionally, the differ-

ent perceptions between farmers and experts (Valros and Barber., 2019) indicate a problem of communication between research and industry (Camerlink and Turner, 2016) that should be considered when designing communication strategies for better implementation of legislation (Kakanis et al., 2019).

CONCLUSION

Tail biting remains one of the major welfare problems for pig industry with direct and indirect economic losses while farmers end up to tail dock on a routine basis as the main preventive measure throughout Europe.

Although a lot of research is undertaken in the last decade, this is mainly focused on some risk factors while some like health status and genetics need further investigation. This could be facilitated through research on the motivational basis of tail biting on a pig level and possible predisposing factors. The chronic consequences of tail amputation and the real functions of pig tails should be clearly defined to the community. There seems to be a potential on early prognosis using Precision Livestock Farming techniques (by means of purpose-built algorithms to evaluate in real time data from sensors in the stable) and more studies are needed on how to recognize an upcoming tail biting outbreak and how to prevent or control it.

Moreover, studies in recent years made prominent the different perspective of farmers and scientists and the possible implications in developing information strategies. The prevalence of the problem could be pointed out to the industry by the development of one concrete unbiased system for recording tail biting lesions in abattoirs that is lacking nowadays while data on economic impact of the behavior is largely missing in literature. Thus academia, industry and policy makers could all benefit by a close collaboration towards raising pigs with intact tails.

CONFLICT OF INTEREST

None declared by the authors.

REFERENCES

Alban, L., Petersen, J. V., Busch, M.E., 2015. A comparison of lesions found during meat inspection of finishing pigs raised under organic/free-range conditions and conventional indoor conditions 85-88.

Averós, X., Brossard, L., Dourmad, J.Y., de Greef, K.H., Edge, H.L., Edwards, S.A., Meunier-Salaün, M.C., 2010. A meta-analysis of the combined effect of housing and environmental enrichment characteristics on the behaviour and performance of pigs. Applied Animal Behaviour Science 127, 73-85.

Becskei Z., Kakanis M.,Janczak A., Sossidou E. 2018. CA 15134 "GROUPHOUSENET" Prevalence of tail biting behavior in Greek pig farms. Proceedings of 25th International Conferecne Animal Protection and Welfare 2018

Becskei Zolt, 2017. Short-Term Scientific Mission (STSM) Report Cost Action CA15134 „Synergy for preventing damaging behaviour in group housed pigs and chicken (GroupHouseNet)" Available online: https://www.grouphousenet.eu/STSM_Becskei%20Zsolt.pdf [accessed 30/07/2020]

Beaudoin, J.M., Bergeron, R., Devillers, N., Laforest, J.P., 2019. Growing pigs' interest in enrichment objects with different characteristics and cleanliness. Animals 9, 1-13.

Benjamin, M., Yik, S., 2019. Precision livestock farming in swinewelfare: A review for swine practitioners. Animals 9, 1-21.

Boyle, L., 2019. Associations between tail directed behaviour, tail lesions and enrichment type in finisher pigs. Book of Abstracts of the 70th Annual Meeting of the European Federation of Animal Science pp 206

Bracke, M.B.M., 2017. Chapter 6. Chains as proper enrichment material for intensively-farmed pigs? Advances in Pig Welfare 1-27.

Bracke, M.B.M., De Lauwere, C.C., Wind, S.M.M., Zonerland, J.J., 2013. Attitudes of Dutch Pig Farmers Towards Tail Biting and Tail Docking. Journal of Agricultural and Environmental Ethics 26, 847-868.

Bracke, M.B.M., Koene, P., 2019. Expert opinion on metal chains and other indestructible objects as proper enrichment for intensively-farmed pigs. PLoS ONE 14, 1-19.

Breuer, K., Sutcliffe, M.E.M., Mercer, J.T., Rance, K.A., O'Connell, N.E., Sneddon, I.A., Edwards, S.A., 2005. Heritability of clinical tail-biting and its relation to performance traits. Livestock Production Science 93, 87-94.

Brunberg, E., Jensen, P., Isaksson, A., Keeling, L.J., 2013. Brain gene expression differences are associated with abnormal tail biting behavior in pigs. Genes, Brain and Behavior 12, 275-281. Brunberg, E., Wallenbeck, A., Keeling, L.J., 2011. Tail biting in fattening pigs: Associations between frequency of tail biting and other abnormal behaviours. Applied Animal Behaviour Science 133, 18-25.

Brunberg, E.I., Bas Rodenburg, T., Rydhmer, L., Kjaer, J.B., Jensen, P., Keeling, L.J., 2016. Omnivores going astray: A review and new synthesis of abnormal behavior in pigs and laying hens. Frontiers in Veterinary Science 3, 1-15.

Buijs, S., Muns, R., 2019. A review of the effects of non-straw enrichment on tail biting in pigs. Animals 9.

Bulens, A., Van Beirendonck, S., Van Thielen, J., Buys, N., Driessen, B., 2018. Rearing finishing pigs with intact tails: Do they benefit from an enriched environment? Journal of Veterinary Behavior: Clinical Applications and Research 24, 1-8.

Calderón Díaz, J.A., Boyle, L.A., Diana, A., Leonard, F.C., Moriarty, J.P., McElroy, M.C., McGettrick, S., Kelliher, D., García Manzanilla, E., 2017. Early life indicators predict mortality, illness, reduced welfare and carcass characteristics in finisher pigs. Preventive Veterinary Medicine 146, 94-102.

Camerlink, I., Ursinus, W.W., Bijma, P., Kemp, B., Bolhuis, J.E., 2014. Indirect Genetic Effects for Growth Rate in Domestic Pigs Alter Aggressive and Manipulative Biting Behaviour. Behavior Genetics 45, 117-126.

Camerlink, I. and Turner, S.P. (2016) Farmers' perception of aggression between growing pigs. Applied Animal Behaviour Science. ISSN 0309-1740

Carroll, G.A., Boyle, L.A., Hanlon, A., Collins, L., Griffin, K., Friel, M., Armstrong, D., O'Connell, N.E., 2018. What can carcass-based assessments tell us about the lifetime welfare status of pigs? Livestock Science 214, 98-105.

Carroll, G.A., Boyle, L.A., Teixeira, D.L., Van Staaveren, N., Hanlon, A., O'Connell, N.E., Staaveren, N. Van, Hanlon, A., Connell, N.E.O., Van Staaveren, N., Hanlon, A., O'Connell, N.E., 2015. Effects of scalding and dehairing of pig carcasses at abattoirs on the visibility of welfare-related lesions. Animal 10, 460-467.

Chou, J.-Y.Y., Drique, C.M.V. V., Sandercock, D.A., D'Eath, R.B., O'Driscoll, K., Chou, J.-Y.Y., Drique, C.M.V. V., Sandercock, D.A., D'Eath, R.B., O'Driscoll, K., 2019a. Rearing undocked pigs on fully slatted floors using multiple types and variations of enrichment. Animals 9, 1-17.

Chou, J.-Y.Y., O'Driscoll, K., D'Eath, R.B., Sandercock, D.A., Camerlink, I., 2019b. Multi-step tail biting outbreak intervention protocols for pigs housed on slatted floors. Animals 9, 582.

Chou, J.Y., D'Eath, R.B., Sandercock, D.A., Waran, N., Haigh, A., O'Driscoll, K., 2018. Use of different wood types as environmental enrichment to manage tail biting in docked pigs in a commercial fully-slatted system. Livestock Science 213, 19-27.

Correia-Gomes, C., Eze, J.I., Borobia-Belsué, J., Tucker, A.W., Sparrow, D., Strachan, D., Gunn, G.J., 2017. Voluntary monitoring systems for pig health and welfare in the UK: Comparative analysis of prevalence and temporal patterns of selected non-respiratory post mortem conditions. Preventive Veterinary Medicine 146, 1-9.

D'Eath, R.B., Arnott, G., Turner, S.P., Jensen, T., Lahrmann, H.P., Busch, M.E., Niemi, J.K., Lawrence, A.B., Sandøe, P., 2014. Injurious tail biting in pigs: How can it be controlled in existing systems without tail docking? Animal 8, 1479-1497.

D'Eath, R.B., Jack, M., Futro, A., Talbot, D., Zhu, Q., Barclay, D., Baxter, E.M., Zhu, Q., Baxter, E.M., D'Eath, R.B., Jack, M., Talbot, D., 2018. Automatic early warning of tail biting in pigs: 3D cameras can detect lowered tail posture before an outbreak. PLoS ONE 13, 1-18.

D'Eath, R.B., Niemi, J.K., Vosough Ahmadi, B., Rutherford, K.M.D., Ison, S.H., Turner, S.P., Anker, H.T., Jensen, T., Busch, M.E., Jensen, K.K., Lawrence, A.B., Sandøe, P., 2016. Why are most EU pigs tail docked? Economic and ethical analysis of four pig housing and management scenarios in the light of EU legislation and animal welfare outcomes. Animal 10, 687-699.

Dalmau, A., Areal, B., Machado, S., Pallisera, J., Velarde, A., 2019. Does the location of enrichment material affect behavior and dirtiness in growing female pigs? Journal of Applied Animal Welfare Science 22, 116-126.

De Briyne, N., Berg, C., Blaha, T., Palzer, A., Temple, D., 2018. "Phasing out pig tail docking in the EU - present state, challenges and possibilities." Porcine Health Management 4, 1-9.

Di Martino, G., Scollo, A., Gottardo, F., Stefani, A.L., Schiavon, E., Capello, K., Marangon, S., Bonfanti, L., 2015. The effect of tail docking on the welfare of pigs housed under challenging conditions. Livestock Science 173, 78-86.

Domun, Y., Pedersen, L.J., White, D., Adeyemi, O., Norton, T., 2019. Learning patterns from time-series data to discriminate predictions of tail-biting, fouling and diarrhoea in pigs. Computers and Electronics in Agriculture 163, 104878.

Edwards, S.A., 2006. Tail biting in pigs: Understanding the intractable problem. Veterinary Journal 171, 198-199.

European Union. Council of the European Union, 2008. Council Directive 2008/120/EC laying down minimum standards for the protection of pigs. Official Journal of the European Union L47/5-13.

European Union. Council Directive 91/630/EEC of 19 November 1991 laying down minimum standards for the protection of pigs. Off. J. Eur. Union 1991,50, 33-38European Union. Commission Recommendation (EU) 2016/336 on the application of Council Directive 2008/120/EC laying down minimum standards for the protection of pigs as regards measures to reduce the need for tail-docking. Off. J. Eur. Union 2016, L62, 20.

European Union. Council Directive 2008/120/EC of 18 December 2008 laying down minimum standards for the protection of pigs (Codified version). Off. J. Eur. Union 2009, L47, 5-13. 17.

European Union. Communication from the commission to the European Parliament, the Council and the Eurpean Economic and Social Committee on the European Union Strategy for the Protection and Welfare of Animals 2012-2015 COM(2012) 6 final

European Food Safety Authority EFSA. The risks associated with tail biting in pigs and possible means to reduce the need for tail docking considering the different housing and husbandry systems. EFSA J. 2007, 5,611.

European Commission, Directorate Health and Food Audits and Analysis. Audit Reports. Available online: https://ec.europa.eu/food/audits-analysis/audit_reports/index.cfm [accessed 30/07/2020]

Fertner, M., Denwood, M., Birkegård, A.C., Stege, H., Boklund, A., 2017. Associations between antibacterial treatment and the prevalence of tail-biting-related sequelae in Danish finishers at slaughter. Frontiers in Veterinary Science 4, 1-8.

Fu, L., Zhou, B., Li, H., Schinckel, A.P., Liang, T., Chu, Q., Li, Y., Xu, F., 2018. Teeth clipping, tail docking and toy enrichment affect physiological indicators, behaviour and lesions of weaned pigs after re-location and mixing. Livestock Science 212, 137-142.

Grümpel, A., Krieter, J., Veit, C., Dippel, S., 2018. Factors influencing the risk for tail lesions in weaner pigs (Sus scrofa). Livestock Science 216, 219-226.

Haigh, A., Yun-Chou, J., O'Driscoll, K., 2019. An investigation into the effectiveness of compressed straw blocks in reducing abnormal behaviour in growing pigs. Animal 1-10.

Harley, S., Boyle, L.A., O'Connell, N.E., More, S.J., Teixeira, D.L., Hanlon, A., 2014. Docking the value of pigmeat? Prevalence and financial implications of welfare lesions in Irish slaughter pigs. Animal Welfare 23, 275-285.

Harley, Sarah, More, S., Boyle, L., O'Connell, N., Hanlon, A., 2012a. Good animal welfare makes economic sense: Potential of pig abattoir meat inspection as a welfare surveillance tool. Irish Veterinary Journal 65, 1.

Harley, S., More, S.J., O'Connell, N.E., Hanlon, A., Teixeira, D., Boyle, L., 2012b. Evaluating the prevalence of tail biting and carcase condemnations in slaughter pigs in the Republic and Northern Ireland, and the potential of abattoir meat inspection as a welfare surveillance tool. Veterinary Record 171, 621.

Heinonen, M., Orro, T., Kokkonen, T., Munsterhjelm, C., Peltoniemi, O., Valros, A., 2010. Tail biting induces a strong acute phase response and tail-end inflammation in finishing pigs. Veterinary Journal 184, 303-307.

Herskin, M.S., Thodberg, K., Jensen, H.E., 2015. Effects of tail docking and docking length on neuroanatomical changes in healed tail tips of pigs. Animal 9, 677-681.

Holling, C., Beilage, E.G., Vidondo, B., Nathues, C., 2017. Provision of straw by a foraging tower - Effect on tail biting in weaners and fattening pigs. Porcine Health Management 3, 1-14.

Hunter, E.J., Jones, T.A., Guise, H.J., Penny, R.H.C., Hoste, S., 2001. The Relationship between Tail Biting in Pigs, Docking Procedure and Other Management Practices. Veterinary Journal 161, 72-79

Kakanis M., Marinou K., Doudounakis S., Sossidou E..2019 Greek farmers' attitudes regarding tail biting and tail docking Book of Abstracts of the 70th Annual Meeting of the European Federation of Animal Science p.p. 203

Keeling, L.J., Wallenbeck, A., Larsen, A., Holmgren, N., 2012. Scoring tail damage in pigs: An evaluation based on recordings at Swedish slaughterhouses. Acta Veterinaria Scandinavica 138, 1.

Klaaborg, J., Kristensen, A.R., Brandt, P., 2019. The effect of pen environment on pen-mate directed behaviour prior to feeding in finisher pigs with intact tails. Livestock Science 219, 35-39.

Kongsted, H., Sørensen, J.T., 2017. Lesions found at routine meat inspection on finishing pigs are associated with production system. Veterinary Journal 223, 21-26.

Kritas, S.K., Morrison, R.B., 2007. Relationships between tail biting in pigs and disease lesions and condemnations at slaughter. Veterinary Record 160, 149-152.

Kritas, S.K., Morrison, R.B., 2004. An observational study on tail biting in commercial grower-finisher barns. Journal of Swine Health and Production 12, 17-22.

Lahrmann, H.P., Busch, M.E., D'Eath, R.B., Forkman, B., Hansen, C.F., 2017. More tail lesions among undocked than tail docked pigs in a conventional herd. Animal 11, 1825-1831. Lahrmann, H.P., Faustrup, J.F., Hansen, C.F., D'eath, R.B., Nielsen, J.P., Forkman, B., 2019. The effect of straw, rope, and bite-rite treatment in weaner pens with a tail biting outbreak. Animals 9, 1-15.

Lahrmann, H.P., Hansen, C.F., D'Eath, R., Busch, M.E., Forkman, B., 2018a. Tail posture predicts tail biting outbreaks at pen level in weaner pigs. Applied Animal Behaviour Science 200, 29-35.

Lahrmann, H.P., Hansen, C.F., D´Eath, R.B., Busch, M.E., Nielsen, J.P., Forkman, B., 2018b. Early intervention with enrichment can prevent tail biting outbreaks in weaner pigs. Livestock Science 214, 272-277.

Larsen, M.L.V., Andersen, H.M.L., Pedersen, L.J., 2019. Changes in activity and object manipulation before tail damage in finisher pigs as an early detector of tail biting. Animal 13, 1037-1044.

Larsen, Mona Lilian Vestbjerg, Andersen, H.M.L., Pedersen, L.J., 2018b. Tail posture as a detector of tail damage and an early detector of tail biting in finishing pigs. Applied Animal Behaviour Science 209, 30-35.

Larsen, M. L.V., Andersen, H.M.L., Pedersen, L.J., 2018a. Which is the most preventive measure against tail damage in finisher pigs: Tail docking, straw provision or lowered stocking density? Animal 12, 1260-1267.

Li, Y., Zhang, H., Johnston, L.J., Martin, W., 2018. Understanding tail-biting in pigs through social network analysis. Animals 8, 1-13.

Li, Y.Z., Zhang, H.F., Johnston, L.J., Martin, W., Peterson, J.D., Coetzee, J.F., 2017. Effects of tail docking and tail biting on performance and welfare of growing-finishing pigs in a confinement housing system. Journal of Animal Science 95, 4835-4845.

Marques, B.M.F.P.P., Bernardi, M.L., Coelho, C.F., Almeida, M., Morales, O.E., Mores, T.J., Borowski, S.M., Barcellos, D.E.S.N., 2012. Influence of tail biting on weight gain, lesions and condemnations at slaughter of liinishing pigs. Pesquisa Veterinaria Brasileira 32, 967-974. Meyer-Hamme, S.E.K., Lambertz, C., Gauly, M., 2016. Does group size have an impact on welfare indicators in fattening pigs? Animal 10, 142-149.

Moinard, C., Mendl, M., Nicol, C.J., Green, L.E., 2003. A case control study of on-farm risk factors for tail biting in pigs. Applied Animal Behaviour Science 81, 333-355.

Munsterhjelm, C., Heinonen, M., Valros, A., 2016. Can tail-in-mouth behaviour in weaned piglets be predicted by behaviour and performance? Applied Animal Behaviour Science 184, 16-24.

Munsterhjelm, C., Heinonen, M., Valros, A., 2015. Effects of clinical lameness and tail biting lesions on voluntary feed intake in growing pigs. Livestock Science 181, 210-219.

Munsterhjelm, C., Nordgreen, J., Aae, F., Heinonen, M., Olstad, K., Aasmundstad, T., Janczak, A.M., Valros, A., 2017. To be blamed or pitied? The effect of illness on social behavior, cytokine levels and feed intake in undocked boars. Physiology and Behavior 179, 298-307. Munsterhjelm, C., Nordgreen, J., Aae, F., Heinonen, M., Valros, A., Janczak, A.M., 2019. Sick and grumpy: Changes in social behaviour after a controlled immune stimulation in group-housed gilts. Physiology and Behavior 198, 76-83.

Munsterhjelm, C., Simola, O., Keeling, L., Valros, A., Heinonen, M., 2013. Health parameters in tail biters and bitten pigs in a case-control study. Animal 7, 814-821.

Nalon, E., De Briyne, N., 2019. Efforts to ban the routine tail docking of pigs and to give pigs enrichment materials via EU law: Where do we stand a quarter of a century on? Animals 9. Naya, A., Gertz, M., Hasler, M., Große Beilage, E., Krieter, J., 2019a. Does a higher content of fibre in the piglet diet have an influence on tail biting in growing pigs? Livestock Science 223, 133-137.

Naya, A., Traulsen, I., Gertz, M., Hasler, M., Burfeind, O., große Beilage, E., Krieter, J., 2019b. Is tail biting in growing pigs reduced by a prolonged suckling period? Applied Animal Behaviour Science 211, 41-46

Niemi, J.K., Sinisalo, A., Valros, A., Heinonen, M., 2012. New challenges for EU agricultural sector and rural areas . Which role for public policy? Market and policy-oriented incentives to provide animal welfare: The case of tail biting. 126th EAAE Seminar 1-13.

Nordkvist E. and Häggblom P.,2014 Fusarium mycotoxin contamination of cereals and bedding straw at Swedish pig farms. Animal Feed Science and Technology 198, 231-237

Palander, P.A., Heinonen, M., Simpura, I., Edwards, S.A., Valros, A.E., 2013. Jejunal morphology and blood metabolites in tail biting, victim and control pigs. Animal 7, 1523-1531.

Pandolfi, F., Edwards, S.A., Maes, D., Kyriazakis, I., 2018. Connecting different data sources to assess the interconnections between biosecurity, health, welfare, and performance in commercial pig farms in Great Britain. Frontiers in Veterinary Science 5, 1-13.

Pandolfi, F., Kyriazakis, I., Stoddart, K., Wainwright, N., Edwards, S.A., 2017. The "Real Welfare" scheme: Identification of risk and protective factors for welfare outcomes in commercial pig farms in the UK. Preventive Veterinary Medicine 146, 34-43.

Paoli , M . A ., Lahrmann , H . P ., Jensen , T . and D ' Eath , R . B . (2016) Behavioural differences between weaner pigs with intact and Copyright © 2016 Universities Federation for Animal Welfare . This manuscript version is made available after the 287-296.

Sandercock, D.A., Barnett, M.W., Coe, J.E., Downing, A.C., Nirmal, A.J., Di Giminiani, P., Edwards, S.A., Freeman, T.C., 2019. Transcriptomics Analysis of Porcine Caudal Dorsal Root Ganglia in Tail Amputated Pigs Shows Long-Term Effects on Many Pain-Associated Genes. Frontiers in Veterinary Science 6.

Sandercock, D.A., Smith, S.H., Di Giminiani, P., Edwards, S.A., 2016. Histopathological Characterization of Tail Injury and Traumatic Neuroma Development after Tail Docking in Piglets. Journal of Comparative Pathology 155, 40-49.

Schrøder-Petersen, D.L., Simonsen, H.B., 2001. Tail biting in pigs. The Veterinary Journal 162, 196-210.

Schmitt, O., O'Driscoll, K., Boyle, L.A., Baxter, E.M., 2019. Artificial rearing affects piglets pre-weaning behaviour, welfare and growth performance. Applied Animal Behaviour Science 210, 16-25.

Scollo, A., Contiero, B., Gottardo, F., 2016. Frequency of tail lesions and risk factors for tail biting in heavy pig production from weaning to 170 kg live weight. Veterinary Journal 207, 92-98.

Scollo, A., Di Martino, G., Bonfanti, L., Stefani, A.L., Schiavon, E., Marangon, S., Gottardo, F., 2013. Tail docking and the rearing of heavy pigs: The role played by gender and the presence of straw in the control of tail biting. Blood parameters, behaviour and skin lesions. Research in Veterinary Science 95, 825-830.

Scollo, A., Gottardo, F., Contiero, B., Edwards, S.A., 2017. A cross-sectional study for predicting tail biting risk in pig farms using classification and regression tree analysis. Preventive Veterinary Medicine 146, 114-120.

Scott, K., Taylor, L., Gill, B.P., Edwards, S.A., 2009. Influence of different types of environmental enrichment on the behaviour of finishing pigs in two different housing systems. 3. Hanging toy versus rootable toy of the same material. Applied Animal Behaviour Science 116, 186-190.

Simonsen, H.B., Klinken, L., Bindseil, E., 1991. Histopathology of intact and docked pigtails. British Veterinary Journal 147, 407-412.

Sinisalo, A., Niemi, J.K., Heinonen, M., Valros, A., 2012. Tail biting and production performance in fattening pigs. Livestock Science 143, 220-225.

Spooner, J.M., Schuppli, C.A., Fraser, D., 2014. Attitudes of Canadian Pig Producers Toward Animal Welfare. Journal of Agricultural and Environmental Ethics 27, 569-589.

Statham, P., Green, L., Bichard, M., Mendl, M., 2009. Predicting tail-biting from behaviour of pigs prior to outbreaks. Applied Animal Behaviour Science 121, 157-164.

Sutherland, M.A., Tucker, C.B., 2011. The long and short of it: A review of tail docking in farm animals. Applied Animal Behaviour Science 135, 179-191.

Taylor, N.R., Main, D.C.J., Mendl, M., Edwards, S.A., 2010. Tail-biting: A new perspective. Veterinary Journal 186, 137-147.

Teixeira, D.L., Harley, S., Hanlon, A., O'Connell, N.E., More, S.J., Manzanilla, E.G., Boyle, L.A., 2016. Study on the association between tail lesion score, cold carcass weight, and viscera condemnations in slaughter pigs. Frontiers in Veterinary Science 3, 1-7.

Telkänranta, H., Bracke, M.B.M., Valros, A., 2014. Fresh wood reduces tail and ear biting and increases exploratory behaviour in finishing pigs. Applied Animal Behaviour Science 161, 51-59.

Thodberg, K., Herskin, M.S., Jensen, T., Jensen, K.H., 2018. The effect of docking length on the risk of tail biting, tail-directed behaviour, aggression and activity level of growing pigs kept under commercial conditions. Animal 12, 2609-2618.

Trickett, S.L., Guy, J.H., Edwards, S.A., 2009. The role of novelty in environmental enrichment for the weaned pig. Applied Animal Behaviour

Science 116, 45-51.
Ursinus, Winanda W., Van Reenen, C.G., Kemp, B., Bolhuis, J.E., 2014a. Tail biting behaviour and tail damage in pigs and the relationship with general behaviour: Predicting the inevitable? Applied Animal Behaviour Science 156, 22-36.
Ursinus, Winanda W., Van Reenen, C.G., Reimert, I., Bolhuis, J.E., 2014b. Tail biting in pigs: Blood serotonin and fearfulness as pieces of the puzzle? PLoS ONE 9.
Ursinus, W. W., Wijnen, H.J., Bartel, A.C., Dijvesteijn, N., van Reenen, C.G., Bolhuis, J.E., 2014c. Damaging biting behaviors in intensively kept rearing gilts: The effect of jute sacks and relations with production characteristics. Journal of Animal Science 92, 5193-5202.
Valros, A., 2017. Tail biting. Advances in Pig Welfare 137-166.
Valros, A., Ahlström, S., Rintala, H., Häkkinen, T., Saloniemi, H., 2004. The prevalence of tail damage in slaughter pigs in Finland and associations to carcass condemnations. Acta Agriculturae Scandinavica - Section A: Animal Science 54, 213-219.
Valros, A., Barber, C., 2019. Producer perceptions of the prevention of tail biting on UK farms: Association to bedding use and tail removal proportion. Animals 9, 1-21.
Valros, A., Heinonen, M., 2015. Save the pig tail. Porcine Health Management 1, 1-7.
Valros, A., Munsterhjelm, C., Hänninen, L., Kauppinen, T., Heinonen, M., 2016. Managing undocked pigs - on-farm prevention of tail biting and attitudes towards tail biting and docking. Porcine Health Management 2, 1-11.
Valros, A., Munsterhjelm, C., Puolanne, E., Ruusunen, M., Heinonen, M., Peltoniemi, O.A.T., Pösö, A.R., 2013. Physiological indicators of stress and meat and carcass characteristics in tail bitten slaughter pigs. Acta Veterinaria Scandinavica 55, 1.
Valros, A., Palander, P., Heinonen, M., Munsterhjelm, C., Brunberg, E., Keeling, L., Piepponen, P., 2015. Evidence for a link between tail biting and central monoamine metabolism in pigs (Sus scrofa domestica). Physiology and Behavior 143, 151-157.
Van de Weerd, H.A., Day, J.E.L., 2009. A review of environmental enrichment for pigs housed in intensive housing systems. Applied Animal Behaviour Science 116, 1-20.
Van De Weerd, H.A., Docking, C.M., Day, J.E.L., Edwards, S.A., 2005. The development of harmful social behaviour in pigs with intact tails and different enrichment backgrounds in two housing systems. Animal Science 80, 289-298.
Van Der Meer, Y., Gerrits, W.J.J., Jansman, A.J.M., Kemp, B., Bolhuis, J.E., 2017. A link between damaging behaviour in pigs, sanitary conditions, and dietary protein and amino acid supply. PLoS ONE 12, 1-21.
van Putten, G., 1969. An investigation into tail-biting among fattening pigs. The British veterinary journal 125, 511-514.
Van Staaveren, N., Calderón Díaz, J.A., Garcia Manzanilla, E., Hanlon, A., Boyle, L.A., 2018. Prevalence of welfare outcomes in the weaner and finisher stages of the production cycle on 31 Irish pig farms. Irish Veterinary Journal 71, 1-9.
Van Staaveren, N., Doyle, B., Manzanilla, E.G., Calderón Díaz, J.A., Hanlon, A., Boyle, L.A., 2017a. Validation of carcass lesions as indicators for on-farm health and welfare of pigs. Journal of Animal Science 95, 1528-1536.
Van Staaveren, N., Teixeira, D.L., Hanlon, A., Boyle, L.A., 2017b. Pig carcass tail lesions: The influence of record keeping through an advisory service and the relationship with farm performance parameters. Animal 11, 140-146.
Van Staaveren, N., Vale, A.P., Manzanilla, E.G., Teixeira, D.L., Leonard, F.C., Hanlon, A., Boyle, L.A., 2016. Relationship between tail lesions and lung health in slaughter pigs. Preventive Veterinary Medicine 127, 21-26.
Veit, C., Büttner, K., Traulsen, I., Gertz, M., Hasler, M., Burfeind, O., Beilage, E. grosse, Krieter, J., 2017. The effect of mixing piglets after weaning on the occurrence of tail-biting during rearing. Livestock Science 201, 70-73.
Vermeer, H.M., Dirx-Kuijken, N.C.P.M.M., Bracke, M.B.M., 2017. Exploration feeding and higher space allocation improve welfare of growing-finishing pigs. Animals 7, 3-11.
Vom Brocke, A.L., Karnholz, C., Madey-Rindermann, D., Gauly, M., Leeb, C., Winckler, C., Schrader, L., Dippel, S., 2019. Tail lesions in fattening pigs: Relationships with postmortem meat inspection and influence of a tail biting management tool. Animal 13, 835-844. Wallenbeck, A., Keeling, L.J., 2013. Using data from electronic feeders on visit frequency and feed consumption to indicate tail biting outbreaks in commercial pig production. Journal of Animal Science 91, 2879-2884.
Wallgren, T., Larsen, A., Gunnarsson, S., 2019a. Tail posture as an indicator of tail biting in undocked finishing pigs. Animals 9.
Wallgren, T., Larsen, A., Lundeheim, N., Westin, R., Gunnarsson, S., 2019b. Implication and impact of straw provision on behaviour, lesions and pen hygiene on commercial farms rearing undocked pigs. Applied Animal Behaviour Science 210, 26-37.
Wallgren, T., Lundeheim, N., Wallenbeck, A., Westin, R., Gunnarsson, S., 2019c. Rearing pigs with intact tails-experiences and practical solutions in Sweden. Animals 9. https://doi.org/10.3390/ani9100812
Wedin, M., Baxter, E.M., Jack, M., Futro, A., D'Eath, R.B., 2018. Early indicators of tail biting outbreaks in pigs. Applied Animal Behaviour Science 208, 7-13.
Zhang, L., Gray, H., Ye, X., Collins, L., Allinson, N., 2019. Automatic individual pig detection and tracking in surveillance videos. Sensors.
Zonderland, J.J., Bracke, M.B.M., den Hartog, L.A., Kemp, B., Spoolder, H.A.M., 2010. Gender effects on tail damage development in single- or mixed-sex groups of weaned piglets. Livestock Science 129, 151-158.
Zonderland, J.J., Kemp, B., Bracke, M.B.M., Den Hartog, L.A., Spoolder, H.A.M., 2011a. Individual piglets contribution to the development of tail biting. Animal 5, 601-607.
Zonderland, J.J., Schepers, F., Bracke, M.B.M., Den Hartog, L.A., Kemp, B., Spoolder, H.A.M., 2011b. Characteristics of biter and victim piglets apparent before a tail-biting outbreak. Animal 5, 767-775. h
Zonderland, J.J., van Riel, J.W., Bracke, M.B.M., Kemp, B., den Hartog, L.A., Spoolder, H.A.M., 2009. Tail posture predicts tail damage among weaned piglets. Applied Animal Behaviour Science 121, 165-170.
Zonderland, J.J., Wolthuis-Fillerup, M., van Reenen, C.G., Bracke, M.B.M., Kemp, B., Hartog, L.A. de., Spoolder, H.A.M., 2008. Prevention and treatment of tail biting in weaned piglets. Applied Animal Behaviour Science 110, 269-281.
Zonderland J.j., Bosma B., Hoste R. 2011c Financiële consequenties van staartbijten bij varkens Wageningen UR Livestock Research, onderdeel van Stichting Dienst Landbouwkundig Onderzoek,2011
Zonderland, J.J., Zonderland-Thomassen, M.A., 2016. Behavioural change by pig producers is the key factor in raising pigs with intact tails. Veterinary Journal 211, 1-2.

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Research article
Ερευνητικό άρθρο

Seroprevalence and associated risk factors for bovine paratuberculosis in dairy cattle

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ABSTRACT: Paratuberculosis is an economically important disease in dairy cows and requires continuous surveillance. The study aimed to investigate the seroprevalence of bovine paratuberculosis (Johne's disease) in one of dairy farm in Egypt. A total of 964 dairy cattle were blood sampled and examined with an ELISA method. One-hundred fifty-five (16.1%) samples reacted positively. The results revealed that age was significantly associated with the prevalence of paratuberculosis in dairy cattle, particularly in animals over 6 years of age. Furthermore, the lactation period, milk yield and pregnancy had non-significant effect on appearance of paratuberculosis in cattle.

Keywords: *Paratuberculosis, Cattle; Seroprevalence: Risk factors; ELISA.*

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INTRODUCTION

Paratuberculosis or Johne's disease (JD) is a chronic debilitating, enteropathy disease of ruminants that is caused by bacterium *Mycobacterium avium subsp. paratuberculosis* (MAP) (Selim and Gaede, 2015; Selim et al., 2019a). The disease is responsible for economic losses worldwide in terms of medication, early culling, reduction in production, severe weight losses and reduced in body weight. The disease can be transmitted through multiple routes either horizontal route by direct transmission through ingestion of contaminated food with feces of adult infected cows or vertical route from dam to calf (Selim et al., 2013). Moreover, milk and its products being a potential source of infection for MAP in humans, causing Crohn's disease or an inflammatory bowel disease (Hruska et al., 2011).

Paratuberculosis has a prolonged incubation period and present slowly in various clinical phases. The infected animals remain in subclinical phase for long period without obvious clinical signs (Gupta et al., 2012). However, animals in advanced clinical phase become weak, emaciated, and suffer from profuse watery diarrhea. Intermandibular oedema or bottle jaw is characteristic of this stage and animals eventually die of dehydration signs and cachexia (Tiwari et al., 2006). Bovine paratuberculosis is worldwide disease, was first reported in European cattle at 1895 then has spread throughout the developed and parts of the developing world, particularly in the bovine dairy industries (Benazzi et al., 1995). Moreover, the herd prevalence level is high could be reach to 50%. Diagnosis of paratuberculosis is established by direct detection of causative agent using selective media or indirectly through detection and estimation of specific antibodies in blood sera or milk, or on the measuring of cellular immunity. The detection of antibodies using absorbed enzyme-linked immunosorbent assay (ELISA) method is considered the method of choice for the diagnosis of paratuberculosis, because of the rapidity of the test and relatively low expenses (Vidić et al., 2011; Selim et al., 2018; Selim et al., 2019b).

In Egypt, the epidemiological information about paratuberculosis is scarce despite few studies have been reported the seroprevalence of MAP among dairy cattle in some localities and detected MAP-DNA in feces of clinical infected cattle (Selim et al., 2019a; Selim et al., 2019b). Paratuberculosis is a challenging and economically important disease not only for the dairy industry, but also from a public health perspective. Thus, it becomes essential to keep on monitoring the status of disease in animals in Egypt. Therefore, this study aimed to investigate the seroprevalence of bovine paratuberculosis among dairy cattle and estimate the associate risk factors for MAP infection.

MATERIAL AND METHODS

Ethics statement

Blood samples were collected under owner's consent, and the study was approved by the Internal Ethics Review Committee of Faculty of Veterinary Medicine, Benha University.

Samples collection and preparation

A total 964 serum samples were collected from 964 cattle to study the seroprevalence of the disease in one of dairy farm in Egypt. All data related to examined animals such as age, lactation period, milk yield and pregnancy stage were collected from farm's owner. Blood samples (5ml) were collected from each animal using vacutainer tube. Serum was separated by centrifugation at 4025 x g for 10 min and stored at -20°C until further serological analysis.

Serological examination using ELISA

The antibody titer against MAP infection was determined using the commercially available IDEXX Paratuberculosis ELISA kit (IDEXX Laboratories, Inc., Westbrook, ME) according the manufacturer's instructions. The optical density (OD) was measured at 450nm using ELISA reader plate (BioTek, USA). The results were expressed based on sample/positive (S/P) ratio.

S/P = (OD of unknown sample - OD of negative-control sample)/(OD of positive-control sample - OD of negative-control sample). Samples having an S/P ratio < 0.2 were considered negative, whereas samples with an S/P ration ≥0.25 were considered a positive.

Statistical analysis

The data were analyzed by the Chi-square using SPSS V17 (IBM, USA). The results were considered significant at a probability level ≤0.05. The relation between each variable and seroprevalence of MAP infection was determined using univariant and then multivariant logistic regression model.

RESULTS

Out of 964 examined sera, the seropositive

cases of MAP infection among dairy cattle were 155 (16.1%). The logistic regression analyses of obtained results showed that the results differed according to the studied factors. The seroprevalence of bovine paratuberculosis was signific antly differed between age groups of examined cattle. The disease was more prevalent in old cattle >6 years of age (23.1%, 95%CI: 16.01-31.96) in comparison with other age groups, as shown in (Table 1). Moreover, the seroprevalence of bovine paratuberculosis showed non-significant relation with stage of milk production or milk yield, (Table 2).

In addition, the pregnancy status of cattle showed significant relationship with number of seropositive MAP cases, where the seroprevalence of MAP infection was more prevalent in non-pregnant cattle (21.7%, 95%CI: 16.34-28.1) and during late stage of pregnancy at third trimester (16.6%, 95%CI: 12.56-21.45), (Table 3).

Two risk factors were fitted for multivariant logistic regression model, (Table 4). The results revealed that the risk of MAP infection increased in cattle of >6 years old (OR=3.5, 95%CI: 1.76-7.08), cattle of middle age (OR=2.7, 95%CI: 1.48-4.94) and in heavy pregnant animal during third trimester of pregnancy (OR=1.9, 95%CI: 0.58-6.79).

Table 1: Age related seroprevalence of bovine paratuberculosis in cattle based on ELISA

Factor	No of examined animals	No of positive (%)	95%CI	P value
Age				
<2 years	180	14 (7.8%)	4.48-12.97	
2-3 years	264	39 (14.8%)	10.8-19.76	0.001*
3-6 years	403	75 (18.6%)	15-22.83	
>6 years	117	27 (23.1%)	16.01-31.96	
Total	964	155 (16.1)	13.85-18.59	

*The results are significant at $p < 0.05$

Table 2: The relation of milk production and stages with seroprevalence of bovine paratuberculosis

Factor	No of examined animals	No of positive (%)	95%CI	P value
Lactation stage				
Early stage (<3months)	210	42 (*20%*)	14.94-26.18	
Mid stage (3-6 months)	62	10 (*16.1%*)	8.41-28.13	0.7*
Late stage (>6 months)	269	53 (*19.7%*)	15.22-25.06	
Dry stage	140	23 (*16.4%*)	10.91-23.85	
Milk yield				
<20	244	54 (*22.1%*)	17.19-27.97	
20-30	233	39 (*16.7%*)	12.31-22.3	0.4*
>30	64	12 (*18.8%*)	10.47-30.85	
Dried cows	140	23 (*16.4%*)	10.91-23.85	

The results are not significant at $p > 0.05$

Table 3: Relation between pregnancy state and seroprevalence of bovine paratuberculosis

Pregnancy state		No of examined animal	No of positive (%)	95%CI
Non-pregnant		203	44 (*21.7%*)	16.34-28.1
pregnant	1st trimester	33	3 (*9%*)	2.38-25.47
	2nd trimester	185	19 (*10.3%*)	6.46-15.8
	3rd trimester	290	48 (*16.6%*)	12.56-21.45
	Total	711	114 (*16%*)	13.45-18.98

The results are significant at P=0.01

Table 4: Risk factors associated with MAP seropositivity in dairy cattle

Risk factors	OR	95% of OR	P value
Age			
<2 years		ref	
2-3 years	2.03	1.06-3.86	0.03
3-6 years	2.7	1.48-4.94	0.001
>6 years	3.5	1.76-7.08	0.001
Pregnancy status			
1st trimaster		ref	
2nd trimaster	1.1	0.31-4.10	0.8
3rd trimaster	1.9	0.58-6.79	0.2
non-pregnant	2.7	0.80-9.49	0.1

OR; Odds ratio
CI; Confidence interval
The result is significant at $P < 0.05$

DISCUSSION

Johne's disease is one of the most economically important diseases of dairy farm and believed to be a potential public health hazard. The infected cows usually suffer from weight loss, diarrhea, decreased milk production and even death. Diagnosis of MAP usually based on detection of MAP itself or the host's immune response against it (Timms et al., 2011; El-haig et al., 2018; Selim et al., 2020b).

The ELISA is the most sensitive and specific test for detection of serum antibodies against MAP-infection in cattle (Speer et al., 2006). ELISA is considered a method of choice for diagnosis of Johne's disease in positive herds. This is due to the ease of sample collection, rapid procedure, low cost and possibility of testing a large number of samples in a short time (Gupta et al., 2012; Abraham et al., 2014; Ali et al., 2019; Selim and Ali, 2020).

A total 964 cattle were examined for anti-MAP antibodies by ELISA. Out of them 155 (16.1%) animals reacted positively. The prevalence of bovine paratuberculosis came in accordance with other previous rate 15% in lower saxony, Germany (Böttcher, 1997). In contrast, the obtained seroprevalence rate was higher than previous rate, 2.4 % in Italy (Lillini et al., 2005), 5.5% in Germany (Donat et al., 2005) and 12.6% in Bhutan, Austria (Gurung et al., 2018) but lower than seroprevalence rate in Austria, 19% (Dreier et al., 2006). The difference of seroprevalence rate in different studies may be attributed to size of sample collected and method of diagnosis (Gurung et al., 2018; Selim et al., 2020a).

The seroprevalence of bovine paratuberculosis had significant disparity between different age groups of examined cattle. The disease was more prevalent among elder cattle (>6 years old) in comparison with young ones. Similar to our findings, other studies reported a relatively higher prevalence of disease in older age animals than that of young ones (Woodbine et al., 2009; Hussain et al., 2018). We believe that the disease was more frequent among animals due to frequent exposure of infection during life and decrease the immunity of animals with advancing of age (Harris and Barletta, 2001; Hailat et al., 2010).

Interestingly, the results of the present study revealed a non-significant relation between stage of milking or milk yield in dairy cattle and seropositivity to MAP infection. The result was disagree with (Hussain et al., 2018), they reported the highest prevalence rate of paratuberculosis in late stage of lactation, that

could be attributed to stress of lactation and increase susceptibility to get MAP infection (Rathnaiah et al., 2017). Further, the seroprevalence of MAP infection more common in lactating animals may be attributed to milk production considered as a stress factor on animals or due to some genetic association in high producing animals (Sorge et al., 2011).

In addition, the assessment of risk factors associated with MAP infection revealed strong relationship between age and pregnancy status of animals and seroprevalence of MAP infection. Similar results were concluded by (Sergeant and Baldock, 2002; Khan et al., 2010; Selim et al., 2019b).

CONCLUSIONS

The present study confirmed the presence of antibodies of MAP among dairy cattle in Egypt and give attention to necessary for application of large-scale screening for bovine paratuberculosis in Egypt. The present study revealed strong association between seroprevalence of MAP and age and pregnancy status of animals. Thus, the periodical screening of old or pregnant animals is very important to identify the infected animals and for early control.

CONFLICT OF INTEREST

None declared by the authors.

REFERENCES

Abraham, A., Thomas, N., Joseph, S., Raghavan, K., 2014. Comparitive study on the efficacy of ELISA and IS900 PCR for the diagnosis of paratuberculosis in goats. Biotechnology in Animal Husbandry 30, 661-667.

Ali, A.-F., Selim, A., Manaa, E.A., Abdelrahman, A., Sakr, A., 2019. Oxidative state markers and clinicopathological findings associated with bovine leukemia virus infection in cattle. Microbial pathogenesis 136, 103662.

Benazzi, S., Berrada, J., Schliesser, T., 1995. First report of paratuberculosis (Johne's disease) in sheep in Morocco. Journal of Veterinary Medicine, Series B 42, 339-344.

Böttcher, M., 1997. Kulturelle und serologische Untersuchungen zur Prävalenz der Paratuberkulose im Regierungsbezirk Arnsberg. Tierärztliche Hochschule Hannover.

Donat, K., Eulenberger, K., Kampfer, P., 2005. Blutserologische Untersuchungen zur Verbreitung von Mycobacterium avium spp. paratuberculosis in sachsischen Rinderbestanden. Tieraerztliche Umschau 60, 497-501.

Dreier, S., Khol, J., Stein, B., Fuchs, K., Gütler, S., Baumgartner, W., 2006. Serological, bacteriological and molecularbiological survey of paratuberculosis (Johne's disease) in Austrian cattle. Journal of Veterinary Medicine, Series B 53, 477-481.

Elhaig, M.M., Selim, A., Mandour, A.S., Schulz, C., Hoffmann, B., 2018. Prevalence and molecular characterization of peste des petits ruminants virus from Ismailia and Suez, Northeastern Egypt, 2014-2016. Small ruminant research 169, 94-98.

Gupta, A., Rani, S.M., Agrawal, P., Gupta, P.K., 2012. Sero-prevalence of paratuberculosis (Johne's Disease) in cattle population of South-Western Bangalore Using ELISA Kit. Open Journal of Veterinary Medicine 2, 196.

Gurung, R.B., Begg, D.J., Whittington, R.J., 2018. A national serosurvey to determine the prevalence of paratuberculosis in cattle in Bhutan following detection of clinical cases. Veterinary medicine and science 4, 288-295.

Hailat, N., Hananeh, W., Metekia, A., Stabel, J., Al-Majali, A., Lafi, S., 2010. Pathology of subclinical paratuberculosis (Johne's Disease) in Awassi sheep with reference to its occurrence in Jordan.

Harris, N.B., Barletta, R.G., 2001. Mycobacterium avium subsp. paratuberculosisin veterinary medicine. Clinical microbiology reviews 14, 489-512.

Hruska, K., Slana, I., Kralik, P., Pavlik, I., 2011. Mycobacterium avium subsp. paratuberculosis in powdered infant milk: F57 competitive real time PCR. Veterinarni Medicina 56, 226-230.

Hussain, S.M., Javed, M.T., Rizvi, F., Qamar, M., 2018. Prevalence of paratuberculosis in cattle and buffaloes in Punjab Pakistan. Pakistan Journal of Agricultural Sciences 55.

Khan, F.A., Chaudhry, Z.I., Ali, M.I., Khan, S., Mumtaz, N., Ahmad, I., 2010. Detection of Mycobacterium avium subsp. paratuberculosis in tissue samples of cattle and buffaloes. Tropical animal health and production 42, 633-638.

Lillini, E., Bitonti, G., Gamberale, F., Cersini, A., 2005. Prevalence of bovine paratuberculosis in the Latium region (Italy). In, Proceedings of the 8th International Colloquium on Paratuberculosis, Copenhagen, Denmark, 17.

Rathnaiah, G., Zinniel, D.K., Bannantine, J.P., Stabel, J.R., Gröhn, Y.T., Collins, M.T., Barletta, R.G., 2017. Pathogenesis, molecular genetics, and genomics of Mycobacterium avium subsp. paratuberculosis, the etiologic agent of Johne's disease. Frontiers in veterinary science 4, 187.

Selim, A., Abdelhady, A., Abdelrahman, A., 2020a. Ovine Paratuberculosis: Seroprevalence and comparison of fecal culture and direct fecal PCR assay. Comparative Immunology, Microbiology and Infectious Diseases, 101526.

Selim, A., Ali, A.-F., 2020. Seroprevalence and risk factors for C. burentii infection in camels in Egypt. Comparative Immunology, Microbiology and Infectious Diseases 68, 101402.

Selim, A., Ali, A.-F., Moustafa, S.M., Ramadan, E., 2018. Molecular and serological data supporting the role of Q fever in abortions of sheep and goats in northern Egypt. Microbial pathogenesis 125, 272-275.

Selim, A., Ali, A.-F., Ramadan, E., 2019a. Prevalence and molecular epidemiology of Johne's disease in Egyptian cattle. Acta tropica 195, 1-5.

Selim, A., El-Haig, M., Galila, E.S., Gaede, W. 2013. Direct detection of Mycobacterium avium subsp. Paratuberculosis in bovine milk by multiplex Real-time PCR. Animal Science Papers and Reports, 2013, 31(4), 291-302.

Selim, A., Gaede, W., 2015. Comparative evaluation of PCR assay for direct detection of Mycobacterium avium subsp. paratuberculosis in Ruminant. Asian J Anim Vet Adv 10, 761-771.

Selim, A., Marawan, M.A., Ali, A.-F., Manaa, E., AbouelGhaut, H.A., 2019b. Seroprevalence of bovine leukemia virus in cattle, buffalo, and camel in Egypt. Tropical animal health and production, 1-4.

Selim, A., Radwan, A., Hamouda, F., 2020b. Seroprevalence and Molecular Characterization of West Nile Virus in Egypt. Comparative Immunology, Microbiology and Infectious Diseases, 101473.

Sergeant, E., Baldock, F., 2002. The estimated prevalence of Johne's disease infected sheep flocks in Australia. Australian veterinary journal 80, 762-768.

Sorge, U.S., Lissemore, K., Godkin, A., Hendrick, S., Wells, S., Kelton, D., 2011. Associations between paratuberculosis milk ELISA result, milk production, and breed in Canadian dairy cows. Journal of dairy science 94, 754-761.

Speer, C., Scott, M.C., Bannantine, J.P., Waters, W.R., Mori, Y., Whitlock, R.H., Eda, S., 2006. A novel enzyme-linked immunosorbent assay for diagnosis of Mycobacterium avium subsp. paratuberculosis infections (Johne's Disease) in cattle. Clin. Vaccine Immunol. 13, 535-540.

Timms, V.J., Gehringer, M.M., Mitchell, H.M., Daskalopoulos, G., Neilan, B.A., 2011. How accurately can we detect Mycobacterium avium subsp. paratuberculosis infection? Journal of microbiological methods 85, 1-8.

Tiwari, A., VanLeeuwen, J.A., McKenna, S.L., Keefe, G.P., Barkema, H.W., 2006. Johne's disease in Canada: Part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. The Canadian Veterinary Journal 47, 874.

Vidić, B., Savić, S., Prica, N., 2011. Paratuberculosis in cattle: The outspread, diagnostics and control. Biotechnology in Animal Husbandry 27, 335-344.

Woodbine, K.A., Schukken, Y.H., Green, L.E., Ramirez-Villaescusa, A., Mason, S., Moore, S.J., Bilbao, C., Swann, N., Medley, G.F., 2009. Seroprevalence and epidemiological characteristics of Mycobacterium avium subsp. paratuberculosis on 114 cattle farms in south west England. Preventive veterinary medicine 89, 102-109.

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Research article
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Assessment of oxidative stress, trace elements, serum biochemistry, and hormones levels in weaned calves with dermatophytosis

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ABSTRACT: In this study, it was aimed to evaluate oxidative stress, serum biochemistry, trace elements, minerals, and testosterone and thyroid hormone levels in weaned calves with dermatophytosis. A total of 28 weaned Holstein calves were used in the study, including 6-8 months old, 14 with dermatophytosis (7 males, 7 females) and 14 healthy (7 males, 7 females). The animals were grouped as the diseased and healthy animals, 14 animals in each group as well as the male diseased and the male healthy animals were grouped as 7 animals in each group for the comparison of testosterone levels. The blood analyses were performed using ELISA kits and biochemistry automatic analyzer. There was a significant difference between the diseased and healthy groups for NO (nitric oxide) ($P<0.05$), TOS (total oxidative stress) ($P<0.001$), TAC (total antioxidant capacity) ($P<0.01$). However, in comparison of the diseased and healthy groups, serum biochemistry with the exception of glucose and triglyceride,trace elements except for manganese, minerals, and thyroid hormone levels were not statistically different ($P>0.05$). In comparison of the diseased and healthy animals for testosterone levels, it was not determined any difference ($P>0.05$). The present study revealed that dermatophytosis could affect oxidant status in calves with dermatophytosis, and that TOC (total oxidant capacity) and NO as oxidative stress marker might be increased for fungicidal effect in the diseased animals with dermatophytosis.

Keywords: Calves with dermatophytosis, oxidative stress, trace elements, serum biochemistry, hormones.

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INTRODUCTION

Dermatophytosis commonly occurs in humans and animals and is an infectious diseases characterized by keratinization in stratum corneum layer of skin and hair loss. This zoonotic disease is of economic importance because of difficult control, contagious, and high cost of treatment (Chermette et al., 2008; Radostits et al., 2007). Dermatophytosis is caused by *Trichophyton*, *Microsporum* and *Epidermophyton* species in domestic animals (Deacon, 1988; Kahn and Line, 2010; Radostits et al., 2007). It is reported that *T. verrucosum* generally causes the disease in cattle (Al-Qudah, et al., 2010; Papini et al., 2009). Factors such as keratinase enzyme, hemolytic activity, humidity, pH, fatty acids of the skin, amino acids, hormones, individual resistance and immune response have important roles in the development of disease (Hashemi and Sarasgani, 2004; Pal and Dave, 2013; Radostits et al., 2007; Schaufuss and Steller, 2003; Xavier et al., 2008).

In calves, dermatophytosis is characterized by non-pruritic lesions around the eye, ear, and dorsum, and sometimes also develops in the generalized form (Kahn and Line, 2010). There are characteristic lesions with scaling patches of hair loss, and gray-white crust formation, but sometimes these lesions have thick crusts with suppuration (Kahn and Line, 2010).

In cattle, deficiency of minerals and trace elements causes growth retardation, immune system deficiency and dermatological lesions (Kahn and Line, 2010; Radostits et al., 2007). Zinc and selenium play roles in numerous metabolic reactions in the body (Shafiei Neek et al., 2011). Zinc deficiency may lead to the development of dermatophytosis, chronic infection and expansion of lesion area (Szczepanik and Wilkolek, 2004).

In recent years, the effect of reactive oxygen species (ROS) on the body defense system has become important in farm animals (Castillo et al., 2003). Reactive oxygen species are produced as by-products due to cellular metabolism in low concentrations for numerous physiological processes including activation of transcription factors, cell immunity, and cellular defense against microorganisms (Miller et al., 1993; Zhang et al., 2016). Reactive oxygen species are increased during diseases and pathological changes in the organism and oxidative stress occurs because of deficiency of antioxidants and increase of oxidants (Lykkesfeldt and Svendsen, 2007; Roth, 1997). In bovine animals oxidative stress has been reported in various diseases caused by pneumonia, enteritis, sepsis, mastitis (Atakisi et al., 2010; Erkilic et al., 2016; Lykkesfeldt and Svendsen, 2007; Schott et al., 2014). In recent years, oxidative status has been investigated to enlighten the pathogenesis of dermatophytosis in animals and humans (Beigh et al., 2014; Karapehlivan et al., 2007; Kurutas and Ozturk, 2016).

In humans, pathogenic fungi and yeasts are reported to be affected by steroids, and this has become special area of interest in clinical research (Brasch, 1997; Clemons et al., 1988). An in vitro study has demonstrated that the growth of *T. rubrum* and *E. floccosum* is suppressed by androgenic hormones but *T. mentagrophytes* and *M. canis* is least responsive to most hormones (Brasch and Flader, 1996). In addition, testosterone levels in patients with dermatophytosis caused by *E. floccosum* decreased compared to healthy subjects but testosterone levels were not different between the patients with *T. rubrum* and healthy subjects (Brasch and Flader, 1996). However, to the best knowledge of authors, the role of androgenic hormones is not known in male calves with dermatophytosis.

Deficiency of minerals and trace elements leads to immune system deficiency, dermatological lesions and dermatophytosis. In addition, oxidative status has been shown in the pathogenesis of infectious diseases and the role of androgenic hormones is not known in male calves with dermatophytosis. In this study, it was aimed to evaluate oxidative stress, serum biochemistry, trace elements, minerals, and testosterone and thyroid hormone levels in calves with dermatophytosis.

MATERIALS AND METHODS

Animals

This study was carried out on total 28 weaned Holstein calves, 6-8 month old, 14 with dermatophytosis (7 males and 7 females) and 14 healthy weaned calves (7 males and 7 females). Each group consisted of 14 weaned calves. Group I was the female and male diseased calves; Group II was the female and male healthy calves. All calves in the study were from one farm and all animals were kept under similar management conditions and were not kept overcrowded (including 96 calves in herd). All calves were healthy at weaning and throughout the study period except for dermatophytosis. Clinical examinations were performed by the same clinician (KS) and took skin scraping and hair samples randomly from the 14 calves of 45 calves with suspected dermatophytosis.

Microbiological analysis

Skin scrapings and hair samples (in calves showed skin lesions) were in part processed for microscopy by use of 10-20% potassium hydroxide (KOH) and after 30 min examined under 400X magnification of the light microscope. Rest of the samples was seeded on the Sabouraud Dextrose agar (SDA, OXOID) supplemented with chloramphenicol (0.05 mg/mL), and plates were incubated at 25°C and 37°C for a period of 1-4 weeks and examined on a daily basis as noted by studies (Larone, 1995; Quinn et al., 1999). The isolated fungal colonies were stained with lactophenol blue. The macro- and microscopic characteristics of isolates were detected as *Trichophyton* sp. (Larone, 1995; Robert et al., 2008).

Blood sampling

Blood samples were obtained from the calves by venipuncture of *vena jugularis* to vacutainer tubes. The blood samples were centrifuged at 3,000 rpm for 10 min and the serum samples were allocated to Eppendorf tubes and stored at -20 °C until analyses.

Total antioxidant capacity (TAC) analysis

Determination of TAC levels was performed using a novel automated colorimetric measurement method (Erel, 2004). The assay has finest quality precision values, lower than 3%. The results were indicated as mmolTrolox Equivalents/L for serum.

Total oxidant capacity (TOC) analysis

Determination of TOC levels was performed using a novel automated measurement method (Erel, 2005). The results were indicated as mmol H_2O_2 Equivalents/L for serum.

Plasma total nitric oxide (NO) analysis

The plasma total NO level measurement was performed by colorimetric method using NO detection kit (Enzo Life Science).

Serum biochemistry analyses

Serum alkaline phosphatase (ALP), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), total protein, albumin, glucose, urea, creatinine, total bilurubin, triglyceride (TG), total cholesterol, low density lipoprotein cholesterol (LDL-C), calcium (Ca), magnesium (Mg), retinol and β-carotene levels were determined by colorimetric method by a biochemistry auto analyzer using commercial test kits (Beckman Coulter, AU5800, USA).

Serum trace elements analyses

The serum copper (Cu), zinc (Zn), iron (Fe) were measured by flame atomic absorption (flame-AA) spectroscopy (Perkin Elmer AAS 800). Selenium (Se) was measured by hydride system atomic absorption (hydride-AA) spectroscopy. Manganese (Mn) levels were measured using atomic absorption spectrometer-graphite furnace system (graphite furnace-AAS).

Hormone analyses

Testosterone hormone, thyroid stimulating hormone (TSH), free triiodothyronine (fT3), and free thyroxin (fT4) levels were analyzed by chemiluminescence method (Beckman Coulter DXI 800, USA).

Statistical analysis

The comparison of the data between the diseased group and the healthy group was performed (SPSS, Version 11.5 Microsoft, Chicago, IL, USA) by using independent samples t test and Mann-Whitney *U* tests in 95% confidence interval. The significance degree between two groups was determined to be $P<0.05$. Data were expressed as mean ± standard error of the mean (SEM) and as median (minimum - maximum).

RESULTS

Oxidative stress markers such as TOC, TAC and NO levels in the weaned calves with dermatophytosis and in the healthy calves are given in Table 1. Total NO, TOC levels in calves with dermatophytosis were significantly increased compared to the healthy calves. TAC levels were significantly decreased in calves with dermatophytosis compared to the healthy calves.

The levels of trace elements and minerals are given in Table 2. The levels of these parameters except for manganese were not significantly different between the diseased and healthy calves. However, manganese levels were significantly lower than those of the healthy calves. Other elements (Cu, Se, Zn, Fe, Ca, Mg) were not found to be statistically important.

The levels of serum biochemistry parameters and hormones such as testosterone, TSH, fT_3, and fT_4 are given in Table 3. The levels of these parameters were not significantly different between the diseased and the healthy calves. The serum glucose and TG levels were significantly increased in the diseased animals over those in the healthy animals.

Table 1. Levels of total oxidant capacity, total antioxidant capacity and nitric oxide in calves with dermatophytosis and in the healthy calves

Parameter	Group I (n=14)	Group II (n=14)
Total NO (µmol/L)	70.09±3.40[a]	50.96±3.54[b]
TAC (mmolTrolox Equiv./L)	2.13 (1.78-2.61)[a]	2.86 (2.11-3.12)[b]
TOC (µmol H_2O_2 Equiv./L)	4.41±0.07[a]	3.54±0.09[b]

Group I: Calves with dermatophytosis; Group II: Healthy calves; NO: Nitric oxide; TAC: Total antioxidant capacity; TOC: Total oxidant capacity. The total NO, and TOC values were given as mean ± SEM for each group including 14 weaned calves, and the TAC values were given as median (minimum - maximum) for each group including 14 weaned calves.
Means with different superscripts in the same row are significantly different (P<0.05).

Table 2. Levels of trace elements (Cu, Zn, Se, Fe, and Mn) and minerals (Ca and Mg) in calves with dermatophytosis and in the healthy calves

Parameter	Group I (n=14)	Group II (n=14)
Cu (ppm)	0.43±0.02	0.45±0.01
Zn (ppm)	0.77±0.06	0.71±0.04
Se (ppb)	13.77±1.31	15.70±0.88
Fe (ppm)	0.50±0.03	0.48±0.02
Ca (mg/dL)	9.5 (6.46-15.15)	9.15 (3.41-9.68)
Mg (mg/dL)	4.39±0.17	4.19±0.20
Mn (ppm)	1.88±0.13[a]	3.05±0.17[b]

Group I: Calves with dermatophytosis; Group II: Healthy calves. The Cu, Zn, Se, Fe, Mg and Mn values were given as mean ± SEM for each group including 14 weaned calves, and the Ca values were given as median (minimum - maximum) for each group including 14 weaned calves.
Means with different superscripts in the same row are significantly different (P<0.05).

Table 3. Levels of serum biochemistry, and thyroid hormones in calves with dermatophytosis and in the healthy calves

Parameter	Group I (n=14)	Group II (n=14)
Triglyceride (mg/dL)	27.71±2.88[a]	19.00±1.99[b]
Total cholesterol (mg/dL)	82.28±5.74	74.84±4.33
LDL-C (mg/dL)	38.07±3.21	35.46±2.53
Urea (mg/dL)	14.28±1.08	15.53±1.35
Creatinine (mg/dL)	0.7 (0.6-0.9)	0.8 (0.6-0.9)
Total bilirubin (mg/dL)	0.18±0.005	0.19±0.008
Glucose (mg/dL)	70.64±2.76[a]	62.84±3.46[b]
Total protein (g/dL)	6.65 (6.30-8.60)	6.70 (4.50-8.10)
Albumin (g/dL)	2.80±0.07	2.81±0.10
AST (U/L)	87±4.59	94.46±5.46
GGT (U/L)	18.85±0.98	17.07±1.04
ALP (U/L)	133.50 (89-222)	102.0 (50-222)
Retinol (mg/dL)	26.19 (17.56-41.04)	23.34 (16.64-37.50)
β-carotene (mg/dL)	12.77 ±1.18	12.85±1.45
TSH uIU/mL	0.18 (0.0-9.0)	0.14 (0.0-2.05)
Free triiodothyronine(fT_3)pg/mL	2.78 (2.39-4.07)	3.19 (1.66-3.88)
Free thyroxin (fT_4) ng/dL	0.79 ± 0.04	0.83 ± 0.05

Group I: Calves with dermatophytosis; Group II: Healthy calves. The triglyceride, total cholesterol, LDL-C, urea, total bilirubin, glucose, albumin, AST, GGT, β-carotene, and free thyroxin values were given as mean ± SEM for each group including 14 weaned calves. The creatinine, total protein, ALP, retinol, TSH, and free triiodothyronine values were given as median (minimum - maximum) for each group including 14 weaned calves.
Means with different superscripts in the same row are significantly different (P<0.05).

Table 4. Serum testosterone levels in the calves with dermatophytosis and in the healthy calves

Parameter	Group I (n=7)	Group II (n=7)
Testosterone (ng/dL)	1.64 ± 0.24	3.66±0.96

Group I: Calves with dermatophytosis; Group II: Healthy calves. The testosterone values were given as mean ± SEM for each group including 7 male weaned calves.

DISCUSSION

Zoophilic dermatophytes induced infections are acute or chronic and highly inflammatory. Keratinocytes, after infected with a zoophilic dermatophyte, express pro-inflammatory genes and secrete cytokines to contribute recruitment of inflammatory cells in the skin, tissue remodeling and wound healing (Martinez-Rossi et al., 2017). Experimental dermatophyte infection in mice has showed that dermal inflammation and histopathologically macrophages, dendritic cells, neutrophils are present. Inflammation also results in cytokine over expression such as transforming growth factor-β, interleukin-1β, and IL-6 (Cambier et al., 2014). The pathogens are cleared in the body by ROS produced by phagocyte cells (Mittal et al., 2014). In addition, during inflammation, macrophages and neutrophils produce NO for microbicidal effect (Mizokami et al., 2016). Comply with these studies, in the present study, significant increase in TOC and NO levels was found in calves with dermatophytosis compared to the healthy calves. This suggested that TOC and NO as oxidative stress marker might be increased for fungicidal effect in the groups with dermatophytosis.

Oxidative stress as revealed by high MDA levels and lower SOD and catalase levels has been reported in dogs with dermatophytosis (Beigh et al., 2014). Similarly, in calves with dermatophytosis, oxidative stress through high MDA and NO, and low antioxidant GSH has been reported (Karapehlivan et al., 2007). In addition, significant oxidative/nitrosative stress revealed by increased MDA, NO and 3-NT levels have been demonstrated in patients with pityriasis versicolor (Kurutas and Ozturk, 2016). Similarly, in the present study, oxidative stress was found in calves with dermatophytosis via increased TOC and NO, and decreased TAC levels. This study suggested that oxidative stress might mediate the fungicidal activity in calves with dermatophytosis.

Trace elements such as Cu, Zn, Se, Mn and Fe have cofactor roles in antioxidant enzymes. Several studies have demonstrated that trace elements have important roles in antioxidant enzymes expression and activities. For example, a selenium, zinc, copper, iron, and manganese deficient diet has been demonstrated to cause a significant decrease in GSH-Px, Cu,Zn-SOD, catalase and GSH-Px, and Mn-SOD activities (Gong and Xiao, 2018; Malecki and Greger, 1996; Prohaska and Brokate, 2001; Toyoda et al., 1989). The deficiencies of trace elements may be caused by dietary imbalances or diseases. In calves with dermatophytosis, trace elements such as whole blood Se, serum Zn, and Cu levels have been significantly decreased in line with significant reduction in antioxidant defense systems including GSH-Px activities and glutathione levels by attributing to possible dermatophyte consumption (Al-Qudah et al., 2010). In other studies, significant decrease in serum zinc levels without changes in blood leukocytes levels (Nisbet et al., 2006), significant decrease in serum Mn and Zn levels and increase in Cu levels in bovine dermatophytosis (Paksoy et al., 2013), and significant decrease in serum Fe in cattle with dermatophytosis (Yildirim et al., 2010) have been reported. In contrast to the results of those studies, in the present study, serum Cu, Zn, Se, Fe levels were not different statistically in the group with dermatophytosis compared to the healthy control group. The serum Mn levels were significantly decreased in calves with dermatophytosis than in the healthy calves in line with the result of Paksoy et al. (2013). The serum Ca and Mg minerals between the group with dermatophytosis and the healthy group were not different statistically. This study did not found any significant difference except Mn in serum trace elements and serum minerals between the diseased group and the healthy group. Similarly, the serum Zn and Cu levels were not statistically different between young cattle with dermatophytosis and the healthy young cattle (Aslan et al., 2010). In addition, a recent study in patients with tinea pedis has revealed that zinc and selenium levels are significantly lower on the lesion site than those on the healthy site, but Cu levels are significantly higher on the lesion site than those on the healthy site. In addition, positive correlation between the lesional area Cu and the lesional area 8-iso-$PGF_{2\alpha}$ (lipid peroxidation product) has been demonstrated (Miraloglu et al., 2016). It is thought that evaluating oxidant status and trace ele-

ments on the lesional site can provide better knowledge in elucidating the dermatophytosis pathogenesis.

Significant decrease in the serum Zn and vitamin A levels has been reported in calves with dermatophytosis (Pasa and Kiral, 2009). Several studies have reported that the levels of vitamin A may be changed in infection conditions (Bendich, 1993; Chew, 1987; Or et al., 2002). Zinc has the effects on vitamin A metabolism such as absorption, transport and utilization through protein synthesis, and Zn-dependent dehydrogenase enzyme (Christian and West, 1998). These studies have determined the important association between zinc and vitamin A. In the present study, contrary to the findings of Pasa and Kiral (2009), retinol and β-carotene levels were also in normal ranges in line with normal serum Zn levels in calves with dermatophytosis.

In addition, the present study found no statistical difference in the serum biochemical parameters such as triglyceride, total cholesterol, LDL, urea, creatinine, total bilirubin, glucose, total protein, albumin, TSH, fT_3, fT_4, AST, GGT, ALP in calves with dermatophytosis compared to healthy calves. This revealed that the calves with dermatophytosis may have normal organ functions. However, in a study (Atakisi et al., 2006) evaluated serum adenosine deaminase and liver function tests in dermatophytic cattle, increased adenosine deaminase, GGT, ALT, AST, and LDH levels have been found and it was thought to be associated with possible liver damage due to the toxic metabolic products of the fungi.

Androgenic hormones can affect fungal growth in male patients with dermatophytosis (Hashemi and Sarasgani, 2004). Serum testosterone levels have been reported to significantly decrease in patients with dermatophytosis caused by *E. floccosum* (Hashemi and Sarasgani, 2004). In contrast to *E. floccosum*, *T. mentagrophytes* and *M. canis* are less susceptible to the androgenic hormones (Brasch and Flader, 1996). In the present study, testosterone levels were not significantly different between male calves with dermatophytosis and the healthy male calves but the male calves with dermatophytosis had non-significant reduction of testosterone levels compared to the male healthy calves.

CONCLUSIONS

The present study revealed that dermatophytosis might affect oxidant status in calves with dermatophytosis and TOC and NO as oxidative stress marker might be increased for fungicidal effect in the groups with dermatophytosis. In addition, serum biochemistry parameters including thyroid and testosterone hormones with the exception of glucose and triglyceride, trace elements except for Mn and minerals were found to be in normal ranges. However, future studies with larger sample sizes are needed to be conducted for changes in testosterone levels.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Al-Qudah KM, Gharaibeh AA, Al-Shyyab MM (2010) Trace minerals status and antioxidant enzymes activities in calves with dermatophytosis. Biol Trace Elem Res136(1):40-47. doi:10.1007/s12011-009-8525-4

Aslan O, Aksoy A, Içа T (2010) Serum concentrations of zinc, copper, manganese in young cattle with dermatophytosis. J Fac Vet Med Univ Erciyes 7(1) 29-33.

Atakisi E, Karapehlivan M, Atakişi O, Kontaş T, Maraşlı Ş. (2006) Adenosine deaminase and biochemical liver function tests in the dermatophytic cattle. Bull Vet Inst Pulawy 50, 481-483.

Atakisi O, Oral H, Atakisi E, Merhan O, Pancarci SM, Ozcan A, Marasli S, Polat B, Colak A, Kaya S (2010) Subclinical mastitis causes alterations in nitric oxide, total oxidant and antioxidant capacity in cow milk. Res Vet Sci89(1):10-13.

Beigh SA, Soodan JS, Singh R, Khan AM, Dar MA (2014) Evaluation of trace elements, oxidant/antioxidant status, vitamin C and beta-carotene in dogs with dermatophytosis. Mycoses 57(6):358-365. doi:10.1111/myc.12163

Bendich A (1993) Physiological role of antioxidants in the immune system. J Dairy Sci 76(9):2789-2794.

Brasch J (1997) Hormones, fungi and skin. Mycoses 40:11.

Brasch J, Flader S (1996) Human androgenic steroids affect growth of dermatophytes in vitro. Mycoses 39(9-10):387-392.

Cambier L, Weatherspoon A, Defaweux V, Bagut ET, Heinen MP, Antoine N, Mignon B (2014) Assessment of the cutaneous immune response during *Arthroderma benhamiae* and *A. vanbreuseghemii* infection using an experimental mouse model. Br J Dermatol170(3):625-633. doi:10.1111/bjd.12673

Castillo C, Hernandez J, Lopez-Alonso M, Miranda M, Benedito JL (2003). Values of plasma lipid hydroperoxides and total antioxidant status in healthy dairy cows: preliminary observations. Archives of Animal Breeding 46, 227-233.

Chermette R, Ferreiro L, Guillot J (2008) Dermatophytoses in animals. Mycopathologia 166(5-6):385-405.

Chew BP (1987) Vitamin A and β-carotene on host defense. J Dairy Sci 70(12):2732-2743.

Christian P, West KP Jr (1998) Interactions between zinc and vitamin A: an update. Am J Clin Nutr 68(2 Suppl):435-441. doi:10.1093/ajcn/68.2.435S

Clemons KV, Schär G, Stover EP, Feldman D, Stevens D (1988) Dermatophyte-hormone relationships: characterization of progesterone-binding specificity and growth inhibition in the genera *Trichophyton* and *Microsporum*. J Clin Microbiol26(10): 2110-2115.

Deacon JW (1988) Introduction to modern mycology, 2nd ed. Oxford: Blackwell Scientific Publications. 1-239.

Erel O (2004) A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem37(4):277-285.

Erel O (2005) A new automated colorimetric method for measuring total oxidant status. Clin Biochem38(12):1103-1111.

Erkilic E, Erdogan H, Ogun M, Kirmizigul A, Gokce E, Kuru M, Kukurt A (2016) Relationship between hepcidin and oxidant/antioxidant status in calves with suspected neonatal septicemia. Vet World 9(11): 1238-1241.

Gong J, Xiao M (2018) Effect of organic selenium supplementation on selenium status, oxidative stress, and antioxidant status in selenium-adequate dairy cows during the periparturient period. Biol Trace Elem Res. 186(2):430-440. doi: 10.1007/s12011-018-1323-0.

Hashemi SJ, Sarasgani M (2004) A study for determination of relationship between serum testosterone concentration and dermatophytosis due to *Epidermophyton floccosum* in patients. Iranian J Publ Health 33(1):10-12.

Kahn C, Line S (2010) Dermatophytosis. In: The Merck Veterinary Manual. USA: Merck Co., Inc. Whitehouse Station, N.J.

Karapehlivan M, Uzlu E, Kaya N, Kankavi O, Ural K, Citil, M (2007) Investigation of some biochemical parameters and the antioxidant system in calves with dermatophytosis. Turk J Vet Anim Sci 31(2):85-89.

Kurutas EB, Ozturk P (2016) The evaluation of local oxidative/nitrosative stress in patients with pityriasis versicolor: a preliminary study. Mycoses 59(11):720-725. doi:10.1111/myc.12522.

Larone DH (1995) Medically important fungi: A guide to identification, 4th Edition, American Society for Microbiology, USA. p. 272-4.

Lykkesfeldt J, Svendsen O (2007) Oxidants and antioxidants in disease: oxidative stress in farm animals. Vet J 173(3):502-511.

Malecki EA, Greger JL (1996) Manganese protects against heart mitochondrial lipid peroxidation in rats fed high levels of polyunsaturated fatty acids. J Nutr 126(1):27-33. doi:10.1093/jn/126.1.27

Martinez-Rossi NM, Peres NT, Rossi A (2017) Pathogenesis of dermatophytosis: Sensing the host tissue. Mycopathologia 182(1-2):215-227. doi:10.1007/s11046-016-0057-9

Miller J, Brzezinska-Slebodzinska E, Madsen F (1993) Oxidative stress, antioxidants, and animal function. J Dairy Sci 76(9):2812-2823.

Miraloglu M, Kurutas EB, Ozturk P, Arican O (2016) Evaluation of local trace element status and 8-Iso-prostaglandin F_2 alpha concentrations in patients with tinea pedis. Biol Proced Online 18:1. doi:10.1186/s12575-015-0030-x

Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB (2014) Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal 20(7):1126-1167. doi:10.1089/ars.2012.5149

Mizokami SS, Hohmann MS, Staurengo-Ferrari L, Carvalho TT, Zarpelon AC, Possebon MI, de Souza AR, Veneziani RC, Arakawa NS, Casagrande R, Verri WA Jr (2016) Pimaradienoic acid inhibits carrageenan-induced inflammatory leukocyte recruitment and edema in mice: Inhibition of oxidative stress, nitric oxide and cytokine production. PLoS One 11(2):e0149656. doi:10.1371/journal.pone.0149656

Nisbet C, Yarim G F, Ciftci G, Arslan HH, Ciftci A (2006) Effects of trichophytosis on serum zinc levels in calves. Biol Trace Elem Res 113(3):273-280. doi:10.1385/bter:113:3:273

Or M, Bakırel, U, Tuncel H, Arun S, Karakoç Y, Dodurka HT, Barutçu UB (2002) Relation of histo-pathological changes with serum zinc and copper levels in dermatologically disease ddogs. J FacVet Med Univ Istanbul 28(2):337-345.

Paksoy N, Ozçelik M, Erkılıç EE, Büyük F, Metin O, Kırmızıgül AH (2013) Serum Copper, Zinc and Manganese Concentrations in Bovine Dermatophytosis in Kars Region. Atatürk Univ J Vet Sci 8(3):210-215.

Pal M, Dave P (2013) Ringworm in cattle and man caused by *Microsporium canis*: Transmission from dog. Int J Livest Res 3(1):100-103.

Papini R, Nardoni S, Fanelli A, Mancianti F (2009) High Infection Rate of *Trichophyton verrucosum* in Calves from Central Italy. Zoo noses Public Health, 56 (2):59-64

Pasa S, Kiral F (2009) Serum zinc and vitamin A concentrations in calves with dermatophytosis. J Fac Vet Med Univ Kafkas15(1):9-12.

Prohaska JR, Brokate B (2001) Lower copper, zinc-superoxide dismutase protein but not mRNA in organs of copper-deficient rats. Arch Biochem Biophys 393(1):170-176. doi:10.1006/abbi.2001.2470.

Quinn PJ, Carter ME, Markey B, Carter GR (1999) Clinical Veterinary Microbiology. p: 381-390. London, England: Mosby-Wolfe.

Radostits OM, Gay CC, Hinchcliff KW, Constable PD (2007) *Ringworm*. Veterinary Medicine. Saunders Elsevier, Edinburgh, 1476-1478.

Robert R, Pihet M (2008) Conventional methods for the diagnosis of dermatophytes. Mycopathologia 166:295-306.

Roth E (1997) Oxygen free radicals and their clinical implications. Acta Chir Hung 36(1-4): 302-305.

Schaufuss P, Steller U (2003) Haemolytic activities of *Trichophyton* species. Med Mycol 41(6):511-516.

Schott C, Cai H, Parker L, Bateman K, Caswell J (2014) Hydrogen peroxide production and free radical-mediated cell stress in *Mycoplasma bovis* pneumonia. J Comp Pathol 150(2-3):127-137.

Shafiei Neek L, Gaeini AA, Choobineh S (2011) Effect of zinc and selenium supplementation on serum testosterone and plasma lactate in cyclist after an exhaustive exercise bout. Biol Trace Elem Res 144(1-

3):454-462. doi:10.1007/s12011-011-9138-2

Szczepanik M, Wilkolek P (2004) Selected parameters of nonspecific immunity in cattle suffering from trychophytosis at different levels of zinc in their serum. Medycyna Weterynaryjna 60(11):1233-1235.

Toyoda H, Himeno S, Imura N (1989) The regulation of glutathione peroxidase gene expression relevant to species difference and the effects of dietary selenium manipulation. Biochim Biophys Acta 1008(3):301-308.

Xavier GA, da Silva LB, da Silva DR, de Moraes Peixoto R, Lino GC, Mota RA (2008) Dermatophytosis caused by *Microsporum canis* and *Microsporum gypseum* in free-living Bradypus variegatus (Schiz, 1825) in the state of Pernambuco, Brazil. Braz J Microbiol 39(3):508-510.

Yildirim M, Cinar M, Ocal N, Yagci B, Askar S (2010) Prevalence of clinical dermatophytosis and oxidative stress in cattle. J Anim Vet Adv 9(14):1978-1982.

Zhang J, Wang X, Vikash V, Ye Q, Wu D, Liu Y, Dong W (2016) ROS and ROS-mediated cellular signaling. Oxid Med Cell Longev 2016:4350965.

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Research article
Ερευνητικό άρθρο

Isolation and characterization of *Pseudomonas putida* caused granulomas in cultured sea bass (*Dicentrarchus labrax*) in Turkey

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ABSTRACT: The present study describes a Pseudomas infection in diseased European sea bass (*Dicentrarchus labrax*) caused by *Pseudomonas putida* in Turkey. Detected symptoms in the diseased fish were internally white nodules in the liver and kidney. Bacteriological samples from the kidney, spleen, liver and blood were streaked onto Tryptic Soy Agar (TSA) and Brain Heart Infusion Agar (BHIA) containing 1.5 % NaCl. After incubation, bacterial colonies produced florescent pigment under the ultraviolet light were observed. The morphological and physiological characteristics of bacterial colonies were determined together with their biochemical characteristics by using API 20E and API 20NE, and isolated bacteria were identified as *Pseudomonas putida*. Furthermore, 16S rRNA gene of one isolate was partially sequenced and showed 99 % identity with the Genbank sequences of *P. putida*. Histopathologically, the granulomatous lesions and presence of Gram-negative basil shaped bacteria in these lesions were observed in the liver and kidney. This study represents the first report of *P. putida* isolation and identification as a primer agent and granulomas in the kidney and liver in the diseased sea bass in the Black Sea, Turkey.

Keywords: API 20E, API 20NE, cultured sea bass, granulomas, Pseudomonas putida, 16S rRNA gene

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INTRODUCTION

Fish pathogenic pseudomonads have been reported increasingly in the past decade. The causative agents of pseudomonas infection such as *Pseudomonas fluorescens* reported from rainbow trout (*Oncorhynchus mykiss)* (Akayli and Timur, 2004; Kaçar and Balta, 2017), Nile tilapia (*Oreochromis niloticus*) (Eissa et al., 2010), sea bream (*Sparus aurata*) (Turk, 2002) *P. luteola* from rainbow trout (Altınok et al., 2007), *P. plecoglossicida* from ayu, (*Plecoglossus altivelis*) (Kobayashi et al., 2000) and rainbow trout (Akayli et al., 2010), *Pseudomonas baetica* sp. nov. from wedge sole, (*Dicologlossa cuneate*) (Lopez et al., 2012), and *P. aureginosa* from Nile tilapia (*Oreochromis niloticus*) (Eissa et al., 2010).

Pseudomonas are ubiquitous inhabitants for oxygenated environments (Palleroni, 1984; Austin and Austin, 2016). *Pseudomonas putida*, a member of genetically related fluorescent pseudomonads, is an aerobic, Gram-negative basil shaped bacterium, and has been isolated and identified from diseased ayu (*Plecoglossus altivelis altivelis*) (Wakabayashi et al., 1996), yellowtail (*Seriola quinqueradiata*) (Kusuda and Toyoshima, 1976), European eel (*Anguilla anguilla)* (Fan, 2001), oyster toadfish (*Opsanus tau*) (Smolowitz et al., 1998), large yellow croaker (*Pseudosciaena crocea*) (Shen et al., 2008), black sea bream (*Sparus macrocephalus*) (Mao et al., 2010), and Nile tilapia (*Oreochromis niloticus*) (Eissa et al., 2010). Moreover, this bacterium is an opportunistic human pathogen responsible for bacteremia (Lombardi et al., 2002).

In Turkey, pseudomonas infection caused by *P. putida* was first diagnosed in scattered mirror carp (*Cyprinus carpio*), gold fish (*Carasius auratus*) (Aydın et al., 1998). After then it was reported from cultured rainbow trout (Altınok et al., 2006). *P. putida* has been not reported as a primer agent in any marine cultured or wild fish species in Turkey. This paper is the first report of *P. putida* infection in the diseased sea bass and histopathologically granulomas in the kidney and liver.

MATERIAL AND METHODS

Fish samples and clinical examination

Ten diseased sea bass (200-250 g) that showed rarely hemorrhagic ulcerative on the ventral body surface and generally white nodules on the liver and kidney were obtained from a floating marine cage farm located on the coast of the Black Sea in Turkey.

Bacteriological examination

Samples of kidney, liver, spleen, and blood were streaked onto Tryptic Soy Agar (TSA) and Brain Heart Infusion Agar (BHIA) supplemented with 1,5% NaCl. Plates were incubated at 20-21 C° for 24-48 hours. The isolates (n=10) recovered from sea bass were characterized by using conventional bacteriological method. In addition, these isolates were determined together with their biochemical characteristics using API 20NE and API 20E (Buller, 2004), however API suspension in 1.5% saline was used as inoculum for marine bacteria as described by Grisez et al., (1991).

Nucleic acid isolation and DNA sequencing

Total DNA extraction was performed with Roche Genomic DNA Purification Kit (11796828001, Germany) according to the manufacturer's instructions and used as template for PCR. An approximately 1400 bp long fragment of the 16S rRNA gene was amplified using the universal bacteria primer sets U8F (5' AGAGTTGATCATGGCTCAG 3') and 1492R (5'GGTTCACTTGTTACGACTT3') as reported by Weisburg et al (1991). PCR product were purified and sequenced by Medsantek (Istanbul, Turkey).

Antimicrobial susceptibility test

Antibacterial susceptibility of the isolates was determined by using KirbyBauer disc diffusion method according to Barry and Thornsberry (1985). For this purpose, 12 commercial antibiotic disc (chloramphenicol (30 µg/disc), kanamycin (30 µg/disc), flumequine (30 µg/disc), erythromycin (5 µg/disc), streptomycin (10 µg/disc), ciproflaxacin (1 µg/disc), sulphamethoxazole (25 µg/disc), ampicillin (10 µg/disc), enrofloxacin (5 µg/disc) florfenicol (30 µg/disc), oxytetracycline (30 µg/disc), furazolidon (50 µg/disc) (Oxoid, England)) were used. Results were carried out according to instruction of the Clinical and Laboratory Standart Institute (2008).

Histopathological examination

Tissue samples from the kidney, liver, spleen, heart, intestine, and gills were immediately fixed in 10% buffered formalin and processed for paraffin embedding. Histologic sections (4-5 µm) were stained with hematoxylin-eosin (H&E) and Brown-Hopps (B&H) and examined under light microscopy (Brown et al., 1973; Bullock, 1978).

RESULTS

Clinical findings

This outbreak occurred in April 2017 with cumulative mortality approximately 15 %. Water temperature was 15-16 C° at the time of sampling. While three sea bass samples externally showed hemorrhagic ulcerative skin lesions on the ventral body surface (Figure 1a), seven fish appeared no external clinical sign on the body surface. Internally, teen sea bass generally exhibited pale liver, enlargement of the spleen, a clear fluid in the intestine and abdominal cavity, liquefaction of the head kidney and generally white nodules on the liver and kidney (Figure 1b).

Figure 1. Diseased sea bass. (a): Hemorrhagic ulcerative skin lesions (*) on the ventral body surface, around the pectoral and ventral fins; (b): White spot on the pale liver (*), and splenomegaly

Bacteriological and molecular identification findings

After the incubation of the bacteriological inoculations from the visceral organs and blood, pure and one dominant colony were isolated on TSA and BHIA (Figure 2a). The isolated bacteria (n=10) produced florescent pigment under the ultraviolet light (Figure 2b). They were motile, basil shaped, Gram-negative, cytochrome oxidase and catalase positive, and oxidative. Therefore, ten isolates were identified as *Pseudomonas* sp. According to the morphological, physiological and biochemical characteristics of the isolates, isolated bacteria were identified as *P. putida* (Table 1). Similarly, our isolates were characterized as *P. putida* by API 20 NE (profile number: 0140057) and API 20E (profile number: 220404643).

Figure 2. *P. putida* (a) produced fluorescent pigment (fluorescein) (b) under the UV light

Molecular identification findings

Gene sequencing with 16S rRNA revealed that the isolate was *P. putida* (99%). This result was derived from the National Center for Biotechnology Information (NCBI) blast database. The sequence obtained in this study is defined as GenBank accession number MH368654.

Antimicrobial susceptibility test findings

P. putida strains were found susceptible to all of the antimicrobials tested, except for oxytetracycline, erythromycin, streptomycin, kanamycin and ampicillin.

Histopathological findings

In the present study, histopathologically rarely melanomacrophage center, discharge of the white pulp and hyperemia in the spleen (Figure 3); epicarditis, the polymorph-nuclear leukocyte infiltration and hypertrophy of the heart muscle cells (Figure 4); degeneration and necrosis of the epithelial cells of kidney tubules periglomerular and tubular oedema (Figure 5); vacuolar degeneration and karyolysis of hepatocyte, congestion (Figure 6a) hemorrhage among the hepatocyte cells (Figure 6b); hyperplasia of the gill filament (Figure 7) were observed. In addition, granulomas were noted in the kidney (Figure 8) and liver section (Figure 9a). Presence of Gram-negative bacteria were found within the necrotic granulomas (Figure 9b, Figure 10).

Table 1. Morphological, physiological and biochemical characteristics of the isolates recovered from diseased sea bass

Characteristics	Isolates (n=10)	Characteristics	Isolates (n=10)
Gram staining	-	Growth at 4 C°	+
Motility	+	Growth at 41 C°	-
Cytochrome oxidase	+	β-galactosidase	-
Catalase	+	H_2S production	-
O/F	O	Arginine dihydrolase	+
Fluorescent pigments	+	Ornithine decarboxylase	-
O/129-10μg	-	Lysine decarboxylase	-
O/129-150 μg	-	Carbohydrate utilization	
Metil Red	-	Rhamnose	-
Voges Proskauer	-	Arabinose	+
Indole	-	Sucrose	-
Citrate	+	Mannitol	-
Nitrate	-	Maltose	-
Aesculine hydrolysis	-	Trehalose	-
Urease	-	Sorbitol	-
Starch hydrolysis	-	Melibiose	+
Gelatin hydrolysis	-	Inositol	-
Growth on MCA	+	Xylose	+
Growth on TSA	+	Lactose	-

+: positive reaction**, -:** negative reaction, **O/F:** Oxidative/Fermentative, **MCA:** MacConkey Agar

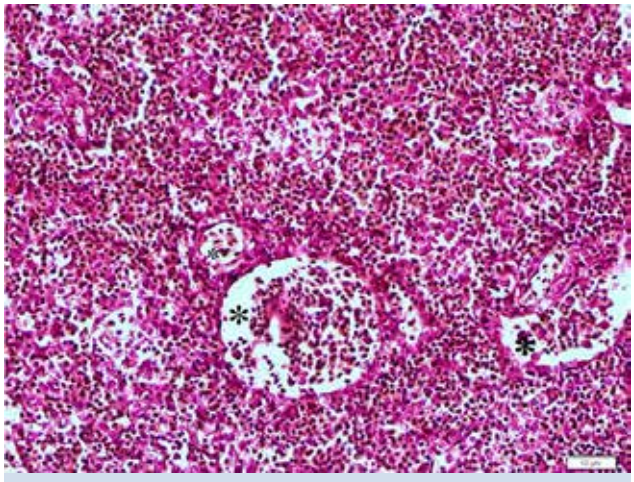

Figure 3. Discharge of the white pulp (arrowed), congestion (*) of spleen vessels (H&E) x1000

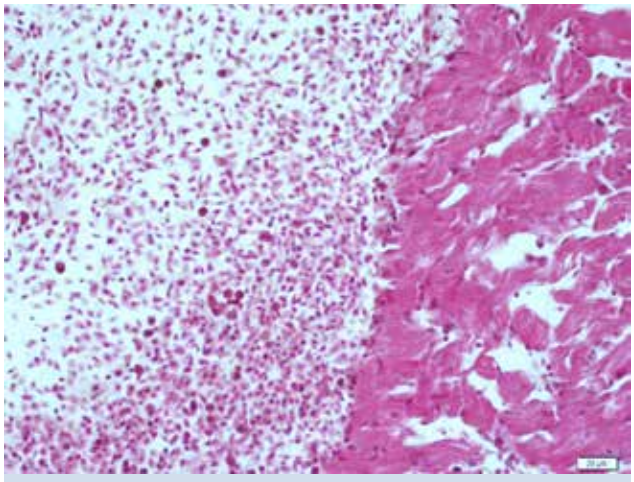

Figure 4. The polymorph-nuclear leukocyte infiltration (arrowed) and hypertrophy of the heart muscle cells (H&E) x400

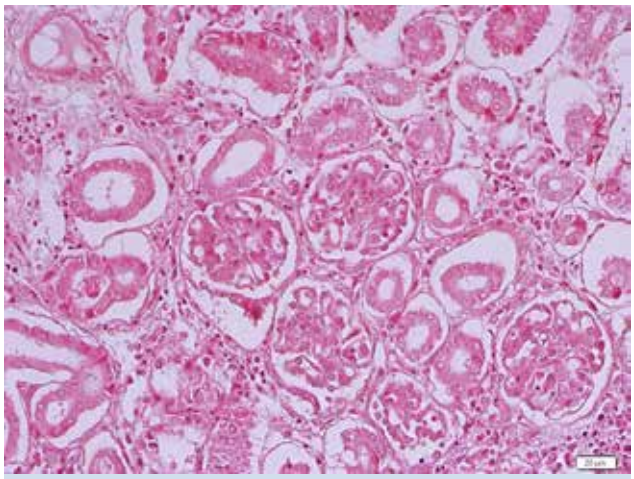

Figure 5. Periglomerular (arrowed), tubular oedema and tubular degeneration (H&E) x400

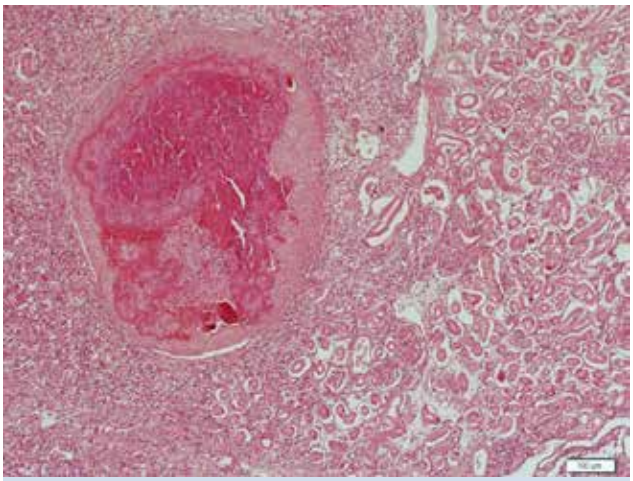

Figure 8. Granuloma in the interrenal haemopoietic tissue of kidney (H&E) X100

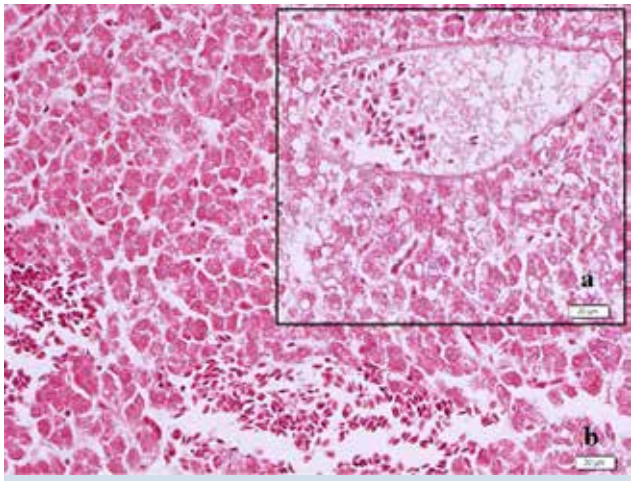

Figure 6. Congestion (a) and hemorrhage (b) among the hepatocyte (H&E) x400

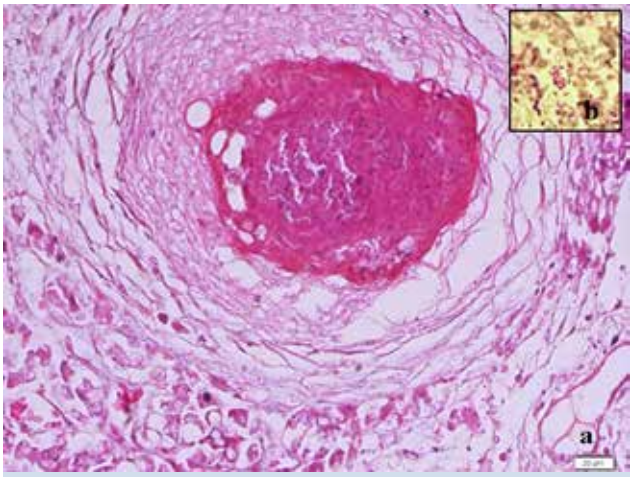

Figure 9. Necrotizing granuloma surrounded by epithelioid in the liver (a) (H&E), Gram negative bacteria in the granuloma (b) (B&H) X400

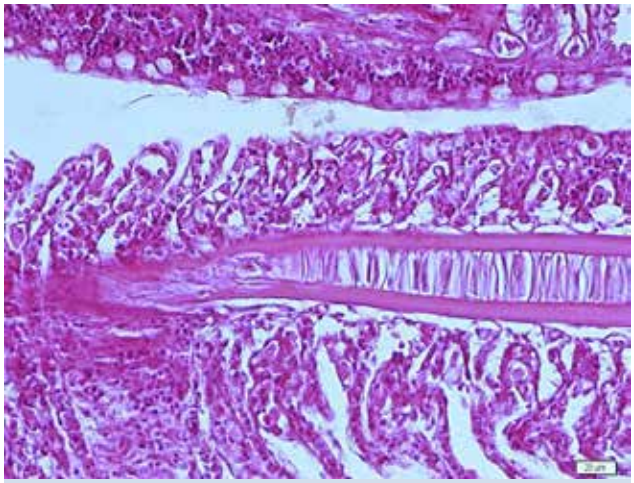

Figure 7. Gills showing hyperplasia of the respiratory epithelium and increased goblet cells (H&E) x400

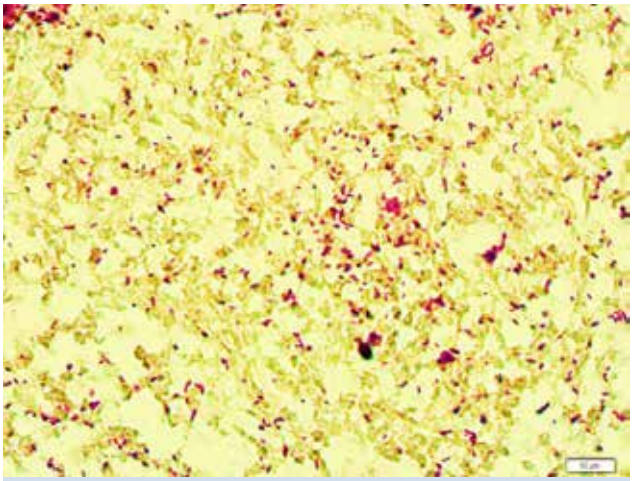

Figure 10. Presence of Gram-negative bacteria caused granuloma in the kidney tissue (B&H) X1000

DISCUSSION

Pseudomonas agents such as *P. fluorescens, P. putida, P. luteola, P. aeroginasa,* and *P. plecoglossicida* have increasingly been reported from the diseased cultured and wild fish species all over the world (Kobayashi et al., 2000; Akayli and Timur, 2004; Altınok et al., 2007; Eissa et al., 2010). However, there is no report about *Pseudomonas* infection caused by *P. putida* as a primer agent in the cultured marine fish species in Turkey.

In this study, diseased sea bass showed either external clinical signs or no external clinical signs as reported in the large yellow croaker (Shen et al., 2008). Although the ulcerative skin lesions on the base of the dorsal fin as reported in rainbow trout (Altınok et al., 2006) have not been observed in this study, three diseased sea bass showed externally ulcerative skin lesions around the pelvic and pectoral fins. Internally the gross pathology such as white nodules in the kidney and liver observed in the sea bass bear similarities to the large yellow croaker infected with *P. putida* (Shen et al., 2008). Similar gross pathological findings have been reported in large yellow croaker infected with *P. plecoglossicida* (Zhang et al., 2014). Altinok et al (2016) reported that there are no internal clinical findings in the rainbow trout. Therefore, it may be considered that the clinical signs of pseudomonas infection caused by *P. putida* is not specific for different fish species.

In the present study, according to Gram staining, presence of cytochrome oxidase and catalase enzyme, oxidation, isolated bacteria were identified as *Pseudomonas* sp. presumptively *P. fluorescens or P. putida* for florescent pigment production under the ultraviolet light. The gelatin hydrolysis, which is the most important biochemical characteristics that distinguishes between these two fluorescent bacteria, has been defined as *P. putida* as it has a negative reaction. (Palleroni, 1984). Moreover, the morphological, physiological and biochemical characteristics of our isolates were very similar to those of *P. putida*, as described in previous reports (Palleroni, 1984; Buller, 2004; Austin and Austin, 2016). It has been reported sufficiency of rapid identification kits (API 20E, API NE) for the identification of *P. putida* isolated from diseased rainbow trout (Altinok et al., 2006). However, misidentification of the fish pathogens can occur when using of the API 20E (Kent, 1982; Topic Popovic et al., 2007). In present study, our isolates with API suspension in 1.5% saline were successfully identified by API 20E and API 20 NE. In contrast to the results described by Altinok et al (2006) in current study we observed that the API 20 NE gave different profile number (0140057) compare with *P. putida* isolated from rainbow trout. Our isolates gave positive reactions at utilization of phenylacetic acid as distinct from rainbow trout isolates. For this reason, to identify and confirmation of *P. putida*, should be made rely heavily on biochemical tests.

In present study, it has been observed that *P. putida* strains were resistant to oxytetracycline, erythromycin, streptomycin, kanamycin, and ampicillin as described Altinok et al (2006). However, Kholil et al (2015) reported that oxytetracycline is highly effective against *Pseudomonas* species. In addition to this report, Saleh et al (2008) reported that *P. anguillaseptica* displayed sensitivity to erythromycin, oxytetracycline, and streptomycin as distinct from our results.

To date, there are no histopathological reports about infection of marine fish species with *P putida*, except for freshwater fish species, rainbow trout. Altinok et al (2006) reported that histopathologically epithelial necrosis in the rainbow trout infected with *P. putida,* however in this study, the most prominent pathological changes were observed in the liver and kidney. The granulomas were observed in the kidney and liver as in the described large yellow croaker infected with *P. putida* and *P. plecoglossicida* (Shen et al., 2008; Zhang et al., 2014). Moreover, hyperemia in the spleen, epicarditis, degeneration and necrosis of kidney tubules, periglomerular and tubular oedema, hemorrhage vacuolar degeneration and karyolysis of hepatocyte, hyperplasia of the gill filament were observed in the diseased sea bass.

CONCLUSIONS

The pathogen causing Pseudomonas infection in the diseased sea bass was identified as *P. putida* for the first time. The gross and histopathological findings of the diseased sea bass indicated that *P. putida* is a potential pathogen of the cultured sea bass. This pathogen bacterium is important risk for the fish health so our further study will be experimentally carried out on pathogenesis and histopathology of *P. putida* infection in the cultured sea bass.

CONFLICT OF INTEREST

None declared by the author.

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This study was approved by the Istanbul University Local Committee on Animal Research Ethics (decision year 2013).

REFERENCES

Akayli T, Timur G (2004) A study on the pseudomonad septicemia in young rainbow trout (*Oncorhynchus mykiss*). Journal of the Faculty of Veterinary Medicine 30 (1): 121-131.

Akayli T, Canak O, Basaran B (2010) A new pseudomonas species observed in cultured young rainbow trout (*Oncorhynchus mykis* Walbaum, 1792): *Pseudomonas plecoglossicida*. Research Journal of Biology Sciences 4(1):107- 111.

Altinok I, Kayis S, Capkin E (2006). *Pseudomonas putida* infection in rainbow trout. Aquaculture 261: 850-855.

Altinok I, Balta F, Capkin E, Kayis S (2007) Disease of rainbow trout caused by *Pseudomonas luteola*. Aquaculture 273(4): 393-397.

Austin B, Austin DA (2016) Bacterial Fish Pathogens. In: Diseases of Farmed and Wild Fish. Springer, Dordrecht: pp 41-135.

Aydin S, Ciltas A, Erman Z (1998) *Pseudomonas putida* infections in scattered mirror carp (*Cyprinus carpio*) and gold fish (*Carassius auratus* l.). In: First International Symposium on Fisheries and Ecology Proceedings, Trabzon, Turkey: pp 259-266.

Barry A L & Thornsberry C (1985). Susceptibility tests: diffusion test procedures. In: *Manual of Clinical Microbiology*, Washington: American Society of Microbiology: pp 978-987.

Brown RC, Hopps HC. (1973) Staining of bacteria in tissue sections: a reliable Gram stain method. American Journal of Clinical Pathology 6 (2): 234-240.

Buller N B (2004). Bacteria from Fish and Other Aquatic Animals: A Practical Identification Manual. CABI Publishing, UK: pp 183-227.

Bullock AM (1978). Laboratory Methods in Fish Pathology. In: Fish Pathology, London: Bailliere Tindall: pp 235-275.

Clinical and Laboratory Standards Institute (CLSI) (2008). Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests For Bacteria Isolated From Animals: Approved Standard-Third Edition M31-A3. CLSI, Waune, PA, USA.

Eissa NM, El-Ghiet, EA, Shaheen AA, Abbass A (2010) Characterization of Pseudomonas species isolated from tilapia "*Oreochromis niloticus*" in Qaroun and Wadi-El-Rayan lakes, Egypt. Global Veterinaria 5(2): 116-121

Fan HP (2001) Study on the gill-rot disease of *Anguilla anguilla* infected by *Pseudomonas putida*. Journal of Fishery Sciences of China 25: 147-150.

Grisez L, Ceusters R, Ollevier F (1991) The use of API 20E for the identification of *Vibrio anguillarum* and *V. ordalii*. Journal of Fish Diseases 14(3): 359-365.

Kacar ZZ, Balta F (2017) Multilocus sequence analysis (MLSA) based identification of *Pseudomonas* spp. isolated from rainbow trout (*Onchorhynchus mykiss*) in the Eastern Black sea region of Turkey using. Fresenius Environmental Bulletin 26 (12): 7506-7512.

Kent ML (1982). Characteristics and identification of Pasteurella and Vibrio species pathogenic to fishes using API-20E multitube test strips. *Canadian Journal of Fisheries and Aquatic Sciences*, 39(12): 1725-1729.

Kholil MI, Hossain MM, Neowajh MS, Islam MS, Kabir M (2015) Comparative efficiency of some commercial antibiotics against Pseudomonas infection in fish. International Journal of Fisheries and Aquatic Research 2(3): 114-117.

Kobayashi T, Imai M, Ishitaka Y, Kawaguchi Y (2000) Histopathological studies of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis* (Temminck and Schlegel). Journal of Fish Diseases 27: 451-457.

Lombardi G, Luzzaro F, Docquier JD, Riccio ML, Perili M, Coli A, Amicosante G, Rossolini GM, Toniolo A (2002) Nosocomial infections caused by multidrug-resistant isolates of *Pseudomonas putida* producing VIM-1 metallo-β-lactamase. Journal of Clinical Microbiology 40: 4051-4055.

Lopez JR, Dieguez AL, Doce A, De-la Roca E, De-la Herran R, Navas JI, Toranzo E, Romalde, JL (2012) Pseudomonas baetica sp. nov., a fish pathogen isolated from wedge sole, *Dicologlossa cuneata* (Moreau). International Journal of Systematic and Evolutionary Microbiology 62(4): 874-882.

Mao Z, Wang M, Chen J, Yang J (2001) Isolation and characterization of *Pseudomonas putida*, a causative agent of gastroenteritis in black sea bream, *Sparus macrocephalus*. Guangdong Agricultural Sciences 1-3.

Palleroni NJ (1984) Genus Pseudomonas. In: Bergey's Manual of Systematic Bacteriology. Williams & Wilkins Co, Baltimore: pp 141-199.

Saleh FM, Sakr MM, Azza MM, El-Rhman A (2008) Contribution on Pseudomonas septicemia caused by *Pseudomonas anguilliseptica* in cultured *Oreochromis niloticus*. In: 8th International Symposium on tilapia in Aquaculture, Egypt: pp: 1177- 1197.

Shen JY, Yu XP, Pan XY, Xu WJ, Yin WL, Cao Z (2008) Isolation and identification of pseudomonassis pathogen from cultured *Pseudosciaena crocea*. Journal of Marine Science: Research and Development 29: 1-6.

Smolowitz R, Wadman E, Chikarmane HM (1998) *Pseudomonas putida* infections of the oyster toadfish (*Opsanus tau*). Biology Bulletin 195: 229-231.

Topić Popović, N., Čož-Rakovac, R., & Strunjak-Perović, I. (2007). Commercial phenotypic tests (API 20E) in diagnosis of fish bacteria. Veterinarni Medicina, 52,(2): 49-53.

Turk N (2002) Çipura (*Sparus aurata*) ve levrek (*Dicentrarchus labrax*) yavrularından bakteri izolasyonu ve antibiyotiklere duyarlılıklarının belirlenmesi. Bornova Veteriner Kontrol ve Araştırma Enstitüsü Dergisi, 27: 7-14.

Wakabayashi H, Sawada K, Ninomiya K, Nishimori E (1996) Bacterial hemorrhagic ascites of ayu caused by *Pseudomonas* sp. Fish Pathology 31: 239-240.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal RNA amplification for phylogenetic study. Journal of Bacteriology 173: 697-703.

Zhang JT, Zhou SM, An SW, Chen L, Wang GL (2014) Visceral granulomas in farmed large yellow croaker, *Larimichthys crocea* (Richardson), caused by a bacterial pathogen, *Pseudomonas plecoglossicida*. Journal of Fish Diseases 37(2): 113-121.

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Research article
Ερευνητικό άρθρο

Determination of the alterations in quality parameters and consumer preference of dry-aged beef based on different periods of ageing using a purposive incubator

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ABSTRACT: The aim of this study was to evaluate beef quality traits including pH, water holding capacity, cooking loss, meat colour, and Warner-Bratzler shear force in dry-aged beef and to determine the most favorable ageing time based on beef quality parameters and consumer acceptability. In this respect, longissimus dorsi samples were obtained from Simmental bulls and stored for an ageing period up to 21-days, and thus, the beef quality evaluation was performed at 0-day, 7-day, 14-day, and 21-day of the experimental period. Results revealed that the lowest shear force value was observed in beef samples on day-14 whereas the lowest pH value was determined in samples on day 21. Moreover, water holding capacity and beef colour values were significantly differentiated based on dry-ageing (P<0.05). In sensory panel evaluation, a significant difference is found only in meat colour rating (P<0.05). There was no significant difference between ageing periods and cooking loss. The most important technical point is that increasing dry-ageing time from 14 to 21 days did not desirably affect quality traits and sensory scores. Hence, dry-ageing for 14 days seemed to be the most economically efficient application. Taken altogether, the present results suggest that the potential for use of dry-ageing should be considered as an alternative method to produce high-quality beef with respect to the optimum ageing process.

Keywords: *Beef, dry ageing, consumer preference, sensory characteristics, meat quality*

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INTRODUCTION

Beef tenderness and palatability are important issues for consumers which can directly influence their acceptance. Ageing meat is one of the most important applications performed to increase the tenderness over time and to enhance the palatability of the product. Moreover, it is obviously required for the development of flavors because true beef flavor is fully developed after approximately 11 days of ageing (Oreskovich et al., 1988; Sitz et al., 2006).

In general, there are two methods of ageing including wet- and dry-ageing (Laster et al., 2008). Beef predominantly sold in food stores is vacuum packaged and aged in a vacuum-bag. This technique is called wet-ageing and it is applicable for 7 to more than 21 days post mortem. A relatively small amount of beef is dry-aged, usually for 14 to 30 days post mortem (Stenström et al., 2014). Dry ageing is the ageing application of carcasses or retail cuts in a refrigerated room or a cooler, where storage temperature, humidity, and air flow are controlled (Sitz et al., 2006; Smith et al., 2008; Dashdorj et al., 2016). The post mortem process of physiological regulation in muscle structure is complex. During the dry ageing period, the key factor is to concentrate the special dry-aged beef flavor. This can only be provided by the juices that are absorbed into beef, biochemical reactions of protein and fat components that enhances the flavor properties. As a result, the enzymatic catalysis leads to more tender beef (Campbell et al., 2001; Dashdorj et al., 2016).

Enhanced flavor and desired palatability characteristics provide the perception of a premium product to dry-aged beef. This situation results in higher prices for dry-aged beef products in the marketplace (Laster et al., 2008). Thus, most dry-aged beef is sold by upscale restaurants, speciality shops or internet and it can be difficult to attain the products in ordinary restaurants or food stores (Stenström et al., 2014). On the other hand, as demand for dry-aged beef increases, the technique of dry ageing has raised its economical importance in meat industry and the food service market. As mentioned above, controlling the storage temperature, humidity, and air flow during the dry ageing process is essential for achieving desired characteristics of dry-aged beef. Recently, specific coolers for dry-aged meat have been developed to provide an optimum ageing process which is easily containable under storage conditions. The majority of earlier studies on the ageing of beef have compared wet ageing and dry ageing with respect to the product quality and consumer sensory preference (Campbell et al., 2001; Laster et al., 2008; Smith et al., 2008; Li et al., 2014; Stenström et al., 2014). However, there are inconsistencies and even contradictions within an optimum ageing time for dry-aged beef. Therefore the aim of this study was to determine beef quality properties and sensory characteristics of beef using a dry ageing incubator and to compare the quality parameters based on different periods of ageing. Furthermore, the consequences for the acceptance by consumers were evaluated.

MATERIALS AND METHODS

Sampling and ageing process

Beef samples were obtained from Simmental bulls (n=3) which were 16 months old and were slaughtered at 610 kg on the same day at a commercial abattoir according to standard practices. Twelve pairs of *m. longissumus dorsi* (obtained from both the left- and right-side from a carcass) were selected for use as samples for evaluation of quality parameters. Primary samples (n=24) were cut transversely at the mid-length (approx. 20 cm in length). Initially, analyses of beef quality parameters including beef colour, water holding capacity (WHC), cooking loss (CL), and Warner-Bratzler shear force (WBSF) were applied to fresh beef samples (as indicated by day 0, control group). Afterwards, the remaining sections were each dry-aged for 7, 14, and 21 days using a dry ageing incubator (Frenox DR6-G, Devon, United Kingdom). Dry ageing was performed for 7, 14 and 21 days at temperature of 2.20 ± 0.40 ºC. Humidity averaged 87 ± 4.60% during the entire experimental period. No ultraviolet lights were used. Regarding all beef quality parameters, three times-repeated measurements were performed and the average was evaluated as the final value for each sample.

pH and weight losses

In order to measure pH values of each sample, a digital pH meter (Testo 205, Lenzkirch, Germany) was used after the calibration with pH 4.01 and pH 7.00 standard buffer solutions (Testo) at 2–4°C. Measurements were conducted in triplicate at random points of each sample. Initially, samples were weighed using an analytical balance (Radwag AS220/C/2, capacity 220 g, readability 0.10 mg, Bracka, Poland), and then cooked in a 75°C water bath (Nuve BM 302, Turkey) for 60 minutes. After cooling in running tap water for 60 minutes, their packages were opened, beef samples

were dried with filter paper to remove excess moisture and weighed again. Ultimately, CL was estimated as a percentage of weight of the samples before and after cooking, according to the following formula: (weight before cooking – weight after cooking) / weight before cooking × 100(Pietrasik and Duda, 2000). The WHC of beef samples was measured by the Grau and Hamm procedure (Grau and Hamm, 1957) and it was considered as the ratio of moisture kept in the sample to the initial moisture content (Pietrasik and Duda, 2000). Briefly, a meat sample weighing 5 g was placed on 10 cm diameter filter papers between two petri plates and was pressed under 2.250 kg for 5 min. Following the removal of the filter papers and the weight, WHC, as a percentage, was calculated as (final filter weight – initial filter weight) / meat sample weight × 100.

Beef colour

The colour of beef samples was measured using a reflectance colourimeter Konica Minolta CM508d (Konica–Minolta Inc., Ramsey, NJ, USA). Samples at least 12 to 15 mm thick were used to absorb non-reflected light. Colour parameters were evaluated based on the CIELAB system with colourimetric coordinates L^* (lightness), a^* (redness), and b^* (yellowness) regarding standard illumination, D65 and 10° standard observer. The device was calibrated with a standard white plate provided by the manufacturer and it was set to make three measurements to take their average. This instrumental evaluation allows beef colour to be expressed in a three dimensional space. Along the a^* axis, a positive a^* represents red, and a negative a^* represents green (scale from +60 for red to −60 for green). Along the Y axis, a positive b^* represents yellow, and a negative b^* represents blue (scale from +60 for yellow to −60 for blue). The third dimension L^* is represented numerically where 100 is white, and 0 is black (Hunt et al., 2012). Three-times-repeated colour measurements were performed from each beef sample of the cut surface and the mean of these measurements was assigned as the final value. Colour measurements were taken so that connective and fat tissue was avoided.

Shear force analysis

After the corresponding ageing time (7, 14, and 21 days) beef samples were prepared for WBSF analysis. The samples cooked for the measurement of CL were used to determine shear force value. Three cylindrical cores (surface area: approx. 1×1 cm, core length: min. 30 mm) parallel to the longitudinal alignment of the muscle fibres were sheared across the widest dimension by using an universal testing machine (Zwick/Roell Z0.5, Germany) equipped with a V-shaped Warner-Bratzler blade with a 60° triangular aperture (Ekiz et al., 2009).WBSF value of each sample were determined by taking the average of the measurements obtained from three cylindrical cores. The device was set to 150 mm/min crosshead speed and 50 kg force (applied to sample). Data were collected with the software provided by the manufacturer.

Consumer sensory panel

Taste panel members (n=17) were chosen among academicians at the Bursa Uludag University Faculty of Veterinary Medicine in Bursa, Turkey. Participants were not to eat or drink for one hour prior to the test. They were also instructed to rinse their mouths with water and to smell the ground coffee before tasting began as well as between samples. Prior to assessment, samples were wrapped individually in aluminium foil and assigned a three-digit code. They were cooked at 200°C using a pre-heated double-plated electric oven (Nuve FN 120, Turkey). Afterwards, the coating fat and connective tissue were removed, they were cut into cubic subsamples (20×20×10 mm) and were kept warm in a heater at 60°C until the taste panel assessment (Gill et al., 2009; Resconi et al., 2010). The panellists scored the beef samples on a 1–8 scale for seven traits, odour, flavour, tenderness, juiciness, colour, general acceptance, and overall liking as described by Gill et al.(2009) with some modifications. Each panelist received three cubes of each sample of four treatments (0 day-control, and dry aged for 7, 14, and 21 days) and thus evaluated 12 samples in the same environmental conditions. Samples were characterized using hedonic scales for beef odour (8=like extremely, 1=dislike extremely), level of beef flavour (8=extremely intense, 1=extremely bland or no flavour), intensity of the tenderness (8=extremely tender, 1=extremely tough), level of juiciness (8=extremely juicy, 1=extremely dry), beef colour (8=like extremely, 1=dislike extremely), general acceptance (8=extremely desirable, 1=extremely undesirable, abnormal flavour or odour), overall like (8=like extremely, very definitely would purchase, 1=dislike extremely, very definitely would not purchase).

Statistical analysis

Measurements were performed in triplicate, and the obtained results were statistically analysed using

the SPSS v23.0 (IBM, Armonk, NY, USA). Testing homogeneity of variances was performed with Levene's test. The data of beef quality parameters were considered as repeated measures (day 0 and 7, 14, and 21 day dry-ageing treatments) and analyzed by using Friedman's test. Post hoc analysis was carried out using Dunn's test.

RESULTS

pH, WBSF, WHC, and weight loss

There was a significant effect of dry ageing on pH, WBSF, and WHC (P<0.05), as shown in Table 1. WBSF and WHC were higher in samples at day 0 compared to dry-aged samples. It is worth noting that dry-aged beef at 14th day had the lowest shear force value (2.61 N/mm). The highest pH was observed in samples at day 7. There was no significant effect of ageing on CL.

Instrumental beef colour

The ageing time had significant effects on all beef colour parameters studied (P<0.05). In this context, the L^* value prominently decreased after 21 days of ageing, and accordingly, the lowest L^* value was determined at the 21st day of dry ageing period (32.47±2.99). Similarly, this period of ageing was significantly associated with the lowest a^* and b^* values (P<0.05). The highest L^* and b^* values were determined on day 0, whereas, the highest value was observed in day 7.

Sensory panel

The influence of dry ageing treatments on consumer preference is shown in Table 2. The only statistical difference among samples that were dry-aged for different lenghts of time was for colour score (P<0.05). Panellists valued day-14 samples significantly higher than other ageing groups with respect to beef colour (Table 2). The greater sensory ratings (based on odour, flavour, tenderness, juiciness, general acceptance, and overall liking scores) for the samples at day 14 contributed to the greater value compared to samples at day 0, 7, and 21. However, this result was not statistically substantiated (P>0.05).

Table 1. Means and their corresponding standard deviations (SD) for pH, Warner–Bratzler shear force values, water holding capacity, cooking loss, and beef colour parameters of beef steaks stratified by dry-ageing periods

Traits	Day 0		Day 7		Day 14		Day 21		Significance
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
pH	5.020^{ab}	0.032	5.207^{a}	0.028	5.081^{ab}	0.049	4.890^{b}	0.240	P<0.05
WB shear force (N/mm)	11.328^{a}	0.866	8.297^{b}	0.941	2.610^{c}	0.010	6.687^{b}	0.577	P<0.05
Water holding capacity	0.660^{a}	0.118	0.190^{ab}	0.094	0.145^{ab}	0.045	0.080^{b}	0.008	P<0.05
Cooking loss (%)	11.550	0.136	8.740	0.021	10.080	0.086	9.00	0.510	NS
L^* (lightness)	45.610^{a}	3.820	40.150^{a}	1.001	43.507^{a}	0.927	32.47^{b}	2.990	P<0.05
a^* (redness)	17.944^{ab}	2.425	20.003^{a}	1.691	13.653^{ab}	0.535	3.053^{b}	1.105	P<0.05
b^* (yellowness)	14.432^{a}	2.451	15.260^{a}	1.250	12.470^{a}	0.481	3.407^{b}	1.366	P<0.05

WB: Warner Bratzler.
^{a,b,c} Different superscripts within a row indicate significant difference.

Table 2. Means and their corresponding standard deviations (SD) for consumer sensory evaluation (n = 17 panellists) for steaks stratified by dry-ageing periods

Traits	Day 0		Day 7		Day 14		Day 21		Significance
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Odour	5.150	1.549	6.067	1.486	6.601	1.352	5.733	1.580	NS
Flavour	4.950	1.588	5.867	1.356	6.200	1.612	6.133	1.767	NS
Tenderness	4.383	1.637	6.067	1.387	6.600	1.765	5.800	1.568	NS
Juiciness	4.183	1.589	5.933	1.486	5.943	1.668	5.333	1.759	NS
Colour	5.483^{b}	1.589	5.801^{b}	1.424	6.733^{a}	1.335	5.533^{b}	1.506	P<0.05
General acceptance*	4.950	1.702	6.200	1.474	6.533	1.506	6.133	1.685	NS
Overall liking	5.033	1.677	6.067	1.486	6.600	0.400	5.933	1.624	NS

*General acceptance was evaluated as the presence of abnormal flavour or odour as indicated by Gill et al. (2009).
^{a,b} Different superscripts within a row indicate significant difference.

DISCUSSION

In the present study, alterations in beef quality parameters were evaluated with respect to samples at 0, 7, 14, and 21 day. Dry ageing method was applied to all samples and the evaluations were performed based on pH, weight losses, WBSF, instrumental colour, and sensory panel. Obviously, the importance of these traits varies according to both attributes of the final product and the consumer choices(Koohmaraie and Geesink, 2006). In many countries, the primary objective of beef production has gradually changed from beef yield to quality. Especially in affluent countries, consumers usually request meat products which are of the high quality. Although there are obvious problems in beef production in many countries (concerning the balance between supply and demand), high quality meat products are increasing its perceived importance. Hence, the different techniques of beef ageing and their economical significance in meat industry should be evaluated in a more detailed and systematic way. In this perspective, this study presents an elaborate analysis of quality parameters of dry-aged beef with respect to ageing time alterations. Due to the significance of quality parameters, various attempts have been made to ensure these parameters of beef when presented to the consumer. Of these beef quality parameters, tenderness is ranked as the most important (Miller et al., 2001; Koohmaraie and Geesink, 2006). Thus, recent studies have tended to focus efforts on evaluating the basis for beef tenderness, primarily because of its economic significance. A wide variation in degree of postmortem tenderization is evident, and hence, the inconsistency of meat tenderness is a prevalent circumstance at the consumer level (Miller et al., 2001; Koohmaraie and Geesink, 2006; Warner et al., 2010). It is worth noting that dry ageing improves WBSF and sensory-panel scores of tenderness (Campbell et al., 2001; Ahnström et al., 2006). This interpretation was substantiated, at least in part, in the present study. WBSF values were significantly affected by dry-ageing process and the beef at day 14 was the most tender (P<0.05). Shear force analysis indicated an obvious increase in corresponding values for day 21 (Figure 1). Moreover, although statistically insignificant, the samples from this type of beef had the highest sensory-panel scores of tenderness (6.60). These results are in accord with the study conducted by Campbell et al. (2001) who suggested that increasing dry ageing time from 14 to 21 day did not appreciably influence flavour or tenderness. Beef colour evaluation also indicated a sharp decrease in the L^*, a^*, and b^* values for 14 to 21 day dry ageing period (Figure 2). Moreover, the 21-day dry-aged beef was significantly associated with the lowest values for pH and WHC (P<0.05). In this study, no statistical differences among treatments for CL were found to demonstrate the effectiveness of the dry-ageing time. These results are of great importance for practical applications, because they allow the implementers in beef sector to decide the optimum dry-ageing with maximum profit.

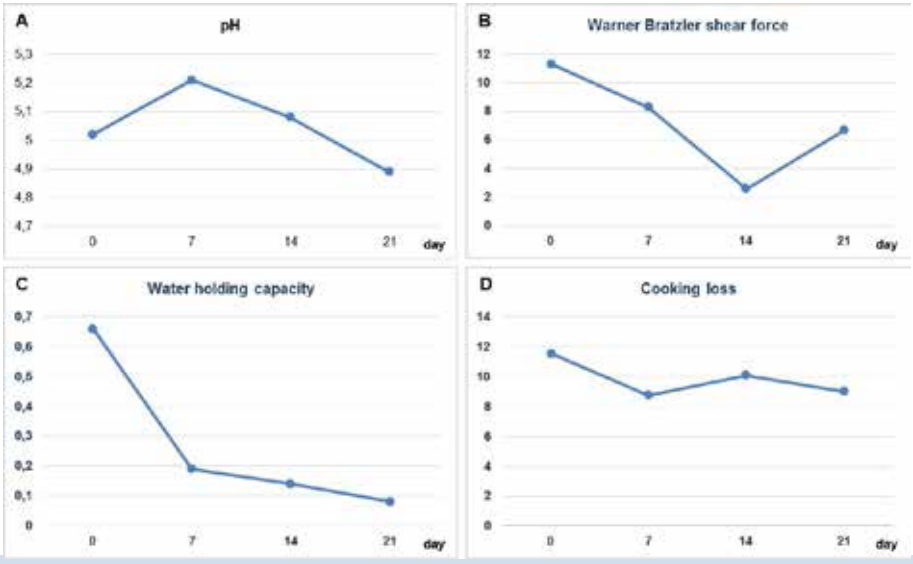

Figure 1. Changes in beef quality parameters including pH, Warner Bratzler shear force (N/mm), water holding capacity, and cooking loss (%) during 21 days of dry-ageing

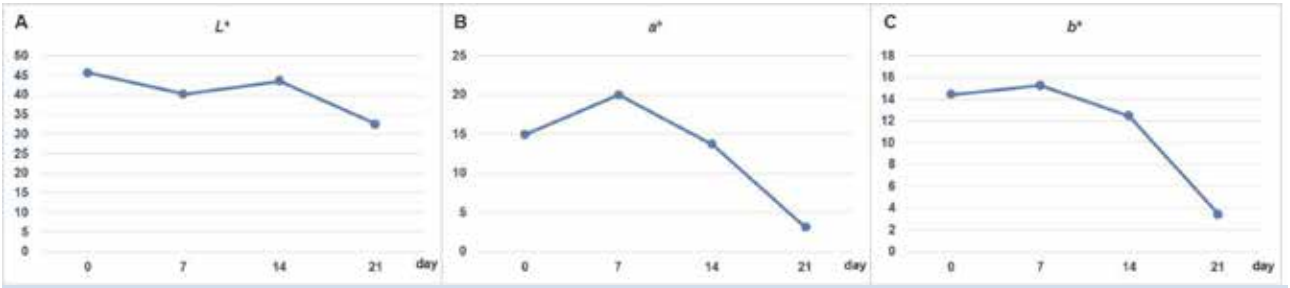

Figure 2. Changes in beef colour parameters during 21 days of dry-ageing

Beef quality is affected by many factors including genetics, management, pre-and post-slaughter factors (Warner et al., 2010). Moreover, evaluation of beef quality through ageing process is complex one. Flavour, juiciness and tenderness influence the palatability of meat and these parameters directly affect the consumer perceptions which include both visual and sensory traits (Koohmaraie and Geesink, 2006; Warner et al., 2010). In the present study, results clearly demonstrated that the most desirable beef is 14-day dry-aged for which panellists gave higher rankings (6.60, 6.20, 6.60, 5.94, 6.53, and 6.60 for odour, flavour, tenderness, juiciness, general acceptance, and overall liking, respectively). However, the only statistical difference among samples was for colour score (P<0.05). The samples at day 14 (6.73) had +1.25, +0.932, and +1.20 higher means of colour score compared to samples at day 0, 7, and 21, respectively. One possible explanation for decrease in colour scores at day 21 may be the higher metmyoglobin percentage at the surface of beef under modified atmosphere after 21 days of dry-ageing together with the decrease that was observed in redness.Beef colour is one of the most important quality parameters because it directly influences the consumer's buying decision (Ardicli, 2018), and thus, evaluation of colour preferences may be crucially important for dry-aged beef which is offered in mostly fine restaurants, upscale grocery stores and gourmet steak companies. Here it should be noted that the application of dry-ageing is usually for 14 to 30 days post mortem (Stenström et al., 2014). Dry ageing process generally improves flavour, sensory, and textural attributes(Warren and Kastner, 1992; Campbell et al., 2001). Increase in dry aged beef flavour is related to several chemical interactions involving proteins, lipids and carbohydrates. In this respect, this particular improvement of the flavour may be relevant to releasement of free amino acids, peptides, reducement of sugars, and the breakdown of ribonucleotides during postmortem ageing. In addition, carbohydrates broken down into sugars can ultimately give sweet taste, while degradation of fats and fat like membrane molecules may contribute to beef aroma (Dashdorj et al., 2016). These chemical alterations give concentration of flavor to dry aged beef. Moreover, during dry ageing process, the natural enzymes in the beef may provide desirable tenderness levels (Warren and Kastner, 1992). However, our study showed that dry ageing for more than 14 days appears to be inadequate with respect to beef quality analyses and sensory panel results.It is apparent that the development of beef quality is a highly dynamic process. Continuing activity of the endogenous hydrolases and proteolytic enzymes which have different substrate specificities and pH optima during the post-mortem ageing period and tenderization process constantly changes the quality parameters and flavor components (Spanier et al., 1997). Improper quality parameters of beef dry-aged for 21 days may be attributed to undesired chemical alterations in the level of numerous reactive chemicals and intermediates during the postmortem ageing period (Warren and Kastner, 1992; Spanier et al., 1997; Dashdorj et al., 2016). Moreover, interactions and reactions between chemical components including peptides and free amino acids, carbohydrates, organic acids, and metabolites of adenine nucleotide metabolism such as ATP are most likely to contribute to the significant results obtained from the present study for 14 days dry-ageing application.

Dry ageing of beef is a costly procedure because of decreased yields due to greater weight, trim losses and time consuming processing (Dashdorj et al., 2016). It is important to note that, a longer ageing time is suggested to be associated with increase in trim loss and decrease in microbiological quality (Ahnström et al., 2006; Karaduman et al., 2018). Therefore, decision on the dry-ageing time seems to be a crucial point to achieve a profitable process.

CONCLUSIONS

Consequently, data from the current study demonstrated that dry ageing process partially enhanced beef quality traits including shear force, colour, particularly colour scores as determined by the sensory panel. A significant improvement in WBSF values for samples at day 14 was also observed. The most important point in the present evaluation is that increasing dry-ageing time from 14 to 21 day did not desirably affect beef quality traits and sensory scores. On the contrary, this period was associated with a sharp decrease in quality parameters. Dry ageing did not adversely impact CL. Of interest for those processors who may plan to attend dry-aged beef applications, this study suggests that dry ageing till 14 days would provide more economically favourable process. On the long view, this implementation may be crucial in dry ageing applications because long period of ageing beef is obviously associated with high risk of contamination, trim loss, ageing shrinkage, requirements of ageing conditions and space. Considering the raise of demand high quality beef products, such as dry-aged beef, studies focusing on this processing need to be performed because corresponding results and recommendations on ageing conditions may help companies, retailers or upscale restaurants who aim to produce a dry aged beef.

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CONFLICT OF INTEREST STATEMENT

None of the authors of this article has any conflict of interest.

REFERENCES

Ahnström ML, Seyfert M, Hunt MC, Johnson DE(2006) Dry aging of beef in a bag highly permeable to water vapour. Meat Sci73(4): 674-679.

Ardicli S(2018) Genetik ve postmortem mekanizmaların sığır eti renk özellikleri üzerine etkisi. Uludag Univ J Fac Vet Med 37(1): 49-59 (in Turkish with an abstract in English).

Campbell R, Hunt M, Levis P, Chambers E(2001) Dry-aging effects on palatability of beef longissimus muscle. J Food Sci66(2): 196-199.

Dashdorj D, Tripathi VK, Cho S, Kim Y, Hwang I(2016) Dry aging of beef; review. J Anim Sci Technol 58(1): 20-30.

Ekiz B, Yilmaz A, Ozcan M, Kaptan C, Hanoglu H,ErdoganI, Yalcintan H (2009)Carcass measurements and meat quality of Turkish Merino, Ramlic, Kivircik, Chios and Imroz lambs raised under an intensive production system. Meat Sci 82(1): 64-70.

Gill JL, Bishop SC, McCorquodale C, Williams JL, Wiener P(2009) Association of selected SNP with carcass and taste panel assessed meat quality traits in a commercial population of Aberdeen Angus-sired beef cattle. Genet Sel Evol 41: 36-47.

Grau R, Hamm R(1957) Über das Wasserbindungsvermögen des Säugetiermuskels. II. Mitteilung. Über die Bestimmung der Wasserbindung des Muskels. Zeitschrift für Lebensmittel-Untersuchung und Forschung 105(6): 446-460.

Hunt M, King A, Barbut S, Claus J, Cornforth D, Hanson D, Lindahl G, Mancini R, Milkowski A, Mohan A, Pohlman F, Raines C, Seyfert M, Sorheim O,Suman S, Weber M (2012) AMSA Meat Color Measurement Guidelines. Champaign, IL: American Meat Science Association, 1–135.

KaradumanTA, Gokce R, Ergezer H, Akcan T(2018) Some physicochemical and sensory properties of dry aged loin, rib and round meat from beef. Journal of Food and Feed Science – Technology 2018(20): 46-54 (in Turkish with an abstract in English).

Koohmaraie M, Geesink G(2006)Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. Meat Sci74(1): 34-43.

Laster M, Smith R, Nicholson K, Nicholson J, Miller R, Griffin DB, Harris KB, Savell JW (2008)Dry versus wet aging of beef: Retail cutting yields and consumer sensory attribute evaluations of steaks from ribeyes, strip loins, and top sirloins from two quality grade groups. Meat Sci 80(3): 795-804.

Li X, Babol J, Bredie WL, Nielsen B, Tomankova J, Lundström K(2014) A comparative study of beef quality after ageing longissimus muscle using a dry ageing bag, traditional dry ageing or vacuum package ageing. Meat Sci 97(4): 433-442.

Miller MF, Carr M, Ramsey C, Crockett K, Hoover L(2001)Consumer thresholds for establishing the value of beef tenderness. J Anim Sci79(12): 3062-3068.

Oreskovich DC, McKeith FK, Carr TR, Novakofski J, Bechtel PJ(1988) Effects of different aging procedures on the palatability of beef. J Food Qual11: 151–158.

PietrasikZ, Duda Z(2000) Effect of fat content and soy protein/carrageenan mix on the quality characteristics of comminuted, scalded sausages. Meat Sci56(2): 181-188.

Resconi VC, Campo MM, i Furnols MF, Montossi F, Sanudo C(2010) Sensory quality of beef from different finishing diets. Meat Sci 86(3): 865-869.

Sitz B, Calkins CR, Feuz DM, Umberge WJ, Eskridge KM(2006)Consumer sensory acceptance and value of wet-aged and dry-aged beef steaks. J Anim Sci84(5): 1221-1226.

Smith R, Nicholson K, Nicholson J, Harris K, Miller R(2008) Dry versus wet aging of beef: Retail cutting yields and consumer palatability evaluations of steaks from US Choice and US Select short loins. Meat Sci 79(4): 631-639.

Spanier AM, Flores M, McMilli KW, Bidne TD (1997) The effect of

post-mortem aging on meat flavor quality in Brangus beef. Correlation of treatments, sensory, instrumental and chemical descriptors. Food Chem 59: 531–538.

Stenström H, Li X, Hunt MC,Lundström K(2014) Consumer preference and effect of correct or misleading information after ageing beef longissimus muscle using vacuum, dry ageing, or a dry ageing bag. Meat Sci 96(2): 661-666.

Warner R, Greenwood P, Pethick D, Ferguson D(2010) Genetic and environmental effects on meat quality. Meat Sci 86(1): 171-183.

Warren K, Kastner C (1992)Acomparison of dry-aged and vacuum-aged beef strip loins. J Muscle Foods 3(2): 151-157.

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Research article
Ερευνητικό άρθρο

Mass mortality of unknown etiology in alpine newts (*Ichtyosaura alperstris veluchiensis*) in an alpine lake in Greece

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ABSTRACT: A mass mortality in alpine newts (*Ichthyosaura alpestris veluchiensis*) was observed in May/June 2013, in Drakolimni lake on Smolikas Mountain, Northwest Greece. 1300 alpine newts were found dead in two events. In 1998 a similar incident was recorded in the nearby lake of Timfi Mt. Newts of every stage and sex were affected, presenting incoordination and inability to float evenly. Ten animals were submitted for complete pathological examination. Field environmental measurements (water temperature, oxygen saturation, pH, conductance, nitric/phosphate concentration) and samples (water, snow, benthos) were collected for ecotoxicological and quality analysis. Necropsy, microbiology (parasitology, bacteriology, mycology), histopathology, molecular investigations (*Ranavirus spp*, *Batrachochytridium dendrobatidis, Batrachochytridium salamandrivorans)*, quality and ecotoxicological examinations did not indicate a causative source for the mortality. To the author´s knowledge this is the biggest mortality of unknown etiology reported in free-living alpine newts in Europe.

Keywords: *alpine newt, mass mortality, Greece, incoordination, unknown etiology*

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INTRODUCTION

This short communication describes the investigation of a mass mortality incident in alpine newts in Greece. The Balkan Peninsula is considered to be a major hotspot for European biodiversity (Griffiths et al., 2004). Balkan alpine lakes are not well studied, due to their small size and difficult access. Greek alpine lakes are small, often fishless and located at high-altitude areas above 1900m, near the summit of mountains. They are called "dragon-lakes" (Drakolimnes in Greek) because their main vertebrate inhabitant, the alpine newt (*Ichthyosaura alpestris*), is reminiscent of the shape and appearance of a small dragon. *Ichthyosaura alpestris* (initially referred to as *Triturus alpestris* and more recently as *Mesotriton alpestris*) is a small animal with a total length of about 80 to 100 mm for males and up to 120mm for females. The Greek alpine newt was classified as a subspecies named *Ichthyosaura alpestris veluchiensis* (Denöel, 2004). Although *I. alpestris* specimens in northwestern Greece are found in various water bodies above 1190m such as ponds, drinking troughs and watering basins, large populations inhabit only the alpine lakes of the region (Denöel and Schabetsberger, 2003).

MATERIALS AND METHODS

The incident took place in the dragon-lake of Mt. Smolikas (40°05'N, 20°54'E, 2140m a.s.l), in the wider region of Ipirus, Northwestern Greece. The lake has a rectangular shape (122m long, 61m wide) and a maximum depth of 3.7 m. Vegetation is limited to small shallow patches. The bottom is muddy, and rocks are rare (Denöel and Schabetsberger, 2003). An older incident of mass mortality was described in 1988 by one author (H. Papaioannou pers.com) in the dragon-lake of Mt. Tymphi (39°59'N, 20°47'E, 2000m a.s.l),which has a somewhat quadratic appearance (max. diameter: 100m) and a maximum depth of 4.95m. It is characterized by rich vegetation along the shoreline, consisting mostly of *Carex* sp (Denöel and Schabetsberger, 2003). The distance between Lake Tymphi and Lake Smolikas is 14.85 km. No exchange exists between newt populations inhabiting the two lakes due to impassable deep valleys that separate them (Figure 1).

A total of 1604 alpine newts were found dead in two events (Mortality rate 0.81% and morbidity rate 0.189%) in May and June 2013. Newts of every stage and both sexes were affected. Clinically affected newts were uncoordinated and unable to float evenly (Figure 2). Otherwise no other symptoms were evident upon observation. The area was searched intensively for the presence of salt, agrochemicals or other possible source of poisoning.

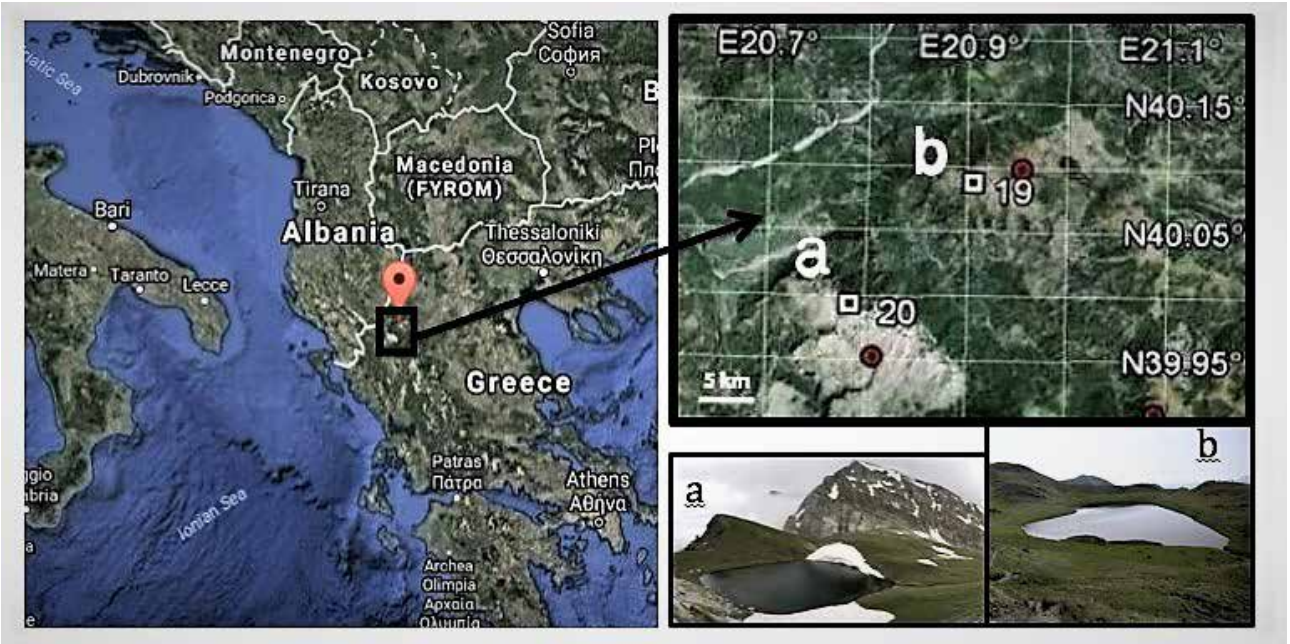

Figure 1. A satellite view of the study area in the mountains of NW Greece. The studied populations are indicated with square shape. Other known newt populations are indicated with red dots. (a: Drakolimni Tymphis, b: Drakolimni Smolika) (Satellite photos by Google-earth)

Figure 2. a) Dead male alpine newt under examination (dorsal view) b) male newt abdominal view c) neotenic newt (close up the external gills)

Environmental measurements included water temperature, oxygen saturation, pH, conductance, nitric and phosphate concentration and were performed on site while samples (water, snow and benthos) were collected using sterilized containers for further ecotoxicological and quality analysis. Samples were kept cool until they were transported to the laboratory and processed. Collected surface water and melted snow were used to detect toxicity. Thamnotox bioassay was performed according to the manufacturer's instructions (MicroBioTests Inc, Kleimoer 15, 9030, Mariakerke (Gent), Belgium). In order to check if the Thamnotox test operates properly we performed a quality control test with the toxicant potassium dichromate ($K_2Cr_2O_7$). For Thamnotox toxicity test we prepared dilutions of surface water and melted snow (6.25% -100%) and we transferred them into the multiwell plate at four replicates. Ten hatched larvae of *Thamnocephalus platyurus* were transferred into each well and the multiwell plate was incubated at 25° C in darkness for 24h. After 24h, using a dissection microscope, we counted live and dead larvae and we calculated the percentage mortality.

Additionally, ten clinical affected newts from different sex and stages (adults, juveniles, and larvae) were collected and transported alive for further veterinary investigations.After humane euthanasia (general anesthesia under isoflurane and pentobarbital injection) (Gentz, 2007),necropsy was performed immediately under standard protocols including wet mount parasitology, bacteriology, mycology and histopathology (one individual). As initial isolation media, Columbia blood agar (Oxoid, Wesel, 46483, North Rhine-Westphalia, Germany), MacConkey agar (Oxoid) and brilliant-green agar (Oxoid) were streak inoculated with the samples and then incubated at 41°C for 24 h under aerobic conditions. If after this time no or scanty growth was present the incubation time was prolonged for another 24 h. Colonies demonstrating distinctive macroscopic appearance were considered separate organisms and isolated on new plates for identification. The isolated bacteria were identified using standard microbiologic techniques including Gram-staining, morphologic characteristics, catalase and oxidase reactions and growth in limiting media. Representative Gram-negative isolates of all distinct

organisms were further identified using the multi-substrate-identification kit BBL Crystal TM E/NF (Becton–Dickinson, Franklin Lakes, 07417-1880,NJ, USA). Among Gram-positive bacteria, cocci and Bacillus were identified to the genus based on colony morphology, catalase and oxidase reactions as well as microscopic appearance after staining. Isolation of Salmonella and determination of Serotypes RV broth (Oxoid) were incubated at 41°C for 24 h, and subsequently a loopful (10 lL) of RV broth culture was streak inoculated onto a xylose lysine tergitol-4 (XLT-4) agar (Oxoid Deutschland GmbH,46483, DE) and a brilliant-green (BGA) agar plate (Oxoid Deutschland GmbH, 46483, DE), respectively. Plates were incubated for another 24 h under aerobic conditions at 41°C. All suspicious colonies were screened for Salmonella with a rapid plate agglutination test using Enteroclon Anti-Salmonella A67, omnivalent (Sifin GmbH, 13088, Berlin, DE). A mycological culture was performed on Sabouraud dextrose agar (Oxoid) and potato dextrose agar (Oxoid) and incubated in 41°C for up to 120 h. One animal was completely fixed in 4% neutral-buffered formalin. Fixed samples were embedded in paraffin, and 5 µm sections were stained with haematoxylin and eosin for histological examination by light microscopy.

Collective samples from nine newts were used for molecular investigations. Total genomic DNA was extracted using the Roche MagNA Pure 96 system with the MagNA Pure 96 DNA and viral RNA small Volume Kit (Roche, Mannheim, 68305, Germany) according to the manufacturer's instructions. A real-time PCR was used to assess the presence/absence of *B. dendrobatidis, B. salamandrivorans* and a conventional PCR to detect *Ranavirus* DNA within the extracted DNA as described previously (Marschang et al., 1999, Boyle et al., 2004, Blooi et al., 2013).

RESULTS

The field investigation by the researchers in the two visits did not reveal human activity, salt disposal or any other source of possible poisoning. The physical parameters measured on site were within the normal limits except the slightly reduced pH (Table 1). Zooplankton concentration was slightly reduced. The acute toxicity assays and the cyanotoxine measurement were negative. No pesticide traces were detected.The values from the reference test were within the limits indicating the good procedure of the bioassay. Percentage mortality of the *Tamnocephalus platyurus* in the samples was very low and comparable to controls. Complete necropsy in three newts revealed no internal or external alterations. Special attention was given to possible skin lesions indicating fungal disease. Parasitology, bacteriology, mycology was unremarkable, and histopathology showed partial autolysis, no signs of infectious process or foreign materials (i.e. sand). Skin histology from 13 sympatric newts for another study some months (October 2013) ago the mortality events, did also not reveal cancerous or skin pathology (Papaioannou et al., 2015). Real-time and conventional PCR for *Ranavirus*, *B. dendrobatidis* and *B. salamandrivorans* were negative.

Table 1. Physical and chemical parameters in alpine dragon-lake of Mt. Smolikas during a newt mass mortality event

Physical chemical parameters in Smolikas Dragon-lake in May 2013	
Temperature	4°C
pH	7.2
S^{2-}	Not detected
NH_4^+	0.02 mg/L
NO^{2-}	0.019 mg/L
NO^{3-}	1.2 mg/L
Ca^{2+}	2.5 mg/L
Mg^{2+}	0.46 mg/L
PO_4^{3-}	Not detected
Na, K, Cl	Not detected
O_2	8 mg/L

DISCUSSION

Mass mortality of free-living newts in Europe, has been reported infrequently with the greatest number recorded being 691 alpine newts in Austria (Sztatecsny and Hodl, 2009). The die-offs so far reported were attributed to road trespass (Mitchell, 2000), ranaviruses (Balseiro et al.,2010, Kik et al., 2011, Martinez-Silvestre et al., 2017) and salt toxicity (Duff et al.,2011).*B. dendrobatidis* and *B. salamandrivorans* were blamed for the decline of Sardinian newts (Bovero et al., 2008) and fire salamanders (Martel et al., 2013) respectively. In alpine newts, which reside in isolated highland niches, only one outbreak has been reported due to ranavirus (Balseiro et al., 2010)and Common Midwife Toad Virus (CMTV)-like ranavirus (Price et al., 2014). Mesomycetozoans fungus-like organisms like *Ichthyophonu* ssp*, Amphibiocystidium* sp cause marked changes in skin and internal organs (Raffel et al., 2008). None of the above-mentioned causes of newt mortality causing skin and internal organ alterations, was detected in the current investigated event (alive specimens, post-mortem examined

specimens and the single histopathologic examined specimen). Additionally,skin histology from 13 sympatric newts for another study five months after the mortality event, did also not reveal cancerous or skin pathology (Papaioannou et al., 2015).Nevertheless, it could not be excluded that other pathogens could be involved such as invertebrate Iridovirus IIV-6 (Stöhr et al., 2016)and Frog Virus-like ranavirus (FV3) (Peace et al.,2019, Vilaca et al., 2019) although the later viruses have been so far affecting anuran populations mostly in North America. Apart from viruses, Perkinsea protists have been associated with cryptic infections and mass mortality of anuran populations and have been found in tadpoles in UK and Czech Republic (Chambouvet et al., 2015).In another scenario biological/environmental parameters could havetriggered the event (e.g. overpopulation, quicker defrosting of the lake with abrupt temperature variation and reduced oxygen concentration). The authors also investigated the option of pneumoconiosis as subsequent to an African red sandstorm in the area. None traces of sand were detected in oropharyngeal cavity, lungs, or internal organs of the examined newts. The limited histopathological samples and the recent molecular sequencing of various rana-like viruses (which were not known at the time of the events) could also have implicated the elucidation of a specific pathogen in this event.The exact date that the mortality started remains unknown, thus the authors could not exclude the possibility that the physical parameters measured on-site could have been changed during the time of initial newt death and the arrival of the investigators. The presence of both dead and alive newts suggests that the events were not acute, but rather spread over a couple of months and if a pathogen or poisoning agent was implicated it would be still active and detectable during the sampling. At the present date of submission of this manuscript the authors have not observed any further mortality in the same alpine lake or any other alpine lake in the area.

CONCLUSIONS

Amphibian medicine can be challenging despite its progress the recent years. In this outbreak sampling was challenging due to the rugged terrain and a possible causative agent/parameter was not identified. Authors stress the importance that similar mortality events in amphibians should be elaborated interdisciplinary (chemical, biological, veterinary, meteorological data) to unveil a possible puzzle of parameters leading to such events. At the time of this manuscript no other mortalities have been reported from these lakes or other alpine lakes in Greece. The authors will further monitor these and other alpine lakes and record possible mortalities in the future.

CONFLICT OF INTEREST

None declared by the authors.

REFERENCES

Balseiro A, Dalton PK, del Cerro A, Marquez I, Parra F, Prieto JM, Casais M (2010) Outbreak of common midwife toad virus in alpine newts (*Mesotritonalpestris cyreni*) and common midwife toads (*Alytes obstetricans*) in Northern Spain: A comparative pathological study of an emerging ranavirus. Vet Journal 186: 256–258

Blooi M, Pasmans F, Longcore JE, Spitzen-van der Sluijs A, Vercammen F, Martel A (2013)Duplex real-time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in Amphibian samples. J Clin Microbiol 51(12):4173-7. doi: 10.1128/JCM.02313-13. Epub 2013 Oct 9.

Bovero S, Sotgiu G, Angelini C, Doglio S, Gazzaniga E, Cunningham AA, Garner TW (2008) Detection of chytridiomycosis caused by Batrachochytrium dendrobatidis in the endangered Sardinian Newt (*Euproctus platycephalus*) in Southern Sardinia, Italy. J Wild Dis 44(3): 712–715

Boyle DG, Boyle DB, Olsen V, Morgan JA, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Organ 60(2):141-8.

Chambouvet A, Gower DJ, Jirků M, Yabsley MJ, Davis AK, Leonard G, Maguire F, Doherty-Bone TM, Bittencourt-Silva GB, Wilkinson M, Richards TA. Cryptic infection of a broad taxonomic and geographic diversity of tadpoles by Perkinsea protists. Proc Natl Acad Sci U S A. 25;112(34): E4743-51. doi: 10.1073/pnas.1500163112.

Denoël M (2004) Distribution and characteristics of aquatic habitats of newts and Yellow-bellied Toads in the district of Ioannina (Epirus, Greece). Herpetozoa 17:49-64.

Denoël M, Schabetsberger R (2003) Resource partitioning in two heterochronic populations of Greek Alpine newts, *Triturusalpestrisveluchiensis*. Acta Oecologica 24:55–64

Duff JP, Colvile K, Foster J, Dumphreys N (2011) Mass mortality of great crested newts (*Trituruscristatus*) on ground treated with road salt. Vet Record 168:282

Gentz EJ (2007) Medicine and Surgery of Amphibians. *ILAR Journal* 48:3:255–259. https://doi.org/10.1093/ilar.48.3.255

Griffiths HI, Krýstufek B, Reed JM (2004) Balkan biodiversity, Pattern and process in the European hotspot. Dordrecht, Boston, London, Kluwer Academic Publishers

Kik M, Martel A, Spitzen-van der Sluijs A, Pasmans F, Wohlsein P, Gröne A, Rijks JM (2011) Ranavirus-associated mass mortality in wild amphibians, The Netherlands, 2010: A first report. Vet Journal 190(2): p.284-286

Marschang, RE, Becher P, Posthaus H, Wild P, Thiel HJ, Müller-Doblies U, Kaleta EF, Bacciarini LN (1999) Isolation and characterization of an iridovirus from Hermann‘s tortoises (*Testudo hermanni*). Archiv Virol144: 1909-1922

Martel A, Spitzen-van der Sluijsb A, Blooi M, Bert W, Ducatelle R, Fischer MC, Woeltjes A, Bosman W, Chiers K, Bossuyt F, Pasmans F

(2013) *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. PNAS 2013; 110 (38): 15325-15329

Martinez-Silvestre A, Montori A, Oromi N, Soler J, Marschang RE (2017) Detection of a ranvirus in introduced newts in Catalonia (NE Spain) Herpetology Notes 10:23-6

Mitchell CP (2000)Mass Mortality of Red-spotted Newts (*Notophthalmus viridescens viridescens* Rafinesque) on a Central Virginia Road *Banisteria* 15: 45-47.

Papaioannou H, Batistatou A, Mavrogiorgou MC, Konidaris C, Frillingos S, Briasoulis E, Vareli K, Sainis I (2015) High incidence of dorsal dark spots in north-western Greek populations of alpine newts *Ichthyosaura alpestris*(Salamandridae, Caudata) Herpetology Notes 8: 589-598

Peace A, O'Regan S, Spatz JA, Reilly PN, Hill RD, Carter ED, Wilkes RP, Waltzek TB, Miller DL, Gray MJ (2019) A highly invasive chimeric ranavirus can decimate tadpole populations rapidly through multiple transmission pathways. Ecol. Model.,410:108777, ISSN 0304-3800,https://doi.org/10.1016/j.ecolmodel.2019.108777.

Price SJ, Garner TW,Nichols RA, Balloux F, Ayres C, de Alba AMC, Bosch J (2014) Collapse of amphibian communities due to an introduced ranavirus. Curr.Biol. 24(21),2586–2591.

Raffel TR, Bommarito T, Barry DS, Witiak SM, Shackelton LA (2008) Widespread infection of the Eastern red-spotted newt (*Notophthalmus viridescens*) by a new species of Amphibiocystidium, a genus of fungus-like mesomycetozoan parasites not previously reported in North America. Parasitol 135: 203–215.

Stöhr AC, Papp T, Marchang RE (2016) Repeated detection of an invertebrate iridovirus in amphibians J Herp Med Surg 26:54-58

Sztatecsny M, Hodl W (2009) Can protected mountain areas serve as refuges for declining amphibians? Potential threats of climate change and amphibian chytridiomycosis in an alpine amphibian population. Eco.mont 1:19-24

Vilaça ST, Bienentreu J-F, Brunetti CR, Lesbarrères D, Murray DL, Kyle CJ (2019) Frog virus 3 genomes reveal prevalent recombination between ranavirus lineages and their origins in Canada. J Virol 93:e00765-19. https://doi.org/10.1128/JVI.00765-19.

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Research article
Ερευνητικό άρθρο

Detection of *Mycobacterium avium* subsp. *paratuberculosis* in liver of slaughtered cattle, sheep and goats by PCR and culture

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ABSTRACT: The presence of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in tissues other than intestines and associated lymph nodes is a potential public health concern. Therefore, the presence of *MAP* in the liver of slaughtered cattle, sheep and goats with unknown Johne's disease status was evaluated. Liver samples were collected randomly from 470 mature animals including 200 cattle, 135 sheep and 135 goats. The existence of *MAP* DNA from obtained liver samples was determined using PCR, thereafter PCR-confirmed samples were cultured. Based on liver PCR results, *IS900* genes were detected in 11/200 (5.5%), 3/135 (2.2%), 2/135 (1.48%) liver samples of cattle, sheep and goats, respectively. In cattle, sheep and goats, 6 (54.5%), 1 (33.3%) and 1 (50%) PCR-positive samples showed positive results in culture, respectively. Our results revealed that *MAP* can be detected and cultured from the liver of slaughtered cattle, sheep and goats with unknown Johne's disease status. Hence, given concerns about a potential zoonotic role for *MAP*, there is a crucial need to detect animals with *MAP* disseminated infection in the liver before slaughter.

Keywords: *Mycobacterium,* Paratuberculosis liver, Johne's disease, Public health, PCR

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INTRODUCTION

The possible role of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in human Crohn's disease, an inflammatory disease of the intestines, has been evaluated for many years (Behr and Kapur 2008; Bull et al. 2003; Uzoigwe et al. 2007). This organism is the causative agent of Johne's disease or paratuberculosis, which is primarily expressed in the gastrointestinal tract. Isolation of *MAP* from extra-intestinal tissues indicates that microbial dissemination via the haematogenous route may occur during the infection.

The role of *MAP* in human Crohn's disease is strongly supported by studies that demonstrate that human beings are exposed to *MAP* within the food, which thus might represent a mode of transmission. Amongst the food products, *MAP* has been already detected in milk and dairy products (Ellingson et al. 2005;Grant et al. 2001;Ikonomopoulos et al. 2005), meat and meat products (Alonso-Hearn et al. 2009; Reddacliff et al. 2010), and liver(Antognoli et al. 2008; Mutharia et al. 2010).

However, the presence of *MAP* in tissues other than intestines and associated lymph nodes; such as meat and liver; have been mostly reported in experimentally infected animals (Bower et al. 2011; Gwozdz et al. 2000) or animals that either showed overt clinical disease or were previously confirmed as fecal shedders by fecal culture (Antognoli et al. 2008; Mutharia et al. 2010).

The liver is a valuable edible organ with high nutritional value and a wide range of use in preparing different food products. Knowing the thermal resistance of *MAP* and the probability of survival of *MAP* in heated foodstuffs (Gao et al. 2002; McDonald et al. 2005; Mutharia et al. 2010), the presence of *MAP* in the liver of the slaughtered animals should be considered more.

The traditional gold standard testing method for *MAP* infection is a bacterial culture of the samples. However, this method has the drawbacks that it takes several weeks or months to yield a result with an estimated sensitivity between 30 and 50% (Whitlock et al. 2000) and unfavorable interlaboratory standardization (Juste et al. 2005). Furthermore, competing bacterial and fungal contamination often overgrow *MAP* cultures, making them unreadable (Stabel et al. 2002). Considering these hurdles, PCR has been widely used to detect *MAP* in suspected samples.

In the present study, the presence of *MAP* in the liver of slaughtered cattle, sheep and goats with unknownJohne's disease status was evaluated by PCR and culture method.

MATERIALS AND METHODS

Collection and preparation of liver samples

During period of six months, a slaughterhouse in the South-western part of Iran was visited once a week and liver samples were collected randomly from 470 mature animals including 200 cattle, 135 sheep and 135 goats. According to the results of the antemortem inspection which was performed by the slaughterhouse veterinarian, the livestock used in the present study for sampling had normal body conditions and also, they did not show any symptoms of Johne's disease. Immediately after opening the carcass, liver samples were taken with sterile single-use surgical blades and transferred to sterile plastic bags.

A pooled liver sample was prepared by cutting liver samples into small pieces and then, a two-gram piece of them was aseptically transferred to a sterile 50 ml falcon tube and gently crushed by a sterile glass rod. Then 18 ml of sterile physiological saline solution was added to the falcon and vortexed for 20 min. The homogenates were filtered through two layers of sterile gauze and centrifuged at 4000 rpm for 30 min. The supernatants were discarded, and the resulting pellets were resuspended in 1 ml of sterile physiological saline solution and divided into two microcentrifuge tubes.

Liver PCR

For DNA extraction, an aliquot of the resuspended pellet (500 µl) was transferred to a microcentrifuge tube and centrifuged at 12000 rpm for 5 min. The pellet was resuspended in 200 µl lysis buffer(100 mM Tris-HCl, pH 8.0; 200 mM NaCl; 0.1 % SDS; 1 % Triton X-100; 5 mM EDTA), stored at room temperature for 30 min and then heated at 100 °C for 10 min and centrifuged at 12000 rpm for 10 min. Subsequently, DNA was extracted with phenol:chloroform:isoamylalcohol (25:24:1), and finally precipitated with the one-tenth volume of sodium acetate (3 M, pH 5.2) and 2.5 volume of chilled absolute ethanol. The precipitated DNA was washed in 80% alcohol, dried and dissolved in 100 µl sterile distilled water. The extracted DNA was frozen until PCR analysis (Nolte et al., 1993).

PCR specific to *MAP* was performed by *IS900*

primers. The *IS900* primers (forward 5′-CCGCTA-ATTGAGAGATGCGATTGG-3′ and reverse 5′-AAT-CAACTCCAGCAGCGCGGCCTCG-3′) amplify a portion of an insertion sequence found 15 to 20 times throughout the *MAP* genome. Amplification condition was as follows: 5 min at 94 °C; 50 cycles of 1 min at 94 °C, 30 s at 65 °C, and 1 min at 72 °C; and a final 10-min extension at 72 °C. A sample was considered positive when an amplified product was observed at 229 bp (Ellingson et al. 1998; Miller et al. 1999; Ellingson et al. 2005).

Liver culture

The remaining aliquot of the resuspended pellet was decontaminated according to Giese and Ahrens (2000). Briefly, 1 ml of 4 % sodium hydroxide solution was added to the sample, stored at room temperature for 15 min and centrifuged at 12000 rpm for 5 min. The supernatant was discarded and the pellet was diluted in 1 ml of 5% oxalic acid solution containing 0.1 % malachite green. After 30 min storage at room temperature, centrifugation was performed and the supernatant discarded. The resulting pellet was diluted in 1 ml of Tryptic Soy Broth (TSB) containing amphotericin B (100 mg/l), chloramphenicol (50 mg/l), vancomycin (50 mg/l), nalidixic acid (50 mg/l). Samples were stored at 4 °C until the PCR results were revealed (Ellingson et al. 1998; Miller et al. 1999; Ellingson et al. 2005).Liver samples that were PCR positive, used formycobacterial culture.

For mycobacterial culture, 250 µl of the above-mentioned TSB was streaked onto two slants of Lowenstein-Jensen medium supplemented with mycobactin J (2 mg/l), amphotericin B (100 mg/l), chloramphenicol (50 mg/l), vancomycin (50 mg/l), nalidixic acid (50 mg/l). Tubes were incubated at 37 °C in a slanted position with loose caps to allow the surface of the mediums to dry. Once the slant surfaces were dry, the tube caps were tightened. Cultures were examined every week for the first 6 weeks for signs of contamination. Contaminated samples were recultured. All cultures without signs of colony growth were held for 18 weeks before results were determined. Positive slants were rinsed with 500 µl of physiological saline solution. DNA extraction and PCR were performed on the slant rinses to confirm the results.

RESULTS

Based on liver PCR results, *IS900*genes were detected in 11/200 (5.5%), 3/135 (2.2%), 2/135 (1.48%) liver samples of cattle, sheep and goats, respectively. In cattle, sheep and goats, 6 (54.5%), 1 (33.3%) and 1 (50%) PCR-positive samples showed positive results in culture, respectively (Table 1).

Table 1. *MAP* culture results of PCR positive livers

		Liver culture	
	No of positive PCR results (Total samples)	**No of positive culture results (%)**	**No of negative culture results (%)**
Cattle	11 (200)	6 (54.5)	5 (45.5)
Sheep	3 (135)	1 (33.3)	2 (66.7)
Goat	2 (135)	1 (50)	1 (50)

DISCUSSION

The cultivation of *MAP* is laborious and time-consuming and does not always lead to bacterial growth; with 30 to 50 % sensitivity (Whitlock et al. 2000). Therefore, in the present study, PCR was used to determine the existence of *MAP* DNA in the liver samples and thereafter liver culture was performed on the PCR-positive samples. PCR targeting the *IS900* gene was considered specific for identification of *MAP* and has frequently been applied to confirm the presence of this organism in the diagnosis of Johne's disease (Ellingson et al. 1998, 2005).

In the present study, liver cultures were positive in 6/11, 1/3 and 1/2 liver samples with a positive result in PCRof cattle, sheep and goats, respectively. Difficulties in the cultivation of *MAP* on culture media (Whittington 2009), the type of culture media (de Juan et al. 2006), the presence of viable but non-culturable cells (Pribylova et al. 2011), and the decontamination die-off are the possible reasons for interpreting these results. According to Reddacliff et al. (2003),routine decontamination protocols in the laboratory were shown to decrease the number of organisms isolated per sample by about 2.7 log and 3.1 log for faeces and tissues, respectively. Hence, samples with low numbers of *MAP* that are subjected to decontamination may show negative culture results. Of course, it should be noted that in the absence of decontamination, competing bacterial and fungal

contamination often overgrow *MAP* cultures, making them unreadable (Stabelet al. 2002).

MAP culture from blood and extra-intestinal tissues in experimentally infected sheep has been evaluated by Bower et al. (2011). They reported disseminated infection in the liver and hepatic lymph nodes of 33.96 % of the 53 sheep which were infected following oral exposure to *MAP.* However*,* the bacterium was isolated from the blood of only 4 of these animals. In addition, they reported that disseminated infection can be detected more frequently from animals with a positive fecal culture result and animals with clinical disease and isolation of *MAP* from blood was difficult in the early stages of the disease while PCR was more effective. A lower lever of *MAP* isolation from the liver of experimentally infected sheep has been reported by Dukkipati et al. (2016). They isolated *MAP* from 9.1% of the livers of experimentally infected New Zealand Merino sheep.

In Denmark, *MAP* has been isolated from the livers of 6.7% and 2.2% of the cows aged 1.6-3.0 and 3.1-8.0 years, respectively (Hasonova et al. 2009). Reddacliff et al. (2010) isolated *MAP* from 59% of the muscle and 85% of peripheral lymph nodes of clinically infected sheep. Samples of liver, kidney, lymph nodes and muscle tissues from carcasses of five cows with advanced Johne's disease have been examined for the presence of viable *MAP* by Mutharia et al. (2010). They recovered viable *MAP* from 7 of 15 liver and mesenteric lymph nodes and 5 of 15 kidney and prescapular lymph node samples. According to Antognoli et al. (2008), 21 out of 40 dairy cows from four different livestock in the United States had *MAP* disseminated infection. They isolated *MAP* from 10 liver samples, 6 kidney samples, 2 heart muscle samples and 4 lung samples. In addition, they reported that 57% of the cows with disseminated infection had average to heavy body condition and no diarrhea and only 9 cows with disseminated infection showed evidence of diarrhea at the time of euthanasia.

In conclusion, the results of this study demonstrated that *MAP* can be detected and cultured from the liver of slaughtered cattle, sheep and goats with unknown Johne's disease status.PCR on liver tissue can be effective in preventing the entry of *MAP* infected livers into the human food supply chain. However, this method is not practical to use for all suspected livers at the slaughterhouse. Given concerns about the potential zoonotic role of *MAP*, it seems crucial to evaluate antemortem resources for the identification of animals with disseminated infection at slaughter and to determine which tissues are most frequently colonized by *MAP*. Having such information can lead to more effective ways to deal with human Crohn's disease.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Alonso-Hearn M, Molina E, Geijo M, Vazquez P, Sevilla I, Garrido JM, Juste RA (2009) Isolation of *Mycobacteriumavium* subsp *paratuberculosis* from muscle tissue of naturally infected cattle. Foodborne Pathog Dis 6:513-518.

Antognoli MC, Garry FB, Hirst HL, Lombard JE, Dennis MM, Gould DH, Salman MD (2008) Characterization of *Mycobacterium avium* subspecies *paratuberculosis* disseminated infection in dairy cattle and its association with antemortem test results. Vet. Microbiol 127:300-308.

Behr MA, Kapur V (2008) The evidence for Mycobacterium paratuberculosis in Crohn's disease. Curr Opin Gastroenterol24:17-21.

Bower KL, Begg DJ, Whittington RJ (2011) Culture of *Mycobacterium avium subspecies paratuberculosis* (*MAP*) from blood and extra-intestinal tissues in experimentally infected sheep. Vet. Microbiol 147:127-132.

Bull TJ, Mcminn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P, Rhodes G, Pickup R, Hermon-Taylor J (2003) Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens fromindividuals with and without Crohn's disease. J Clin Microbiol 41:2915-2923.

de Juan L, Alvarez J, Romero B, Bezos J, Castellanos E, Aranaz A, Mateos A, Dominguez L (2006) Comparison of four different culture media for isolation and growth of type II and type I/III *Mycobacterium avium* subsp. *paratuberculosis* strains isolated from cattle and goats. Appl Environ Microbiol 72(9):5927-5932.

Dukkipati VSR, Ridler AL, Thompson KG, Buddle BM, Hedgespeth BA, Price-Carterb M, Begg DJ, Whittington RJ, Gicquel B et al (2016) Experimental infection of New Zealand Merino sheep with a suspension of *Mycobacterium avium subspecies paratuberculosis* (*Map*) strain Telford: Kinetics of the immune response, histopathology and Map culture. Vet. Microbio 195:136-143.

Ellingson JL, Anderson JL, Koziczkowski JJ, Radcliff RP, Sloan SJ, Allen SE, Sullivan NM (2005) Detection of viable *Mycobacterium avium* subsp. *paratuberculosis* in retail pasteurized whole milk by two culture methods and PCR. J Food Prot 68(5):966-972.

Ellingson JL, Bolin CA, Stabel JR (1998) Identification of a gene unique to *Mycobacterium avium* subspecies *paratuberculosis* and application to diagnosis of paratuberculosis. Mol Cell Probes 12:133-142.

Gao A, Mutharia L, Chen S, Rahn K, Odumeru J (2002) Effect of pasteurization on survival of *Mycobacterium paratuberculosis* in milk. J Dairy Sci 85:3198-3205.

Giese SB, Ahrens P (2000) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected cows by PCR and culture. Vet. Microbiol **77**:291-297.

Grant IR, Rowe MT, Dundee L, Hitchings E (2001)*Mycobacterium avium* ssp. *paratuberculosis*: its incidence, heat resistance and detection in milk and dairy products. Int J Dairy Technol 54:2-13.

Gwozdz JM, Thompson KG, Murray A, West DM, Manktelow BW (2000) Use of the polymerase chain reaction assay for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in blood and liver biopsies from experimentally infected sheep. Aust Vet J 78:622-624.

Hasonova L, Trcka I, Babak V, Rozsypalova Z, Pribylova R, Pavlik I (2009) Distribution of *Mycobacterium avium* subsp. *paratuberculosis* in tissues of naturally infected cattle as affected by age. Vet Med 54(6):257-269.

Ikonomopoulos J, Pavlik I, Bartos M, Svastova P, Ayele WY, Roubal, P, Lukas J, Cook N, Gazouli M (2005) Detection of *Mycobacterium avium* subsp. *Paratuberculosis* in retail cheeses from Greece and the Czech Republic.Appl Environ Microbiol 71:8934-8936.

McDonald WL, O'Riley KJ, Schroen CJ, Condrom RJ (2005) Heat inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk. Appl Environ Microbiol 71:1785-1789.

Miller JM, Jenny AL, Ellingson JL (1999) Polymerase chain reaction identification of *Mycobacterium avium* in formalinfixed, paraffin-embedded animal tissues. J Vet Diagn Invest 11:436-440.

Mutharia LM, Klassen MD, Fairles J, Barbut S, Gill CO (2010)*Mycobacterium avium* subsp. *paratuberculosis* in muscle, lymphatic and organ tissues from cows with advanced Johne's disease. Int J Food Microbiol 136:340-344.

Nolte FS, Metchock B, McGowan Jr JE, Edwards A, Okwumabua O, Thurmond C, Mitchell PS, Plikaytis B, Shinnick T (1993). Direct Detection of *Mycobacteriumtuberculosis* in Sputum by Polymerase Chain Reaction and DNA Hybridization. J Clin Microbiol 31(7):1777-1782.

Pribylova R, Slana I, Kralik P, Kralova A, Babak V, Pavlik I (2011) Correlation of *Mycobacterium avium* subsp. *paratuberculosis* counts in gastrointestinal tract, muscles of the diaphragm and the masseter of dairy cattle and potential risk for consumers. Int J Food Microbiol 151:314-318.

Reddacliff LA, Marsh IB, Fell SA, Austin SL, Whittington RJ (2010) Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from muscle and peripheral lymph nodes using acid-pepsin digest prior to BACTEC culture.Vet. Microbiol145:122-128.

Reddacliff LA, Vadali A, Whittington RJ (2003) The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces. Vet. Microbiol95:271-282.

Stabel JR, Wells SJ, Wagner BA (2002) Relationships between fecal culture, ELISA, and bulk tank milk test results for Johne's disease in US dairy herds. J Dairy Sci 85:525-531.

Uzoigwe JC, Khaitsa ML, Gibbs PS (2007) Epidemiological evidence for *Mycobacterium avium* subspecies *paratuberculosis* as a cause of Crohn's disease. Epidemiol Infect 135(7):1057-1068.

Whitlock RH, Wells SJ, Sweeney RW, van Tiem J (2000) ELISA and fecal culture for paratuberculosis (Johne's disease): Sensitivity and specificity of each method. Vet. Microbiol77:387-398.

Whittington RJ (2009) Factors affecting isolation and identification of *Mycobacterium avium* subsp. *paratuberculosis* from fecal and tissue samples in a liquid culture system. J. Clin. Microbiol 47:614-622.

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Research article
Ερευνητικό άρθρο

Survival of Shiga Toxin-Producing *Escherichia coli* (STEC) Serogroups During Production and Storage of Yogurt

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ABSTRACT: In this study, the survival of *Escherichia coli* O157:H7 and non-O157 STEC serogroups of O26, O111, O103, and O145 were investigated during production and storage of yogurt. For this purpose, pathogens were individually inoculated into milk after pasteurization along with the starter culture (approximately 7.00±1.00 $\log_{10}$ cfu/g). After incubation at 44°C (about 180 min), yogurt samples were capped and stored at 4°C for 20 days. Pathogens were enumerated at 0, 5, 10, 15, and 20th days of storage. Lactic acid content (%) and pH of the samples were also screened. Moreover, mesophilic *Lactococcus* spp. and mesophilic *Lactobacillus* spp. were enumerated during production of yogurt.After incubation, the number of *E. coli* O157, O26, O103, O145, O111were 6.76±0.45, 6.64±0.53, 7.12±0.43, 6.00±1.39, 5.89±1.37 $\log_{10}$ cfu/g, respectively. A significant decrease was determined in all groups during the storage of yogurt samples at 4°C ($p<0.05$). It was detected on the 20th day of storage that the number of *E. coli* O157:H7 and non-O157 STEC serogroups of O103 and O145 were under the detection limit. However, STEC O26 and O111 were viable around 1.51±0.98 and 1.18±0.62 $\log_{10}$ cfu/g respectively. Results of the study showed that *Escherichia coli* O157:H7 and non-O157 STEC serogroups might pose a potential health risk during production and storage of yogurt.

Keywords: Yogurt, *E. coli*, VTEC, Acid Adaptation, Dairy Products

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INTRODUCTION

Escherichia coli (*E. coli*) is a natural member of the intestinal flora of all warm-blooded animals, including humans. Additionally, some *E. coli* strains can cause gastrointestinal illnesses in humans (ECDC and EFSA, 2011). Shiga-like toxin-producing *E. coli* serogroups are pathogenic bacteria which are known as Shiga Toxin-Producing *E. coli* (STEC) or Vero-Toxin-Producing *E. coli* (VTEC). Some STEC strains cause serious diseases including hemorrhagic colitis, hemolytic uremic syndrome (HUS), and kidney failure (CDC, 2015).

STEC are generally non-pathogenic to ruminants including cattle, goat, sheep, deer, and elk; on the contrary, these organisms are pathogenic for humans. The major vector of STEC is cattle. Other animals including pigs and birds can spread STEC to the environment. Shiga toxigenic *E. coli* O157 is responsible for most of the STEC outbreaks. On the otherhand, non-O157 STEC outbreaks are recently increasing. Non-O157 serogroups of O26, O103, O111, O121, O145, and O45 were listed as ''Big Six'' by the United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) due to the increasing incidence of food-borne diseases caused by these serogroups (USDA FSIS, 2010). Each year 265.000 STEC infections occur in the USA, 36% of which are the infections caused by STEC O157 and the remaining part is caused by non-O157 STEC. Some food products pose a high risk of *E. coli* O157:H7 infection (CDC, 2014), such as raw milk and soft cheeses made from raw milk. *E. coli* O157:H7 and O26:H11 are the most common serogroups of STEC outbreaks from dairy products (Baylis, 2009; Farrokh et al., 2013).

There are studies which show that *E. coli* O157:H7 can survive in yogurt, Colby, Romano and Feta cheese, Kashar cheese, sour cream, buttermilk, white cheese, and goat cheese fora couple of days to weeks (Arocha et al., 1992; Ioanna et al., 2017; Bellio et al., 2018; Oztekin 2019). According to Arıcan and Andıç (2011) *E. coli* O157:H7 could survive the storage of set yogurt in pH values of 4.0 and 4.6. In several studies, it was stated that since the pathogen can survive for several weeks in different dairy products, *E. coli* O157:H7 poses a food safety risk even in low levels (Arocha et al., 1992; Tosun et al., 2007).

Yogurt is consumed widely in Turkey and annually approximately 2.293.431 tons are produced (AERI, 2005). Likewise, it is one of the most consumed dairy products in the world (Sahar and Rahman, 2019). The report of a yogurt-borne *E. coli* O157:H7 outbreak (Morgan et al., 1993) shows the possibility of contamination (Massa et al., 1997). The decrease in pH may provide an advantage for *E. coli* serogroups during the production of yogurt. The pH ranges of yogurts that are produced in the world are approximately between 3.5 and 4.6 (Glass et al., 1992). There is no available data in the literature about the survival of non-O157 serogroups during the production and storage of yogurt. Thus, the aim of the present study is to investigate the survival of *E.coli* O157:H7 and non-O157 serogroups of O26, O111, O103, and O145 during the production and storage of yogurt.

MATERIALS AND METHODS

The raw cow milk was purchased from a local market that sells products from nearby villages and was shortly brought to the laboratory in a thermobox at 4°C (15-30 minutes). Approximately 5.5 liters of milk were used for each repetition and a total of approximately 16.5 liters of milk were used for the study. Pre-made yogurt was used as the starter culture and 2% (w/v) of it was added to the pasteurized cow milk. This pre-made yogurt was provided from a local plant in Tunceli (Simge Süt Ürünleri LTD. ŞTİ.).

Preparing of STEC strains for inoculum

All STEC strains were obtained from a proficiency test held by Istituto Superiori di Sanita (Italy) and stored at -20°C as reference material. Additionally, for daily use STEC serogroups were preserved on agar slants at 4°C. Strains from these slants were inoculated to Tryptic Soy Broth (10 ml) (LABM, UK) and incubated at 37°C for 24 hours (repeated twice). After that the cultures were centrifuged for 5 min at 3500 rpm. The cells were washed with sterile 0.9% NaCl solution and centrifuged once more. Those cultures were diluted with 0.1% sterile peptone water to obtain a cell density of 10^7-10^8 cfu/ml.

The production of contaminated yogurt

5.5 liters of raw cow milk were transferred to a large steel pot and pasteurized at 95°C for 5 minutes. After the pasteurization process, the milk was cooled to 48°Cby using a water-bath. At this stage, 100 ml of milk were sampled for determination of the acidity. Pre-made yogurt was used as the starter and milk was inoculated with the pre-made yogurt at a ratio of

2% (w/v) in the steel pot. Microbiological sampling of 2x25 ml samples for Lactic acid bacteria quantification. After this stage, milk was aseptically divided into five sterile beakers (1500 ml). STEC strains [O157:H7 (ATCC 43894), O26, O111, O103, and O145] were inoculated individually to each beaker and stirred about 5 minutes by sterile spoons. In order to determine the inoculation level (Fermentation Pre) 2×25 ml of this mixture was sampled from each beaker and transferred to the sterile stomacher bags. The contaminated milk samples in beakers were transferred to each of the prepared 250 mL plastic containers for approximately 200 mL each, and allowed to incubate at 44°C. When the pH of the samples reached to pH 4.7, the containers were removed from the incubator and stored at 4°C. In the meantime, 2×25 g samples were taken from different plastic containers (Fermentation Post) and pathogens were enumerated after incubation. The rest of the microbiological and chemical analyses were conducted at 5, 10, 15, and 20 days of the storage.

Microbiological analysis

Each yogurt sample was aseptically stirred by using sterile glass stirrers before sampling. For each strain, 25 g of sample was weighted into sterile stomacher bags and 225 ml of sterile 0.1% peptone water (LABM, Lancashire, UK) was added and then mixed for 2 min by a bag mixer. Analyses were made by spread plate technique.

Sorbitol MacConkey agar (LABM, Lancashire, UK) was used for enumeration of *E. coli* O157:H7 and STEC non-O157 strains. Plates were incubated at 35°C for 24 hours (Dikici et al., 2015).

De Man, Rogosa, Sharpe agar (MRS agar, LABM, Lancashire, UK) and M17 agar (LABM, Lancashire, UK) were used for enumeration of mesophilic *Lactobacillus* spp., *Lactococcus* spp. respectively. Surface plated plates were incubated at 37°C for 48 hours for the enumeration of *Lactococcus* spp. (Rogga et al., 2005). *Lactobacillus* spp. was enumerated by the double layered petri dishes technique after incubation at 30°C for 72 hours (ISO 15214, 2015).

Chemical analysis

For each experimental group, the pH values were measured by a pH-meter (Termo Scientific, Orion-3Star, Singapore) at 5, 10, 15, and 20 days of storage.

The lactic acid content (%) was determined according to AOAC920.124 methods at each experiment day (AOAC, 1990). The analyses were performed separately for each experimental group and repeated twice.

Statistical analysis

Bacterial counts were converted to $\log_{10}$ cfu/g. The data for each pathogen were subjected to the ANOVA test in accordance with repetition x number of samplesxtime to determine fixed effects and interactions between variables. The mean values were separated using Fisher's least squares method according to the General Linear Models (GLM) procedures and the statistical significance level was accepted as 5%. Analysis of the data was made by using the Statistical Analysis System (SAS) (SAS, 1999).

RESULTS

In this study, the survival of STEC serogroups *E. coli* O157:H7, O26, O111, O103, and O145 were investigated during the production of yogurt. The pH value of pasteurized milk samples was 6.81± 0.11.The pH value of pasteurized milk after adding starter culture was 6.36±0.11 (Table 1). The pH of the milk, in which pre fermentation yogurt was used as starter culture, was 4.77±0.01 after incubation(Table 1). Significant decreases in pH values were observed in all groups between 0 and 5 days of the storage($p<0.05$). After 5 days of storage, although the pH value of all samples decreased, there was no significant difference among pH values ($p>0.05$). The pH value of yogurt samples ranged between 4.16-4.03 at the end of the storage (Table 1).

Table 1. The changes in pH values during the production and storage of contaminated yogurt samples ($\log_{10}$ cfu/g) (n:6)

Serogroups	The pH values during production and storage					
	Fermentation Pre	Fermentation Post	5	10	15	20
O157	6.36 ± 0.11^{A}	4.77 ± 0.01^{B}	4.18 ± 0.05^{C}	4.17 ± 0.06^{C}	4.16 ± 0.06^{C}	4.11 ± 0.06^{C}
O26	6.36 ± 0.11^{A}	4.77 ± 0.01^{B}	4.20 ± 0.05^{C}	4.13 ± 0.04^{C}	4.12 ± 0.05^{C}	4.05 ± 0.09^{C}
O103	6.36 ± 0.11^{A}	4.77 ± 0.01^{B}	4.26 ± 0.07^{C}	4.23 ± 0.03^{C}	4.17 ± 0.04^{C}	4.16 ± 0.04^{C}
O145	6.36 ± 0.11^{A}	4.77 ± 0.01^{B}	4.17 ± 0.03^{C}	4.13 ± 0.03^{C}	4.12 ± 0.05^{C}	4.06 ± 0.02^{C}
O111	6.36 ± 0.11^{A}	4.77 ± 0.01^{B}	4.19 ± 0.08^{C}	4.14 ± 0.05^{C}	4.11 ± 0.06^{C}	4.03 ± 0.11^{C}

A-C: Means in the same line with different superscripts are statistically different ($P<0.05$)

Although statistically significant decline ($p<0.05$) was observed before and after incubation, no significant change occurred in the lactic acid content of the samples on the other days of storage ($p>0.05$) (Table 2).The lactic acid content of all samples from experimental groups was similar. Therefore the obtained values from experimental groups were averaged and given in Table 2.

The high levels of spiking with STEC serogroups can be explained as to monitor the dramatic changes of numbers of pathogens during incubation and storage. The number of STEC serogroups increased during incubation of yogurt (Table 3); however, this increase was not statistically significant ($p>0.05$). It was determined that the number of STEC O157, O103, and O145 decreased about 6.00+1.00 $\log_{10}$ cfu/g during storage and were below the detectable level at the end of the storage period. However, the number of STEC O26 and O111 were determined as 1.51±0.98 and 1.18±0.62 $\log_{10}$ cfu/g, respectively at the end of the 20 days of storage (Table 3).

Table 2. The lactic acid % values of during the production and storage of contaminated yogurt samples (n:6)

Serogroups	Fermentation		Storage Day			
	Pre	Post	5	10	15	20
O157	0.15±0.02^{A}	0.84±0.01^{B}	0.81±0.12^{B}	0.91±0.01^{B}	0.90±0.10	0.87±0.12^{B}
O26	0.18±0.02^{A}	0.71±0.01^{B}	0.85±0.08^{B}	0.93±0.01^{B}	0.87±0.18	0.82±0.10^{B}
O103	0.16±0.10^{A}	0.73±0.01^{B}	0.79±0.16^{B}	0.95±0.01^{B}	0.85±0.15^{B}	0.88±0.14^{B}
O145	0.17±0.02^{A}	0.72±0.01^{B}	0.80±0.14^{B}	0.90±0.01^{B}	0.82±0.17^{B}	0.83±0.07^{B}
O111	0.19±0.04^{A}	0.70±0.01^{B}	0.80±0.10^{B}	0.96±0.01^{B}	0.81±0.15^{B}	0.85±0.11^{B}

AB: Means in the same line with different superscripts are statistically different ($P<0.05$).

Table 3. The microbiological changes during the production and storage of contaminated yogurt samples ($\log_{10}$ cfu/g) (n:6).

Serogroups	Fermentation		Storage Day			
	Pre	Post	5	10	15	20
O157	6.79±0.45^{A}	7.01±0.31^{A}	4.59±1.59^{B}	3.14±0.47^{B}	3.55±1.24^{B}	< 1.0
O26	6.64±0.53^{A}	7.32±0.37^{A}	5.07±0.98^{B}	3.42±0.73^{C}	2.27±0.72^{CD}	1.51±0.98^{D}
O103	7.12±0.43^{A}	7.29±0.43^{A}	5.47±1.18^{B}	4.28±1.08^{BC}	2.52±1.32^{C}	<1.0
O145	6.00±1.39^{A}	7.41±0.35^{A}	4.66±1.03^{A}	1.95±1.15^{B}	1.83±1.47^{B}	<1.0
O111	5.89±1.37^{A}	7.35±0.45^{A}	4.95±1.03^{A}	3.20±1.87^{B}	2.36±1.04^{B}	1.18±0.62^{B}

A-D: Means in the same line with different superscripts are statistically different ($P<0.05$)

.Pre-Fermentation *Lactobacillu*s spp. and *Lactococcus* spp counts were5.02+0.22, 4.93+0.31 $\log_{10}$ cfu/g respectively. The number of mesophilic *Lactobacillus* spp. and *Lactococcus* spp. of experimentally contaminated yogurt samples were 7.04±0.51, 7.42±0.21 $\log_{10}$ cfu/g respectively after incubation. The numbers of these bacteria were around the targeted number of starter bacteria in yogurt (7 $\log_{10}$ cfu/g).There were no significant changes in the populations of mesophilic *Lactobacillus* spp. and *Lactococcus* spp. The number of mesophilic *Lactobacillus*spp. in the samples of experimental groups O157, O26, O103, O145, O111 were 7.82±0.46, 7.88+0.31, 7.65±0.53, 7.62±0.40 and 7.80±0.38 $\log_{10}$ cfu/g, respectively. The number of *Lactococcus* spp. in the same groups were7.91±0.22, 7.90±0.19, 7.88±0.27, 7.94±0.51 and 7.93±0.42 $\log_{10}$ cfu/g, respectively.

DISCUSSION

Although yogurt is a heat-treated dairy product, a possible post-process contamination might alter the safety of the product and cause serious health hazards for consumers. According to the study of Dehkordi et al. (2014), 50 out of the 600 dairy products were contaminated with *E. coli* and yogurt was the most contaminated one. The most prevalent *E. coli* serogroups were O157 and O26. Since several studies reported that STEC serogroups are acid resistant pathogens that can survive in various dairy products for several weeks to several months (Govaris et al., 2002; Solomakos et al., 2009; Miszcyzcha et al., 2012; Ioanna et al., 2017; Bellio et al., 2018), the reliability of acidic foods such as yogurt is questionable.

In this study, it was determined that *E. coli* O157:H7 and non-O157 STEC serogroups can survive in yogurt environment (Table 3). *E. coli* O157:H7, non-O157 STEC O103, and O145 had the lowest survival rate and viable counts dropped below the detection lim-

it at day 20 of storage (Table 3). It was reported that the inhibitory effect of starter cultures on *E. coli* O157 and non-O157 was dependent on strain and species. Similar results were reported in goat milk fermented with yogurt starter cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarus* subsp. *thermophilus*) that inhibited non-O157 *E.coli* strains (Fayemi and Buys, 2017). In another study it was shown that yogurt starters had no adverse effect on *E.coli* O157:H7 individually but showed an antimicrobial effect when used together (Dineen et al., 1998). The reason for that is reported to be the high antimicrobial substances produced from the beginning (Ogueke, 2008). In the studies conducted on fermented milk and dairy products, the reasons for the decrease in the number of pathogenic microorganisms during fermentation and storage were reported as the number and type of lactic acid bacteria as well as the substances which are produced during acidic fermentation such as organic acid, hydrogen peroxide, bacteriocin, diacetyl, ethanol (Dineen et al., 1998; Leistner, 2000; Rogga et al., 2005; Dikici, 2008; Ogueke, 2008; Callon et al., 2016; Fayemi and Buys, 2017; Bellio et al., 2018).

STEC O26 and O111 maintained their viability during storage of 20 days. STEC O26 and O111 counts in the inoculated milk were 6.64±0.53 $\log_{10}$ cfu/mL and 5.69±1.37 $\log_{10}$ cfu/mL respectively. Significant decreases of viable counts were determined during storage ($p<0.05$) and STEC O26 and O111 counts at the end of the storage were determined as 1.51±0.98 $\log_{10}$ cfu/g and 1.18±0.62 $\log_{10}$ cfu/g respectively (Table 3). Therefore, it can be concluded from these results that during the production and storage of yogurt, STEC O26 and O111 were more resistant than the other serogroups in this study. Since STEC O26 is the second most prevalent STEC serogroup (Lajhar et al., 2017), it is expected that these bacteria are more resistant to stressors.

There is no report on the survivability of non-O157 STEC serogroups in yogurt in the literature and such reports regarding dairy products are limited. The studies carried out by Miszczycha et al. (2012) and Bellio et al. (2018) show that the number of STEC increased during the first step of cheese production. In these studies researchers determined that the increase of LAB and the decrease of pH were not effective on the inhibition of *E. coli* O157:H7. In both studies, the number of STEC increased and then decreased as a result of ripening at the first step of cheese making.

In the study of Arıcan and Andıç (2011), the number of *E. coli* O157:H7 was found to be similar to that of our study during the fermentation and storage of yogurt (Table 3). Likewise, in the study of Tosun et al. (2007), the viability of acid-adapted and non-adapted *E. coli* O157:H7 were investigated in fermented dairy products. In this study, non-adapted *E. coli* O157:H7 maintained its viability during storage of symbiotic yogurt for 26 days at 4°C. Massa et al. (1997) investigated the viability of O157:H7 in traditional and bifido yogurt. They reported that O157:H7 was still detectable at the end of 7 daysof storage. As a consequence, it can be concluded from the study of Akdemir and Evrendilek (2007) that *E. coli* O157:H7 can survive in acidic foods. This pathogen can survive in several acidic foods such as sweet pickles (pH: 2.8) (Tsai and Ingham, 1997), yogurt (pH: 4.5) (Massa et al., 1997), and mayonnaise (pH: 3.65) (Weagant et al, 1994). Although yogurt is considered as a rather safe product due to its natural acidic environment, this pathogen can survive in acidic foods such as yogurt through its resistance to the low pH (Bracket et al., 1994; Conner and Kotrola, 1995; Dineen et al., 1998; Bellio et al., 2018; Yousef and Courtney, 2003).

The number of studies on the behavior of non-O157 STEC serogroups in foods is quite low. Especially, the studies on dairy products are very rare. The possible contamination to any product after the heat treatment may pose a potential risk to the safety of food and public health. Therefore, in this study the safety of yogurt was investigated to demonstrate the consequences of a post-heat treatment contamination with STEC.

CONCLUSIONS

According to the results obtained from the present study, *E.coli* O157:H7 and non-O157 strains of O26, O111, O103, and O145 could survive yogurt environment. Even though *E.coli* O26 and O111 were still detectable on the 20th day whereas O157, O103 and O145 were not; the differences in the survivor numbers were not very significant. Therefore, it cannot be concluded that these strains were more resistant to yogurt production and storage steps than other strains used in this study, without further investigation.

The incubation step did not decrease the number of pathogens; on the contrary, the number of all pathogens increased at this step. The possible risks resulting from post-process contamination of yogurt can be seen clearly from these results that STEC O157:H7 and non-O157 STEC serogroups could survive the production and storage of yogurt. Considering the fact that the infective dose of STEC pathogens

can be very low, consumption of contaminated yogurt could be threatening. Various studies have shown that pathogens, which are capable of adapting to acidic environments, are more likely to cause diseases.

As a zero tolerance bacteria to be listed by the authorities, STEC, the public health hazard should be taken into account and additional countermeasures should be taken. foods should be monitored in terms of STEC contamination throughout production and storage period. Risk assessment of non-O157 STEC serogroups should be made by each country and the STECshould be included in the legislation; the isolation and identification procedures must be improved, rapid and reliable screening systems need to be developed.

CONFLICT OF INTEREST

None declared by the authors.

REFERENCES

AERI(2005) Agricultural Economics Research Institute of Turkey Dairy Situation and Outlook 2007-2008 Publication.Ankara pp 22.

AOAC(1990) AOAC Official Methods, fifteenth ed. Methods 948.12, 920.124, 983.14,947.05, 920.123.

Arıcan A, Andıc S(2011) Survival of *E.coli* O157:H7 in yogurt incubated until two different Ph values and stored at 4°C. Kafkas Univ Vet Fak Derg 17:537-542.

Arocha MM, McVey M, Loder SD, Rupnow JW, Bullerman L(1992)Behaviour of hemorrhagic *Escherichia coli* O157:H7 during the manufacture of Cottage cheese. J Food Protect55:379-381.

Baylis CL(2009) Raw milk and raw milk cheeses as vehicles for infection by Verocytotoxin-producing *Escherichia coli*. Int J Dairy Technol 62:293-307.

Bellio A, Bianchi DM, Vitale N, Vernetti L, Gallina S, Decastelli L(2018) Behavior of *Escherichia coli* O157:H7 during the manufacture and ripening of Fontina Protected Designation of Origin cheese. J Dairy Sci 101:4962-4970.

Callon C, Ariliguie C, Montel M(2016) Control of Shigatoxin-producing *Escherichia coli* in cheese by dairy bacterial strains. Food Microbiol 53:63-70.

Centers for Disease Control and Prevention (CDC)(2014) What are Shiga toxin-producing *E.coli*. December 1 https://www.cdc.gov/ecoli/general/index.html.

Centers for Diseases Control and Prevention (CDC)(2015)*E.coli* (*Escherichia coli*) general information. November 6http://www.cdc.gov/ecoli/general/index.html.

Conner DE, Kotrola JS(1995) Growth and survival of *Escherichia coli* O157:H7 under acidic conditions. Appl Environ Microbiol 61:382-385.

Dehkordi FS,Yazdani F, Mozafari J, Valizadeh Y (2014) Virulence factors, serogroups and antimicrobial resistance properties of *Escherichia coli* strains in fermented dairy products. BMC Research Notes 7:217.

Dikici A(2008) Development of acid adaptation in *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* in Savak tulumi cheese during production and ripening. PhD thesis, Firat University, Institute of Health Sciences, Elazig, Turkey.

Dikici A, Koluman A, Calicioglu M(2015) Comparison of effects of mild heat combined with lactic acid on Shiga toxin producing *Escherichia coli* O157:H7, O103, O111, O145 and O26 inoculated to spinach and soybean sprout. Food Control 50:184-189.

Dineen SS, Takeuchi K, Soudah JE, Boor KJ, (1998)Persistence of *Escherichia coli* O157:H7 in dairy fermentation systems. J Food Prot 61:1602-1608.

Evrendilek GA(2007) Survival of *Escherichia coli* O157:H7 in yogurt drink, plain yogurt and salted yogurt: Effects of storage time, temperature, background flora and product characteristic. Int J Dairy Technol60:118-122.

Farrokh C, Jordan K, Auvray F, Glass K, Oppegaard H, Raynaud S, Thevenot D, Condron R, De Reu K, Govaris A, Heggum K, Heyndrickx M, Hummerjohann J, Lindsay D, Miszczycha S, Moussiegt S, Verstraete K, Cerf O(2013) Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. Int J Food Microbiol 162:190-212.

Fayemi OE, Buys EM (2017) Effect of *Lactobacillus plantarum* on the survival of acid-tolerant non-O157 Shiga toxin-producing *E. coli* (STEC) strains in fermented goat's milk. Int J Dairy Technol70:399-406.

Glass KA, Loeffelholz JM, Ford JP, Doyle MP(1992) Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. Appl Environ Microbiol 58:2513-2516.

Govaris A, Koidis P, Papatheodorou K (2002) Behaviour of *Escherichia coli* O157[ratio]H7 in sour milk, cows' milk yogurt and ewes' milk yogurt. J Dairy Res 69:655-660.

Ionna F, Quaglia NC, Storelli MM, Castiglia D, Goffredo E, Storelli A, De Rosa M, Normanno G, Jambrengi AC, Dambrasio A(2017) Survival of *Escherichia coli* O157:H7during the manufacture and ripening of Cacioricotta goat cheese. Int J Food Microbiol 70:200-205.

ISO 15214, (2015)Microbiology of food and animal feeding stuffs - Horizontal method for the emumeration of mesophilic lactic acid bacteria - Colony-count technique at 30 °C. International Standart Organization.

USDA Food Safety and Inspection Service (FSIS)(2010) Detection and isolation of non-O157 Shiga-toxin producing *Escherichia coli* strains (STEC) from meat products. In: Microbiological Laboratory Guidebook, version 5B.00, Washington, DC.

Lajhar SA, Brownlie J, Barlow R(2017) Survival capabilities of *Escherichia coli* O26 isolated from cattle and clinical sources in Australia to disinfectants, acids and antimicrobials. BMC Microbiol 17: 47.

Leistner L(2000) Basic aspects of food preservation by hurdle technology. Int J Food Microbiol 55:181-186.

Massa S, Altieri C, Quaranda V, De Pace R(1997)Survival of *E. coli* O157:H7 in yoghurt during preparation and storage at 4°C. Let Appl Microbiol 24:347-350.

Miszczycha SD, Perrin F, Ganet S, Jamet E, Tenenhaus-Aziza F, Montel MC, Thevenot-Sergentet D (2012) Behavior of different shiga toxin-producing *Escherichia coli* serotypes in various experimentally contaminated raw-milk cheese. Appl Environ Microbiol 79:150-158.

Morgan D, Newman CP, Hutchinson DN, Walker AM, Rowe B, Majid F(1993)Verotoxin-producing *Escherichia coli* O157:H7 infections associated with consumption of yoghurt. Epidemiol Infect 111:181-187.

Ogueke CC(2008) The effect of metabolites of *Lactobacillus*sp. in fermented milk on the growth of hospital isolates of *E. coli*. Life Sci J 5:90-94.

OztekinD (2019) Presence of *Escherichia coli* O26, O111 and O157 in milk and dairy products, antibiotic susceptibilty and molecular typing of isolates. Master Thesis, Institute of Health Science, Department of Veterinary Food Hygiene and Technology, University of Erciyes.

Rogga KJ, Samelis J, Kakouri A, Katsiari MC, Savvaidis IN, Kontominas MG(2005) Survival of *Listeria monocytogenes* in Galotyri, a traditional Greek soft acid-curd cheese, stored aerobically at 4°C and 12°C. Int Dairy J 15:59-67.

SAS(1999) Version 6.1. SAS Institute. Cary, Nort Caroline, USA.

Sahar A, Rahman U (2019) Contribution of animal origin foods in the human diet (Eds: Khan MI, Sameen A), Animal Sourced Foods for Developing Economies: Preservation, Nutrition, and Safety, CRC Press, USA.

Solomakos N, Govaris A, Angelidis AS, Pournaras S, Burriel AR, Kritas SK, Papageorgiou DK (2009) Occurrence, virulence genes and antibiotic resistance of Escherichia coli O157 isolated from raw bovine, caprine and ovine milk in Greece. Food Microbiol, 26 (8):865-871.

Tosun H, Seçkin AK, Gönül-Aktuğ Ş (2007) Acid adaptation effect on survival of *Escherichia coli* O157:H7 in fermented milk products. Turkish J Vet An Sci 31:61-66.

Tsai YW, Ingham SC(1997) Survival of *Escherichia coli* O157:H7 and *Salmonella* spp. in acidic condiments. J Food Prot 60:751-755.

Weagant SD, Braynt ML, Park DH(1994) Survival of *E. coli* 157:H7 in mayonnaise-based sauces at room and refrigerated temperatures. J Food Prot 57:629-631.

Yousef AE, Courtney PD(2003)Basics of Stress Adaptation and Implications.In New-Generation Foods. CRC Press LLC, Boca Raton, pp 1-30.

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Research article
Ερευνητικό άρθρο

A clinical survey on the electrocardiogram after intravenous granisetron hydrochloride administration in clinically normal dogs

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ABSTRACT: Cardiac side effect of 5-hydroxytryptamine-3 receptor antagonists has been a concern for clinicians. There is a substantial need for greater clarity about the safety of granisetron hydrochloride, an antiemetic agent of this class used in oncological and parvoviral gastroenteritis with acute vomiting in dogs.This study aimed to assess the electrocardiographic effects of a single dose of intravenous granisetron. We randomly assigned 16 adult crossbreed female healthy dogs into two groups of intervention and control and injected them intravenously with granisetron and normal saline, respectively, at a dose of 0.5 ml/kg over one minute. Standard electrocardiography (ECG) was recorded at the baseline, as well as 5, 15, 30, 60, 120, 360, and 720 minutes after the intervention. Heart rate and ECG parameters (PR intervals, QRS duration, ST-segment, T-wave amplitudes, QT, JT, QTc and JTc intervals) were evaluated in lead II.No significant difference was observed between the intervention and the control groups in any of the measured variables at any of the time-points. Mean values of measured parameters showed no significant difference compared with baseline values in the control group, while the granisetron group saw statistically significant but clinically asymptomatic changes in heart rate, PR, QRS, QT, JT, and QTc at different time-points, compared to the baseline values ($P<0.05$). In conclusion, Granisetron administration was not associated with clinically significant adverse effects on ECG variables or heart rate. Thus, it can be regarded as a relatively safe drug.

Keywords: Granisetron hydrocholride, QTc, JTc, Electrocardiogram, Dog.

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INTRODUCTION

Nausea and vomiting are among the commonly encountered side effects in patients undergoing chemotherapy, occurring in most cytostatic chemotherapeutic regimens among cancer patients (Harris 2010; Janelsins et al. 2013; Schnell 2003; Yamakuni et al. 2000).Moreover, severe gastroenteritis especially in puppies with parvoviral infection can be a major cause of nausea and vomiting in animals (Goddard and Leisewitz 2010; Nandi and Kumar 2010; Woods et al. 1980). These emesis occurring within 24 hours, is now known to be mediated via 5-hydroxythriptamin3 (5-HT3) receptors and thus can be controlled with the specific 5-HT3 receptor antagonists (Grunberg 1993; Hsu 2010). Granisetron, ondansetron, andtropisetron, having been considered the most powerful antiemetics so far, have been proven at least equally efficacious as conventional treatments (Chevallier 1993; Gomez-de-Segura et al. 1998; Gregory and Ettinger 1998; Hsu 2010; Janelsins et al. 2013; Schnell 2003).

5-HT3 receptor antagonists, however, have been reportedly associated with an increased risk of abnormalities in cardiac repolarization. For instance, some studies have reported adverse electrocardiographic effects from granisetron and raised concerns over the possibility of this drug causing arrhythmia (Buyukavci et al. 2005; Pınarlı et al. 2006). Previous studies have stated that efficacy of 5-HT3 receptor antagonists when used intravenously outweighs their risk and small, transient electrocardiographic abnormalities that might appear are clinically insignificant (Cakir et al. 2012; Coop 2003; Ghaffari et al. 2010; Keefe 2002; Navari and Koeller 2003).

However, available evidence is not sufficient to warrant their safety, especially in patients with preexisting cardiovascular disorders or those receiving cardiotoxic drugs e.g. chemotherapeutic regimens. Potential cardiovascular adverse effects of 5-HT3 receptor antagonists have attracted growing research interest and studies have investigated the safety of some of these agents. Nevertheless, an interventional animal study to evaluate the electrocardiographic effects of granisetron and its safety compared with placebois lacking (Dennis et al. 2007; Ghaffari et al. 2010; Tricco et al. 2016). Therefore, the present study was aimed to assess the electrocardiographic effects of single-dose intravenous administration of granisetron on healthy dogs, as animal models.

MATERIALS AND METHODS

Study Design

The "The first evaluation of the electrocardiographic effects of iv granisetron (As hydrochloride) 3 mg (Caspian Tamin, Iran) in healthy dogs» was an experimental, randomized, placebo-controlled study. The study was approved by the Iran Society for Prevention of Cruelty to Animals in accordance with Iranian ethical codes for studies on laboratory animals. The contract between the sponsor (Ferdowsi University of Mashhad) and the guide investigator provided that the latter would have full access to all results and the right to independently publish the study regardless of the outcome.

Animals

In this experimental study, 16 adult crossbreed female dogs with a mean weight of 23.32±0.78 kg were included. The study was performed completely according to the principles of the World Medical Association (WMA) statement on animal use in biomedical research. For a period of two weeks before the intervention, all included dogs were housed individually in cages in an animal room with standard and monitored conditions for environmental adaptation. Water and commercial dry dog foods were given to the dogs ad libitum during the study. The dogs were vaccinated and antiparasitic agents were given to them.

All dogs underwent thorough physical examination, blood and fecal analysis before the study and standard electrocardiography at the baseline and their condition were confirmed as healthy based on the results.

Exclusion criteria: Dogs were excluded from the study if they had cardiovascular disease and clinically relevant systemic disease.

We removed the dogs from their home cages and took them to a quiet room two hoursbeforethe intervention so that they can acclimatize to the environment.

The dogs were randomly assigned to two different groups of intervention (N=8) and control (N=8). The intervention group received intravenous granisetron,injected slowly at a dose of 0.5 ml/kg over one minute, while the control group received intravenous normal saline at the exact same dose and condition.

A 6-lead standard electrocardiogram (ECG) comprising leads I, II, III, aVR, aVL, and aVF was performed using a single channel digital electrocardiograph (ECG 110, Kenzo®, Suzuken, Japan) and recorded at 50 mm/s and 10 mm/mv for all dogs, while they were in right lateral recumbency position on a table. All ECGs were performed by one single

expert operator observing the standard conditions.

One-minute ECGs of lead II were recorded in all dogs at baseline and 5, 15, 30, 60, 120, 360, and 720 minutes after the intervention. In all ECGs taken, heart rate, PR interval, QRS duration, ST segment, T-wave amplitude, QT interval, and JT interval were recorded in checklists for each dog. The JT interval was calculated by subtracting QRS duration from the QT interval, measured from the endpoint of QRS complex (known as j-point) to the endpoint of T wave, marking the point that T wave returns to the isoelectric line. Moreover, we calculated the rate-corrected QT and JT intervals, being known as QTc and JTc, respectively, using the formulas below.

$$JTc = \frac{JT}{\sqrt[3]{RR}} \qquad QTc = \frac{QT}{\sqrt[3]{RR}}$$

Statistical Methods

Statistical analysis was performed using the statistical package for social sciences (SPSS; version 12.0 for Windows, IBM Statistics, Chicago, IL, USA). One-sample Kolmogorov-Smirnov test was used to assess the normal distribution of data. Independent samples t-test was used to compare the measured parameters in each time-point between the two groups. Paired samples t-test was used to compare data with the baseline values in each group. P<0.05 was considered as statistically significant.

RESULTS

Overall, 16 dogs in two groups of intervention (N=8) and control (N=8) were studied. The electrocardiographic parameters of dogs in both groups are shown in Table-1. As the table implies, in the control group, none of the measured parameters changed significantly at any of the time-points, compared with their baseline values. However, in the intervention group, several parameters were changed significantly at different time-points, compared with baseline values.

Mean heart rate of the intervention group was significantly different from their baseline heart rate 30, 60, and 120 minutes after the intervention (P=0.033, P=0,033, and P=0.015, respectively). In the intervention group, mean QRS duration 5 minutes after the intervention and mean QTc interval 15 minutes after the intervention was significantly different from the corresponding baseline values (P=0.011 and P=0.037). Moreover, mean values of PR, QT, and JT intervals 5, 15, 30, 60, 120, and 360 minutes after the intervention was significantly different from the baseline value in the intervention group (P<0.05). Nevertheless, mean values of ST segment, JTc interval, and T wave amplitude had no significant change over the course of study in the intervention group.

Table 1. Mean values of electrocardiographic parameters in intervention and control groups at different time-points

Parameter		baseline	5 min	15 min	30 min	60 min	120 min	360 min	720 min
HR	I	90.00±5.34	82.50±4.53	85.00±6.26	**80.00±5.34**	**80.00±5.34**	**78.75±6.10**	82.50±5.90	92.50±7.50
	C	90.00±8.16	85.00±7.18	80.00±8.56	86.66±7.60	78.33±4.77	81.66±5.42	93.33±9.54	88.33±7.03
PR	I	0.106±0.007	**0.117±0.010**	**0.117±0.009**	**0.120±0.008**	**0.120±0.009**	**0.117±0.009**	**0.119±0.008**	0.110±0.007
	C	0.101±0.005	0.087±0.006	0.095±0.005	0.099±0.004	0.099±0.005	0.100±0.007	0.0091±0.008	0.100±0.005
QRS	I	0.093±0.003	**0.098±0.003**	0.099±0.003	0.095±0.003	0.094±0.003	0.094±0.004	0.094±0.003	0.090±0.004
	C	0.954±0.005	0.096±0.005	0.097±0.006	0.098±0.005	0.096±0.003	0.096±0.005	0.099±0.005	0.100±0.004
ST	I	0.064±0.019	0.083±0.007	0.075±0.022	0.061±0.021	0.065±0.021	0.087±0.005	0.077±0.009	0.085±0.006
	C	0.052±0.026	0.033±0.031	0.034±0.031	0.060±0.026	0.031±0.035	0.055±0.027	0.062±0.027	0.060±0.026
QT	I	0.273±0.011	**0.290±0.011**	**0.295±0.009**	**0.288±0.009**	**0.293±0.014**	**0.292±0.013**	**0.292±0.012**	0.277±0.009
	C	0.272±0.006	0.279±0.005	0.276±0.007	0.275±0.007	0.276±0.006	0.280±0.006	0.274±0.009	0.273±0.007
JT	I	0.180±0.009	**0.192±0.009**	**0.196±0.008**	**0.193±0.007**	**0.198±0.011**	**0.197±0.010**	**0.198±0.009**	0.187±0.007
	C	0.176±0.009	0.182±0.007	0.178±0.010	0.177±0.009	0.179±0.008	0.184±0.009	0.174±0.009	0.173±0.008
QTc	I	0.309±0.008	0.318±0.007	**0.322±0.006**	0.312±0.008	0.311±0.010	0.314±0.012	0.315±0.009	0.311±0.008
	C	0.306±0.007	0.314±0.009	0.301±0.007	0.303±0.008	0.308±0.008	0.311±0.004	0.313±0.006	0.315±0.007
JTc	I	0.204±0.007	0.210±0.007	0.213±0.006	0.209±0.005	0.288±0.025	0.211±0.009	0.213±0.007	0.209±0.007
	C	0.198±0.010	0.205±0.008	0.199±0.011	0.195±0.009	0.199±0.007	0.204±0.008	0.199±0.008	0.198±0.005
TWA	I	0.100±0.106	0.007±0.111	0.001±0.111	-0.003±0.104	0.024±0.127	0.044±0.011	0.065±0.119	0.050±0.083
	C	0.068±0.171	0.219±0.173	0.053±0.207	0.089±0.118	-.034±0.173	0.028±0.175	0.044±0.151	-.004±0.165

HR: heart rate; TWA: T wave amplitude; I: intervention; C: control

*Values shown in bold were significantly different from the baseline value in that group, according to paired samples t-test (P<0.05)

Figure-1 shows the alterations in mean values of heart rate, PR interval, QRS duration, ST segment, QT interval, JT interval, QTc, and JTc in each of the intervention and control groups and compares the trend of changes between the dogs in these two groups.Figure-2 compares the changes in mean T wave amplitude between the intervention and the control groups. The intervention and the control groups showed no significant differences in any of the measured parameters at any of the time-points.

Regarding the side effects of the treatment, a second-degree ventricular block was seen in one dog (12.5%) in the intervention group, which is presented in Figure-3. In contrast, none of the dogs in the control group showed any notable change in cardiac rhythm.

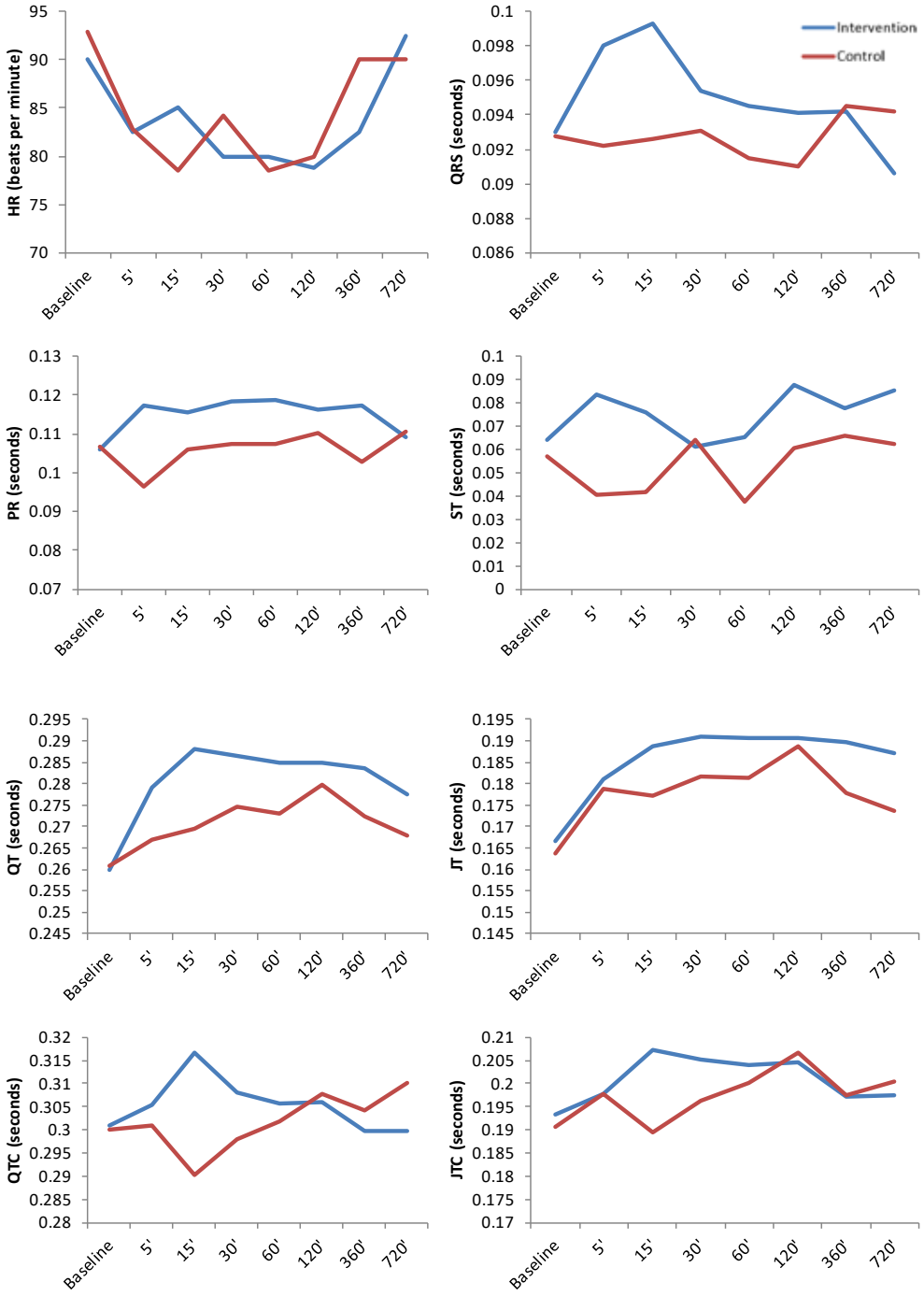

Figure.1. Comparison of the changes in mean values of some measured parameters over the first twelve hours after the intervention between the intervention and the control groups

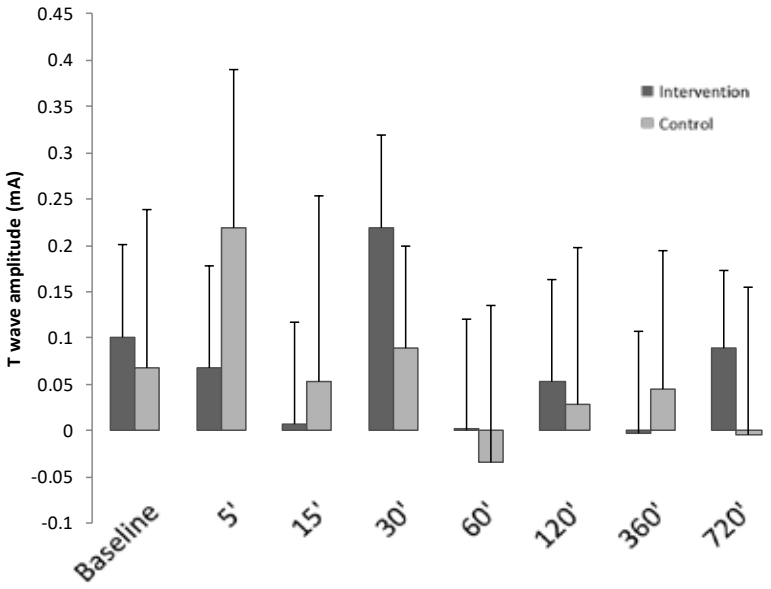

Figure.2. Comparison of the changes in mean T wave amplitude between the intervention and the control groups

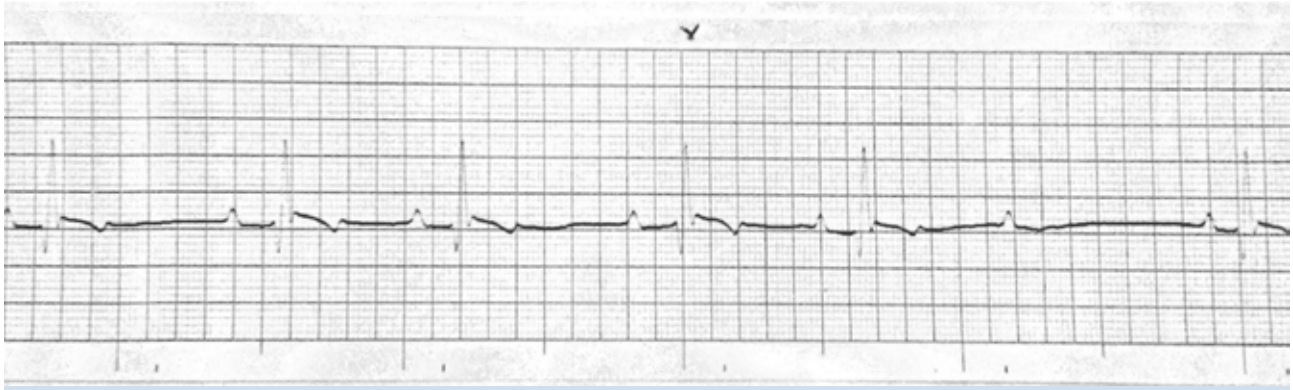

Figure.3. Second-degree ventricular block in one dog in the intervention group

DISCUSSION

Granisetron has been emerged as a potent antiemetic agent with manifold indications in different conditions. However, there is not enough evidence to draw a firm conclusion on the safety of this drug. The present work is one of the few studies investigating the electrocardiographic safety of this drug and probably the first study to investigate this issue in animal models. In this study we only included female dogs because female dogs possesses larger potassium channels in the epicardium and endocardium, which in turn provides a greater repolarization gradient and longer QT intervals compared with male dogs (Xiao et al. 2006).

We found no significant difference regarding any of the measured variables at any of the time-points between the intervention and control group. This finding is in line with several other studies comparing the cardiac effects of granisetron with placebo among healthy adults (Boike et al. 1997; Mason et al. 2013; Mason et al. 2012).

Mason and colleagues in a double-blinded randomized placebo-controlled crossover trial on healthy subjects compared subcutaneous and intravenous granisetron with placebo over a 49-hour period in terms of ECG changes. They found no clinically significant difference in any of the electrocardiographic parameters between granisetron and control groups (Mason et al. 2014).

Similarly, Mason et al. in another randomized controlled crossover trial, evaluated 12-lead ECGs in healthy subjects receiving subcutaneous APF530 (sustained-release granisetron) and intravenous gran-

isetron with placebo and found no clinically significant difference in electrocardiographic parameters of the subjects (Mason et al. 2013).

We also found that in the control group, none of the measured parameters changed significantly from the baseline at any of the time-points. However, in the intervention group, statistically significant changes in measured parameters were seen compared with baseline at some points in the study, including reduced heart rate as well as an increase in QRS duration and PR, QT, QTc, and JT intervals. However, the changes were not clinically significant, with the exception of a second-degree ventricular block in the group receiving granisetron.

In fact, granisetron can potentially affect PR, QT, and JT intervals and QRS duration by blocking both the sodium and rapid potassium channels. Therefore, we can attribute the changes in ECG parameters in our study to blockade of sodium and potassium channels in myocytes, both of which can lead to ventricular dysrhythmias (Jantunen et al. 1996; Kuryshev et al. 2000).

As our findings indicate increased QT interval in some time-points among the intervention groups, given that QT interval comprises QT and QRS, we can state that both depolarization and repolarization of ventricles are affected by granisetron.

Although no previous study has investigated the electrocardiographic side effects of granisetron in animal models, our results were comparable to human studies on this subject, which reported statistically significant changes in parameters of ECG including PR interval, QT interval, QRS duration, and QT interval (Boike et al. 1997; Hunt et al. 1995; Schnell 2003; Tricco et al. 2016).

Moreover, some studies have reported the electrocardiographic effects of ondansetron, another 5-HT3 receptor antagonist, in animal models. Williams et al. reported that ondansetron can increase the QTc interval in a dose-dependent manner in anesthetized dogs. They also suggested that this effect could be attributed to the effects of this drug on potassium channels (Williams et al. 1991). In another animal study by Ghaffari and colleagues, standard doses of ondansetron were introduced to eight crossbreed dogs and the results indicated a significant increase in QT interval along with significantly decreased heart rate (Ghaffari et al. 2010).

In the present study, we observed a decrease in heart rate of dogs in the granisetron group, started 30 minutes after the intervention and continuing for 2 hours, though being insignificant. We hypothesize that granisetron can decrease the heart rate through blocking serotonin receptors on the afferent vagus nerve in the gastrointestinal tract and the feedback mechanism (Buyukavci et al. 2005; Spartinou et al. 2017).

Our study had some limitations. One of the limitations was our small sample size, which increases the chance of biases. Another limitation was using a single dose and only one mode of delivering the drug. Evaluating the effects of divided doses during the day and other modes of delivery such as subcutaneous granisetron could have been of benefit.

Since over 85% of cardiovascular toxicities have been predicted by animal studies on dogs, it can be posited that there are physiologic proximities between canine and human species (Gralinski 2003). Therefore, our findings can be of cardinal importance in directing further human research regarding the cardiotoxic effects of granisetron by providing several useful insights into the safety of this drug when used in healthy dogs.

CONCLUSIONS

Our findings indicate that despite granisetron at 0.5 mg/kg b.w. intravenously can lead to some electrocardiographic changes, which are mostly mild, trivial, and clinically insignificant. Therefore, it can be said that granisetron has transient and subclinical effects on the electrocardiographic parameters.Clinical electrocardiographic manifestations of granisetron can thus be possibly seen in patients with history of arrhythmia, conductive heart disorders, electrolyte disturbances (especially hypokalemia, which can be caused by viral gastroenteritis), those receiving cardiotoxic chemotherapy regimens, or patients with physiologic long QT intervals.

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REFERENCES

Boike SC, ILson B, Zariffa N, Jorkasky DK (1997) Cardiovascular effects of iv granisetron at two administration rates and of ondansetron in healthy adults American journal of health-system pharmacy 54:1172-1176

Buyukavci M, Olgun H, Ceviz N (2005) The effects of ondansetron and granisetron on electrocardiography in children receiving chemotherapy for acute leukemia American journal of clinical oncology 28:201-204

Cakir F, Yapar O, Canpolat C, Akalin F, Berrak S (2012) Cardiac effects of granisetron in a prospective crossover randomized dose comparison trial Supportive Care in Cancer 20:2451-2457

Chevallier B (1993) on behalf of the Granisetron Study Group: The control of acute cisplatin-induced emesis. A comparative study of granisetron and a combination regimen of high-dose metoclopramide and dexamethasone Br J Cancer 68:176-180

Coop AJ (2003) Comment: electrocardiographic and cardiovascular effects of the 5-hydroxytryptamine-3 receptor antagonists Annals of Pharmacotherapy 37:1918-1918

Dennis S, Wotton P, Boswood A, Flaherty D (2007) Comparison of the effects of thiopentone and propofol on the electrocardiogram of dogs The Veterinary Record 160:681-686

Ghaffari MS, Moghaddassi AP, Khorami N (2010) The acute effects of ondansetron on the electrocardiogram in clinically normal dogs Comparative clinical pathology 19:499-502

Goddard A, Leisewitz AL (2010) Canine parvovirus Veterinary Clinics: Small Animal Practice 40:1041-1053

Gomez-de-Segura IA, Grande AG, De Miguel E (1998) Antiemetic effects of Lerisetron in radiation-induced emesis in the dog Acta Oncologica 37:759-763

Gralinski MR (2003) The dog's role in the preclinical assessment of QT interval prolongation Toxicologic pathology 31:11-16

Gregory RE, Ettinger DS (1998) 5-HT3 receptor antagonists for the prevention of chemotherapy-induced nausea and vomiting Drugs 55:173-189

Grunberg SM (1993) Potential for combination therapy with the new antiserotonergic agents European Journal of Cancer 29:S39-S41

Harris DG (2010) Nausea and vomiting in advanced cancer British medical bulletin 96:175-185

Hsu ES (2010) A review of granisetron, 5-hydroxytryptamine3 receptor antagonists, and other antiemetics American journal of therapeutics 17:476-486

Hunt TL, Cramer M, Shah A, Stewart W, Benedict CR, Hahne WF (1995) A Double-Blind, Placebo-Controlled, Dose-Ranging Safety Evaluation of Single-Dose Intravenous Dolasetron in Healthy Male Volunteers The Journal of Clinical Pharmacology 35:705-712

Janelsins MC, Tejani MA, Kamen C, Peoples AR, Mustian KM, Morrow GR (2013) Current pharmacotherapy for chemotherapy-induced nausea and vomiting in cancer patients Expert opinion on pharmacotherapy 14:757-766

Jantunen IT, Kataja VV, Muhonen TT, Parviainen T (1996) Effects of granisetron with doxorubicin or epirubicin on ECG intervals Cancer chemotherapy and pharmacology 37:502-504

Keefe DL (2002) The cardiotoxic potential of the 5-HT3 receptor antagonist antiemetics: is there cause for concern? The Oncologist 7:65-72

Kuryshev YA, Brown AM, Wang L, Benedict CR, Rampe D (2000) Interactions of the 5-hydroxytryptamine 3 antagonist class of antiemetic drugs with human cardiac ion channels Journal of Pharmacology and Experimental Therapeutics 295:614-620

Mason J, Moon TE, O'Boyle E, Dietz AJ (2013) Comparison of the effects of subcutaneous APF530, intravenous granisetron, moxifloxacin, and placebo on the QT interval in humans. American Society of Clinical Oncology,

Mason JW, Moon TE, O'Boyle E, Dietz A (2014) A randomized, placebo-controlled, four-period crossover, definitive QT study of the effects of APF530 exposure, high-dose intravenous granisetron, and moxifloxacin on QTc prolongation Cancer management and research 6:181

Mason JW, Selness DS, Moon TE, O'Mahony B, Donachie P, Howell J (2012) Pharmacokinetics and repolarization effects of intravenous and transdermal granisetron Clinical Cancer Research 18:2913-2921

Nandi S, Kumar M (2010) Canine parvovirus: current perspective Indian Journal of Virology 21:31-44

Navari RM, Koeller JM (2003) Electrocardiographic and cardiovascular effects of the 5-hydroxytryptamine3 receptor antagonists Annals of Pharmacotherapy 37:1276-1286

Pınarlı F, Elli M, Dağdemir A, Baysal K, Acar S (2006) Electrocardiographic findings after 5-HT3 receptor antagonists and chemotherapy in children with cancer Pediatric blood & cancer 47:567-571

Schnell FM (2003) Chemotherapy-induced nausea and vomiting: the importance of acute antiemetic control The oncologist 8:187-198

Spartinou A, Nyktari V, Papaioannou A (2017) Granisetron: a review of pharmacokinetics and clinical experience in chemotherapy induced-nausea and vomiting Expert opinion on drug metabolism & toxicology 13:1289-1297

Tricco AC et al. (2016) Comparative safety and effectiveness of serotonin receptor antagonists in patients undergoing chemotherapy: a

systematic review and network meta-analysis BMC Medicine 14:216 doi:10.1186/s12916-016-0761-9

Williams PD, Cohen ML, Turk JA (1991) Electrocardiographic effects of zatosetron and ondansetron, two 5HT3 receptor antagonists, in anesthetized dogs Drug development research 24:277-284

Woods C, Pollock R, Carmichael L (1980) Canine parvoviral enteritis Journal of the American Animal Hospital Association 16:171-179

Xiao L, Zhang L, Han W, Wang Z, Nattel S (2006) Sex-based transmural differences in cardiac repolarization and ionic-current properties in canine left ventricles American Journal of Physiology-Heart and Circulatory Physiology 291:H570-H580

Yamakuni H et al. (2000) Probable Involvement of the 5-Hydroxytryptamine4Receptor in Methotrexate-Induced Delayed Emesis in Dogs Journal of Pharmacology and Experimental Therapeutics 292:1002-1007

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Research article
Ερευνητικό άρθρο

Antimicrobial effects of fruit sauces on some pathogenic bacteria in vitro and on chicken breast meat

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ABSTRACT: The use of natural food additives is currently a rising trend. In the present study, the aim was to determine the antimicrobial effects of plum, pomegranate, Seville orange and sumac sauces on *E. coli* O157:H7,*E. coli* type I,*Listeriamonocytogenes*, *Listeria ivanovii*, *Salmonella* Typhimurium and *Staphylococcus aureus.* Different concentrations (1%, 10%, 100%, v/v) of the sauces were tested on the studied bacteria *in vitro* using the agar diffusion and minimal inhibition concentration (MIC) methods. The results showed that the sumac sauce had the highest antimicrobial activity. The Seville orange, plum and pomegranate sauces also exerted antimicrobial activity in descending order. The antimicrobial activity of the fruit sauces was more effective at a concentration of 100% than at 10% and 1%, v/v. The most inhibitory effect was recorded for sumac sauce at a concentration of 100% (v/v) on *L.monocytogenes*and *E. coli* O157:H7. The findings of the MIC method aligned with the agar diffusion method. In addition, the *in situ*(food method) antimicrobial effect of the sauces on the indigenous microflora of chicken breast samples sold in stores was determined. Chicken samples hosting aerobic mesophilic bacteria, coliforms and *E. coli* were treated for two hours at 4 °C with plum, pomegranate, Seville orange and sumac sauces and were then monitored. The findings revealed that the Seville orange and sumac sauces were the most effective in reducing the indigenous microbial growth on the chicken samples. The plum sauce showed higher antimicrobial activity than pomegranate sauce. The phenolic content and acidity of the samples significantly ($P< 0.05$) affected the antimicrobial activity both *in vitro* (agar diffusion and MIC) and *in situ* (chilled chicken breast). In conclusion, the sumac and Seville orange sauces were found to be the most promising natural antibacterial agents, and their use could be recommended, for example, in catering services to reduce the risk of foodborne illness.

***Keywords*:** Antimicrobial effect, Chicken, Pomegranate, Seville orange, Sumac

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INTRODUCTION

Economic losses resulting from food spoilage and foodborne illnesses are a global problem. To decrease foodborne illnesses, the growth of microbial organisms should be prevented during the food production chain. The Centers for Disease Control and Prevention (CDC) estimates that, each year, roughly 48 million people become ill, 128.000 are hospitalised and 3.000 die from foodborne diseases in the United States. Unspecified agents contribute to 80% of the total number of illnesses, and the remaining 20% are caused by 31 known pathogens. *Salmonella* species and *Staphylococcus aureus* are among the top five pathogens contributing to domestically acquired foodborne illnesses. In the United states, *Escherichia coli* (STEC) O157:H7 is among the top pathogenic bacteria resulting in hospitalisation and *Listeria monocytogenes* in death (Anonymous, 2018). Catering services are one of the sources of foodborne outbreaks. A recent survey reported that, among 28 European Union member states, catering services followed household environments as the primary source of foodborne salmonellosis. Various facilities such as hospital restaurants, takeaway restaurants, ethnic restaurants, hotels, and in-flight catering services were among the sources of infections (Osimani et al., 2016).

Chicken meat is one of the major sources of food poisoning cases because of the frequent presence of *Salmonella* spp., *E. coli* and *Campylobacter* spp. (Kim et al., 2019; Li et al., 2019; Vetchapitak and Misawa, 2019; Dantas et al., 2020; Saad et al., 2020; Shen et al., 2020). For instance, several virulence genes of *Salmonella* spp. were detected in chicken samples from retail markets in South Korea (Dantas et al., 2020).

To minimise the prevalence of foodborne diseases, some slaughter establishments have begun to add antimicrobial chemicals (e.g. acidified sodium chloride, chlorine, sodium hypochlorite) to rinse solution for chicken carcasses (Ebel et al., 2019). However, because of consumer concerns over synthetic additives in food production, recent research has focused on the use of natural antimicrobial agents. Concentrated fruit sauces, which have mostly been used in salad dressings, have been widely studied because of their antimicrobial activity. For example, pomegranate sauces were previously found to inhibit the growth of *S. aureus* and *E. coli* O157:H7 (Karabiyikli and Kisla, 2012). Plum sauce displayed a strong antimicrobial effect on the growth of coliform bacteria and *E. coli* on ground beef and minced beef samples (Yapar, 2006). Sour orange juice completely inactivated *Salmonella* Typhimurium and *L. monocytogenes* at the end of the seventh day of incubation at 37 °C (Karabıyıklı et al., 2014). Sumac sauce inhibited the growth of *E. coli* O157:H7 and *L. monocytogenes* in vitro conditions (Kunduhoğlu and Pilatin, 2004). Hence, the use of fruit sauces could be a practical way of decreasing the microbial load and maintaining food safety in households and catering services. The aim of the present study was to determine the antimicrobial effects of fruit sauces on some pathogenic bacteria both *in vitro* (minimal inhibition concentration [MIC] and agar diffusion tests) and *in situ* (by placing chicken breasts in each fruit sauce at 4 °C for 2 h).

MATERIALS AND METHODS

Materials

Nutrient agar (NA), nutrient broth (NB), peptone water (0.1%), Mueller-Hinton broth (MB), Violet Red Bile agar (VRB), Brillant Green Bile Broth (BGLB), Fluorocult Lauryl Sulphate Broth (Merck), KOVACS's indole reagent and Fluorocult *E. coli* O157:H7 agar were purchased from Merck (Darmstadt, Germany). Chemicals used in physicochemical analyses were purchased from Sigma-Aldrich (United Kingdom). Fresh pomegranates (*Punica granatum*) were purchased from local markets in the Antakya province of Turkey. Sumac (*Rhus coriaria* L.) fruits were purchased in the Gaziantep province. Plums (*Prunus domestica* cv. French) and Seville oranges (*Citrus aurantium*) were purchased in the Mersin province. Chicken samples were purchased in their original packages from two different suppliers in the Adana province and were immediately transferred to the laboratory and kept under refrigeration (4°C).

Preparation of fruit sauces

The pomegranate, plum and Seville orange samples were first cleaned and washed under tap water. Then, the fruits were pressed using a lab-type juice press (Waring, US). The obtained fruit juices were filtered through filter paper (Isolab, Germany). The filtrates were gently boiled in a kitchen saucepan, filtered, boiled again for 1 h and left to reach ambient temperature. However, the sumac fruits were not subjected to a heat treatment and were left at room temperature until sediments were observed by the naked eye. The filtrate was then sun dried until reaching a dark colour, after which it was filtered and bottled. All sauce types were bottled in sterile glass jars and

stored at 4 °C until further use.

Test microorganisms

E. coli O157:H7,*E. coli* type I,*L.monocytogenes*, *Listeria ivanovii*, *S.*Typhimurium and *S. aureus* were acquired from former foodborne isolates in our laboratory (Çukurova University Agricultural Faculty Food Engineering Department, Turkey) collection. Reference strains were purchased from local companies to control the studied bacterial types.

Antibacterial assay (*in vitro*) using the agar-well diffusion method

The agar-well diffusion method was used following the modifications of Ahmad and Beg (2001) to determine the antimicrobial activity of the fruit sauces. Freshly grown cultures were first propagated in 5 mL of NB in tubes and incubated at 30 °C for 24 h. Following incubation, tubes were centrifuged at 3,000 *g* for 10 min at 4 °C and washed twice in NB. The supernatant was discarded. Bacterial pellets of the test microorganisms were diluted in 3 mL of sterile distilled water and used as stock cultures. Appropriate concentrations (0.5 McFarland units, corresponding with approximately 1.5×10^8 CFU/mL) of the bacteria were determined with a densitometer and further diluted to 10^5 CFU/mL. One millilitre of prepared cells was spread onto the surface of nutrient agar plates and left to dry at ambient conditions for 30 min. A sterilised stainless steel borer was used to punch wells in the agar medium 6 mm in diameter. Each well was then filled with 0.5 mL of fruit sauce diluted in sterilised distilled water to achieve different final concentrations (1%, 10%, 100% v/v, water basis). The plates were left at ambient conditions to allow the diffusion of the sauces in the agar plates. The plates were then incubated at 37 °C for 24-48 h until visible growth of microorganisms was evident in the control plates. The diameter of the inhibition zone was measured using callipers and expressed in millimetres. The values were taken as the average of five repetitions. The sensitivities of the bacteria were classified by the diameter of the inhibition zone, as described by Ponce et al. (2003): not sensitive (−) for diameters less than 8 mm, sensitive (+) for diameters 9-14 mm, very sensitive (+ +) for diameters 15-19 mm and extremely sensitive (+ + +) for diameters larger than 20 mm.

Minimal inhibitory concentration test

The minimum inhibitory concentrations (MICs) were evaluated by the broth microdilution method (M26-A) in accordance with the guidelines of the National Committee for Clinical Laboratory Standards (2003). Each fruit sauce was serially diluted two-fold in Mueller Hinton Broth (Merck, Darmstadt/Germany) to achieve increasingly more diluted concentrations (1:0.125, 1:0.25, 1:0.5, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 µg/mL). Each dilution and a control tube (containing no fruit sauce) was inoculated with 5.0 log CFU/mL of the test microorganisms. Tubes were then incubated at 37 °C for 24-48 h. The MIC was assigned to the lowest concentration of each antimicrobial fruit sauce that prevented bacterial growth. The test was conducted five times per fruit sauce.

Antimicrobial activity (*in situ*) of fruit sauces on chicken samples

To determine the antimicrobial effect of the fruit sauces on the naturally existing bacterial microflora of the chicken samples, the following procedures were applied. Twenty grams of chicken breast samples were treated with 15 mL of each fruit sauce separately and mixed well in Petri plates with their lids. Samples were left to rest at 4 °C for 2 h to ensure that each sauce had coated the surface of the chicken samples. Then, 10 g of each chicken sample was diluted in 90 mL of peptone water, homogenized in a stomacher (BagMixer 400 P, Interscience, France) and serial decimal dilutions were made. Dilutions were surface plated on VRB agar to monitor coliform bacteria and incubated at 35 °C for 18-24 h according to the solid medium method (SMM).

To confirm the coliforms, at least 10 colonies were selected and incubated at 35 °C in BGLB broth, and Gram stains were made (Anonymous, 2002). The most probable number (MPN) method was used with Fluorocult Lauryl Sulphate Broth to identify the amount of bacteria. After incubation at 35 °C for 48 h, the tubes were checked for gas formation, which confirms the presence of coliform bacteria. The gas-positive tubes were checked for light blue fluorescence under UV light (366 nm). Afterwards, the tubes showing both positive and negative fluorescence were subjected to an indole test using KOVACS's indole reagent. Positive indole and fluorescence samples were marked as *E. coli* type I (BGA, 1992). Meanwhile, negative fluorescence and positive indole samples were marked as *E. coli* O157:H7. Fluorocult *E. coli* O157:H7 agar was used to confirm *E. coli* O157:H7, and the plates were incubated at 35 °C for 18-24 h (Szabo et al., 1986). NA (Nutrient Agar) was surface plated to carry

out the total aerobic mesophilic bacteria counts, and plates were incubated at 30 °C for 24-48 h (Beuchat et al., 1991).

Physicochemical analyses

The pH and total acidity of the fruit samples were measured in duplicate. A Mettler Toledo Seven Compact pH Meter (Port Melbourne, Victoria,Australia) was used for pH determination. The titrimetric method with NaOH (0.1 N) was used to measure the total acidity of the samples. The results were expressed as the citric acid percent (%) of the samples taken as the total acidity (Ting and Rouseff, 1986). To determine the total phenolic compounds, 2 mL of each fruit sauce was mixed with 8 mL of ethanol (80%, v/v) and then centrifuged at 4,000 rpm for 20 min. Fifty µL of supernatant phase was mixed with 100 µL of Folin-Ciocalteau solution and 1,500 µL of distilled water for 10 min. Then, 50 µL Na_2CO_3 (20%, v/v) was added, and the mixture was left in the dark for 2 h. The optical density of samples was then measured against the blank at a wavelength of 765 nm using a spectrophotometer (Shimadzu UV-1700 Pharmaspec, Japan). A standard curve was created using gallic acid to measure the total phenolic content of samples in mg gallic acid/L (Abdullakasim et al., 2007).

Statistical analyses

Data were analysed by ANOVA (one-way analysis) using Statistical Package for the Social Sciences (SPSS) software, version 21 (IBM, USA)). Duncan's post-hoc test was applied at a significance level of $P< 0.05$.

RESULTS

Physicochemical analyses

The pH of the sumac sauce was the lowest among the sauces and that of pomegranate was the highest. These findings were accompanied with the titration acidity of the fruit sauces (Table 1). The total phenolic content of the pomegranate sauce was the lowest and that of the sumac sauce was the highest.

Microbial inhibition analyses

The agar diffusion test results showed that all bacteria were extremely sensitive to plum, Seville orange and sumac sauces at a concentration of 100%, v/v (Table 2). *Escherichia coli* type I, *S.*Typhimurium and *E. coli* O157:H7 were also extremely sensitive to pomegranate sauce at a concentration of 100%, v/v. Sumac sauce at 10% (v/v) concentration showed high antimicrobial activity against test microorganisms. The sensitivity of the bacteria to the remaining sauces at a concentration of 10% (v/v) varied, but in general, the studied bacteria were not sensitive at this concentration. The lowest concentration (1%, v/v) of the sauces was ineffective in inactivating microbial growth. Only *L. monocytogenes* was sensitive to sumac sauce at a concentration of 1%, v/v (Table 2).

The findings of the MIC method (Table 3) in general aligned with the those of the agar diffusion method, as shown in Table 2. The sumac sauce had a significantly ($P< 0.05$) higher antimicrobial activity than the remaining sauces on all of the studied bacteria except for *L. ivanovii*. The MIC value of sumac sauce and Seville orange sauce was the same for this latter bacterium (Table 3).

The pomegranate sauce had a significantly ($P< 0.05$) higher antimicrobial activity than the Seville orange and plum sauces against *S.*Typhimurium. The same sauce had significantly ($P< 0.05$) less activity than the other sauces (plum and Seville orange) against *L. ivanovii* and *S. aureus* and no activity against *L. monocytogenes*. The plum, pomegranate, and Seville orange sauces showed no differences in their antimicrobial activity against *E. coli* type I and *E. coli* O157:H7. The Seville orange sauce had a significantly ($P< 0.05$) higher antimicrobial activity against *L. ivanovii* and *S. aureus* compared to the plum and pomegranate sauces (Table 3).

According to the *in situ* (food method) test results, the Seville orange and sumac sauces both completely inactivated the indigenous microbial (total aerobic mesophilic bacteria, coliform bacteria and *E. coli* type I) growth in the chicken breast samples. The amount of coliform bacteria inactivated by the plum and pomegranate sauces was higher than the total aerobic mesophilic bacterial counts (Table 4).

DISCUSSION

Previous studies have shown that the antimicrobial activity of pomegranates (González et al., 2002; Duman et al., 2009; Tağı, 2010), plums (ShyamalaGowri and Vasantha, 2010; Mehta et al., 2014), Seville oranges (Değirmenci and Erkurt, 2020), and sumac (Ali-Shtayeh et al., 2013) depends on the high acidity and rich phenolic content of the fruits. Similarly, we found that the fruits with higher phenolic content and lower pH (Table 1) displayed significantly ($P< 0.05$) higher antimicrobial activity, as shown in Tables 2, 3, and 4.

Table 1 Physicochemical analyses

	Plum	Pomegranate	Seville orange	Sumac
pH	3.36±0.01[a]	5.12±0.02 [b]	2.47±0.01 [c]	2.13±0.00 [d]
Titration Acidity (% citric acid)	3.268±0.17[a]	0.098±0.00[b]	16.195±0.00[c]	36.84±1.12[d]
Total phenolic content (mg gallic acid/L)	260.32±3.85[a]	74.30±0.94[b]	153.99±1.37[c]	2637.59±9.95[d]

Results are presented as means of two observations ± standard deviation.
Data in the same row bearing different superscript letters are significantly different (P<0.05).

Table 2 Agar diffusion test results (mm)

	E.coli Type I	*S.*Typhimurium	*E.coli* O157:H7	*L.monocytogenes*	*L.ivanovii*	*S.aureus*
Plum						
100%	28[ax]	24[abx]	29[axy]	25[abx]	20[bx]	24.2[abx]
10%	1.0	1.0	2.0	2.0	12	1.0
1%	1.0	1.0	1.0	1.0	1.0	1.0
Pomegranate						
100%	32[ax]	36.4[ay]	26[bx]	1.0[dy]	9.0[cy]	6.0[cdy]
10%	17	3.0	3.0	1.0	6.0	10
1%	5.0	1.0	1.0	1.0	1.0	1.0
Seville orange						
100%	33.8[ax]	37[ay]	33.8[ay]	24[bx]	25[bx]	24[bx]
10%	2.0	7.0	2.0	3.0	22	12.2
1%	6.0	2.0	6.0	1.0	2.0	2.0
Sumac						
100%	35.2[abx]	38.2[aby]	40.2[bz]	40.7[bz]	32.5[az]	37.5[abz]
10%	22.5	21.5	20	15	21.2	25
1%	1.0	5.0	1.0	9.0	3.7	1.0

Results are presented as means of five observations.
Different superscript letters (a, b, c) within the same row indicate significant (p< 0.05) differences among the bacterial types as affected by each fruit sauces at 100% concentration.
Different superscript letters (x, y, z) within the same column indicate significant (p< 0.05) differences among the fruit sauces at 100% concentration against each bacterial types.

Table3 MIC test results (µg/mL)

	E.coli Type I	*S.*Typhimurium	*E.coli* O157:H7	*L.monocytogenes*	*L.ivanovii*	*S.aureus*
Plum	1:32 (0.031)[a]	1:32 (0.031)[a]	1:32 (0.031)[a]	1:32 (0.031)[a]	1:16 (0.062)[a]	1:32 (0.031)[a]
Pomegranate	1:28 (0.035)[a]	1:64 (0.015)[b]	1:32 (0.031)[a]	<1:2 (0.5)[b]	1:8 (0.125)[a]	1:8 (0.125)[b]
Seville orange	1:32 (0.031)[a]	1:32 (0.031)[a]	1:32 (0.031)[a]	1:32 (0.031)[a]	1:256 (0.003)[b]	1:128(0.007)[c]
Sumac	1:256 (0.003)[b]	1:256 (0.003)[c]	1:256 (0.003)[b]	1:256 (0.003)[c]	1:256 (0.003)[b]	1:256 (0.003)[d]

Results are presented as means of five observations.
Different superscript letters within the same column indicate significant (p< 0.05) differences.

Table4 Antimicrobial activity of fruit sauces on chicken breast meat samples

	TVC	Coliform (SMM)	Coliform (MPN)	*E.coli*
Supplier 1				
Control	5.49±0.05^{d}	3.73±0.08^{c}	> 1100	> 1100
Plum	3.04±0.04^{c}	2.84±0.06^{b}	26.7	26.7
Pomegranate	4.59±0.01^{b}	2.84±0.00^{b}	35	35
Seville orange	< 0.1±0.00^{a}	< 0.1±0.00^{a}	< 3.0	< 3.0
Sumac	< 0.1±0.00^{a}	< 0.1±0.00^{a}	< 3.0	< 3.0
Supplier 2				
Control	5.64±1.04^{g}	5.25±0.35^{h}	> 1100	> 1100
Plum	3.62±0.32^{f}	2.72±0.12^{g}	15	15
Pomegranate	4.87±0.02^{fg}	3.65±0.18^{f}	46	46
Seville orange	< 0.1±0.00^{e}	< 0.1±0.00^{e}	< 3.0	< 3.0
Sumac	< 0.1±0.00^{e}	< 0.1±0.00^{e}	< 3.0	< 3.0

Results are presented as means of two observations.
Different superscript letters within each supplier sample columns indicate significant (p< 0.05) differences for TVC and Coliform (SMM), and shown in log CFU/g.
No statistical analyses were performed on Coliform (MPN) and *E.coli*.

The phenolic compounds in plants are present in the form of monophenols, diphenols or triphenols, which are called simple phenolic compounds. In addition, phenolic acids such as gallic acid, caffeic acid, ferulic acid and furocoumarins are among the antimicrobial compounds (Daglia, 2012; Quinto et al., 2019).

Polyphenols are generally divided into two classes as flavonoids and nonflavonoids, which are important for their potential antimicrobial activity (Daglia, 2012). The freeze-dried arils of pomegranate fruits (FDAPs) contain both flavonoids (proanthocyanidin trimers and degradation of procyanidin dimers of flavan-3-ols) and nonflavonoids (caffeic acid and ferulic acid) in addition to palmitic and stearic acids, as determined by Uzunlu and Niranjan (2017). That study found that polycaprolactone (PCL)-incorporated pomegranate methanolic extract films had higher antimicrobial activity against *E. coli* and *S. aureus* than PCL-FDAP active films (Uzunlu and Niranjan, 2017).

Naz et al. (2007) directly isolated the bioactive phenolic compounds of pomegranate fruits and determined their antimicrobial activity. Of the identified phenolic compounds, gallic acid showed the highest antibacterial activity against tested Gram-positive and Gram-negative bacteria (Naz et al., 2007).

Duman et al. (2009) found that both *E. coli* and *S.* Typhimurium cells were highly affected by pomegranate sauce at a concentration of 100% (v/v), and *S. aureus* was less affected. Var et al. (2016) and Kunduhoğlu and Pilatin (2004) also reported that *E. coli* and *Salmonella* cells were highly inactivated by pomegranate sauce at a concentration of 100%, v/v. Another study examined the antimicrobial effect of traditional and commercial pomegranate sauces in Turkey and found traditional sauces to be more effective than commercially produced sauces (Karabiyikli and Kisla, 2012).

An extensive review reported that commercial pomegranate juices from the whole fruit have higher antioxidant activity than from the arils only (Kalaycıoğlu and Erim, 2017). In addition, the use of different solvents (water, ethanol, petroleum ether, chloroform, acetone, methanol) to extract bioactive compounds from different parts (aril, peel, seed) of the pomegranate fruit resulted in differences in antimicrobial activity (Tanveer et al., 2015). For instance, the aqueous extract of pomegranate displayed lower (0.20 mg/mL) MIC value than the ethanolic extract against *E. coli* (Voravuthikunchai et al., 2004). González et al. (2002) documented a high content of polyphenolic compounds in pomegranate peel and seeds with a high antimicrobial activity.

Our pomegranate sauce had a lower total phenolic content (74.3 mg GAE/L) and higher pH than earlier reported studies (see Kalaycıoğlu and Erim, 2017). One study reported a similar phenolic content (88.5 mg GAE/kg) for a pomegranate fruit clone '351' grown in south-eastern Spain. The MIC values of the

present pomegranate sauce were found to be in the range of 0.015-0.5 μg/mL (Table 3), with inhibition zones of 9.0-36.4 mm against the tested microorganisms (Table 2).

Prashanth et al. (2001) found that the aqueous extract of pomegranate rind had a MIC value against *Salmonella* Typhi of 0.025 μg/mL. This was similar to our findings for *S.*Typhimurium, for which we found a MIC value of 0.015 μg/mL. We also determined, interestingly, that pomegranate sauce had higher antimicrobial activity against *S.*Typhimurium than the other pathogens in both the agar diffusion and MIC assays (Tables 2 and 3).

Pradeep et al. (2008) also found that methanolic extracts of pomegranate pericarp showed the highest antimicrobial activity against *S.* Typhimurium and *Shigella dysenteriae* serotype 2, with an inhibition zone of 25 mm (for each) among other *Salmonella* types and *E. coli*. In addition, Pérez and Anesini (1994) determined that the pericarp extract of pomegranate showed strong antimicrobial activity against the multidrug-resistant typhoid fever-causing *S.* Typhi. An *in vivo* study to treat salmonellosis successfully found that pomegranate peel extract effectively inhibited the growth of *S.*Typhimurium and significantly reduced mouse mortality (Choi et al., 2011).

However, the comparison of the phenolic contents and antimicrobial activity of fruits and their cultivars is not always possible because the variety, climate, growing and processing conditions of the studied fruits and methods used to extract the bioactive compounds from fruits can result in differences in the data (Weerakkody et al., 2010; Değirmenci and Erkurt, 2020).

For instance, Orak (2009) found a total phenol content of 9,870 μg/mL in a sour concentrate of pomegranate, which was about one-third lower that of the fruit's juice. Conventional evaporation for nearly 8 h to produce sour concentrate mainly resulted in differences in the phenolic content of the fruit. Orak (2009) also stated that the difference in phenolic composition depends on the studied material and determination methods.

For instance, Shyamala Gowri and Vasantha (2010) found that the aqueous extracts of black plum (*Syzygium cumini* L.) leaves contained higher phenols than the methanolic extracts. Leaves of black plum (*S.cumini* L.) extracted in both methanol and water have been found to inhibit *Bacillus subtilis*, *S.*Typhi, *Pseudomonas aeruginosa*, *E. coli* and *Proteus vulgaris* (Shyamala Gowri and Vasantha, 2010). The authors referred to the antibacterial effect of tannins and other phenolic constituents present in black plum (*S.cumini*) leaves.

The present MIC values of plum sauce were the same (0.031 μg/mL) for the tested bacteria except for *L. ivanovii*, which had a value of 0.062 μg/mL, showing its resistance (Table 3). The inhibition zones were aligned with the MIC values, showing similar sensitivity of the bacteria. In the agar diffusion tests (Table 2), *L. ivanovii* was more resistant than the remaining bacteria, similar to the MIC test (Table 3).

Our research group previously documented that plum sauces have a high antibacterial effect on *Salmonella enteritidis*, *S.*Typhimurium, *E. coli*, *S. aureus* and *Bacillus* spp. at a concentration of 100%, v/v (Var et al., 2016). Fung and Thompson (2001) have reported that dried plum diluted at various concentrations inhibited the growth of *E.coli* O157:H7, *S.* Typhimurium and *S. aureus* on raw and cooked pork meat. In addition, Mehta et al. (2014) previously assessed extracts of dried plum samples against four bacterial pathogens, namely *S. aureus*, *Staphylococcus epidermidis*, *B. subtilis* and *Proteus mirabilis*. These researchers determined that dried plum extracts exercised an inhibitory effect against most of the tested bacteria, although no inhibition was detected against *B. subtilis.* Furfural and eugenol were determined as the antibacterial agents (Mehta et al., 2014).

Citrus aurantium (Seville orange) essential oil contains oxygenated monoterpenes, aliphatic hydrocarbons, monoterpene hydrocarbons and esters. Of a total of 77 different compounds, the main compound and dominant chemical class was linalool. Several chemical compounds present in fruits show antimicrobial activity against microorganisms (Değirmenci and Erkurt, 2020). For example, Karabıyıklı et al. (2014) examined the antimicrobial activity of sour orange juice against *L.monocytogenes* at different concentrations. At a concentration of 100% (v/v), *L. monocytogenes* was completely inactivated following incubation at 37 °C for 3 h, and a concentration of 10% (v/v) had a similar effect after 3 days of incubation and, at a concentration of 1% (v/v), after 1 day of incubation at 37 °C. *Salmonella* Typhimurium cells were totally inactivated at a concentration of 100% (v/v) after incubation at 37 °C for 3 h, and a concentration of 10% (v/v) also inactivated this bacterium after 2 days of incubation at 37 °C. However, at a concentration of 1% (v/v), this bacterium was inactivated after 7 days of incubation (Karabıyıklı et al., 2014).

The *in vitro* data revealed that both *L. monocytogenes* and *L. ivanovii* were extremely sensitive to Seville orange sauce at a concentration of 100%, v/v. *Listeria ivanovii* was also extremely sensitive to a concentration of 10% (v/v), although *L. monocytogenes* was not. There was no antimicrobial effect at a concentration of 1%, v/v. *Salmonella* Typhimurium cells were highly affected at a concentration of 100% (v/v), but were not affected at concentrations of 10% (v/v) and 1%, v/v (Table 2). Al-Oqaili et al. (2014) also reported that bitter orange juice at a concentration of 100% (v/v) showed very high antimicrobial activity against *S. aureus* using the agar diffusion method.

Sumac is used as astringent agent and for indigestion, anorexia, diarrhoea, haemorrhagia and hyperglycaemia in traditional folk medicine (Nasar-Abbas and Halkman, 2004; Fazeli et al., 2007). The bioactivity of sumac was previously documented and found to contain 211 phytochemicals. In particular, sumac is an abundant source of phenolic components, mainly hydrolysable tannins (Abu-Reidah et al., 2015).

The present findings for sumac sauce showed that the inhibition zones for *L. monocytogenes* and *E. coli* O157:H7 were 40.7 mm and 40.2 mm, respectively, at a concentration of 100% (v/v). Kunduhoğlu and Pilatin (2004) reported inhibition zones of 32 and 28 mm for *L. monocytogenes* and *E. coli* O157:H7, respectively, at a concentration of 75%, v/v. Another study found that the water extracts of sumac were effective against 12 bacterial strains, including *L. monocytogenes*, *S. aureus*, *E. coli* type I, *E. coli* O157:H7 and *S. enteritidis* (Nasar-Abbas and Halkman, 2004). The authors also studied a neutralised extract (pH 7.2) of sumac to exclude the inhibitory effect of the high citric and malic acid content, and the same inhibition patterns were found against all tested bacterial strains except *P. vulgaris* (Nasar-Abbas and Halkman, 2004). Another study (Digrak et al., 2001) extensively investigated the antibacterial and antifungal activities of medicinal plants, including sumac. The antimicrobial effect of the sumac samples was in the range of 35-51 mm based on the disc diffusion method (Digrak et al., 2001). Overall, our findings are in consistent with the literature.

Antimicrobial activity (*in situ*) of fruit sauces on chicken samples

The *in situ* antimicrobial effect of sauces on the indigenous microflora of the chicken samples showed that the Seville orange and sumac sauces completely inactivated the growth of total aerobic mesophilic bacteria, coliform bacteria and *E. coli* type I after 2 h at 4 °C. Meanwhile, the plum and pomegranate sauces decreased the growth of all tested bacteria by one to two log units (Table 4). Similarly, Yapar (2006) found that plum and pomegranate sauces at a concentration of 100% (v/v) completely inactivated coliform bacteria and *E. coli* growths on minced beef meat and ground beef meat. Total aerobic mesophilic bacteria decreased by 3 logs on minced beef meat dipped in plum and pomegranate sauces after 2 h at 4 °C; a 2-log decrease was found for ground beef samples. Coagulase-negative *Staphylococci* decreased by one log unit on minced beef samples dipped in plum sauce. Finally, a very slight (~0.5 logs) decrease was reported for both ground and minced beef samples dipped in pomegranate sauce (Yapar, 2006).

Consistent with our data, Bazargani-Gilani et al. (2015) reported the initial total viable count (TVC) of chicken breast meat samples as 4.85 log CFU/g. These authors stated that pomegranate juice retarded the growth of TVC and extended the shelf life of chicken up to 15 days at 4 °C storage. Lytou et al. (2016) studied the effects of marination (with pomegranate juice, olive oil, dried thyme and honey) and chilling at 4 °C on chicken breast fillets. The initial population of TVC of the samples was 5.1 log CFU/g, which decreased by *ca*. 1.0 log units on the first day. Taking into account that the upper acceptability limit of the microbial load for fresh meat is 7 logs (Senter et al., 2000), these authors reported that the growth of TVC and *Pseudomonas* spp. on chicken breast fillets marinated with pomegranate juice and stored at 4 °C was controlled, extending the time required to reach this upper limit by up to 8 days and 4 days, respectively (Lytou et al., 2016). Notably, *Pseudomonas* spp. is one the dominant genera of bacteria in chicken meat in addition to *Moraxella*, *Brochotrix* and *Carnobacterium* species and plays a significant role in meat spoilage (Kim et al., 2019).

CONCLUSIONS

In summary, all of the studied fruit sauces showed antimicrobial activity against the test microorganisms. Sumac sauce exhibited the highest antimicrobial activity both *in vitro* and *in situ* (on chilled chicken breasts). We propose the use of fruit sauces (4 °C for 2 h) as a practical way for catering services and households to decrease the existing microbial population of chicken breast meat.

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REFERENCES

Abdullakasim P, Songchitsomboon S, Techagumpuch M, Balee N, Swatsitang P, Sungpuag N (2007) Antioxidant capacity, total phenolics and sugar content of selected Thai health beverages. Int J Food SciNutr 58:77-85.

Abu-Reidah IM, Ali-Shtayeh MS, Jamous RM, Arráez-Román D, Segura-Carretero A (2015) HPLC-DAD-ESI-MS/MS screening of bioactive components from *Rhuscoriaria* L. (Sumac) fruits. Food Chem 166:179-191.

Ahmad I, Beg AZ (2001) Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. J Ethnopharmacol 74:113-123.

Ali-Shtayeh MS, Al-Assali AA, Jamous RM (2013) Antimicrobial activity of Palestinian medicinal plants against acne-inducing bacteria. African J Microbiol Res 7:2560-2573.

Al-Oqaili RMS, Al-Alak SK, Mohammed BB (2014) Antibacterial activity of citrus juices against methicillin resistant *Staphylococcus aureus*. J BiolAgric Healthcare 4:7-10.

Anonymous (2002) Official Methods of Analysis, 17th ed., Bacteriological Analytical Manual. Chapter 4: Enumeration of *Escherichia coli* and the Coliform Bacteria. Feng, P. Weagant, S. D. Grant, M. A. and W. Burkhardt. Retrieved on July 18, 2019 from: https://www.fda.gov/food/laboratory-methods-food/bam-4-enumeration-escherichia-coli-and-coliform-bacteria.

Anonymous (2018) Burden of foodborne illlness: findings. National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Foodborne, Waterborne, and Environmental Diseases (DFWED).

Bazargani-Gilani B, Aliakbarlu J, Tajik H (2015) Effect of pomegranate juice dipping and chitosan coating enriched with *Zataria multiflora*Boiss essential oil on the shelf-life of chicken meat during refrigerated storage. Innov Food SciEmergTechnol 29:280-287.

Beuchat LR, Nail BV, Brackett RE, Fox TL (1991) Comparison of the Petrifilm™ yeast and mold culture film method to conventional methods for enumerating yeasts and molds in foods. J Food Protect 54:443-447.

BGA (1992). Bundesgesundheitsamt: Amtliche Sammlung von Untersuchungsverfahren
nach § 35 LMBG - Loseblattsammlung. BeuthVerlag Berlin, Köln.

Choi J-G, Kang O-H, Lee Y-S, Chae H-S, Oh Y-C, Brice O-O, Kim M-S, Sohn D-H, Kim H-S, Park H, Shin D-W, Rho J-R, Kwon D-Y (2011) In vitro and in vivo antibacterial activity of *Punicagranatum* peel ethanol extract against *Salmonella*. Evidence-Based Complementary and Alternative Medicine 690518:1-8.

Daglia M (2012) Polyphenols as antimicrobial agents. CurrOpin Biotech 23:174-181.

Dantas STA, Camargo CH, Tiba-Casas MR, Vivian RC, Pinto JPAN, Pantoja JCF, Hernandes RT, Júnior AF, Rall VLM (2020) Environmental persistence and virulence of Salmonella spp. isolated from a poultry slaughterhouse. Food Res Int 129:108835.

Değirmenci H, Erkurt, H (2020) Relationship between volatile components, antimicrobial and antioxidant properties of the essential oil, hydrosol and extracts of *Citrus aurantium* L. flowers. J Infect Public Heal 13:58-67.

Digrak M, Hakki Alma M, Ilçim A (2001) Antibacterial and antifungal activities of Turkish medicinal plants. Pharm Biol 39:346-350.

Duman AD, Ozgen M, Dayisoylu KS, Erbil N, Durgac C (2009) Antimicrobial activity of six pomegranate (*Punica granatum* L.) varieties and their relation to some of their pomological and phytonutrient characteristics. Molecules14:1808-1817.

Ebel ED, Williams MS, Tameru B (2019) Relatedness of *Salmonella* contamination frequency on chicken carcasses and parts when processed in the same establishment. Food Control 100:198-203.

Fazeli MR, Amin G, Attari MMA, Ashtiani H, Jamalifar H, Samadi N (2007) Antimicrobial activities of Iranian sumac and avishan-e shirazi (*Zataria multiflora*) against some food-borne bacteria. Food Control 18:646-649.

Fung DYC, Thompson L (2001) Effects of dried plums on suppression of growth of foodborne pathogens in liquid medium and ground meat. Report to the California Dried Plum Board, Sacramento, CA, 27 pp.

González CN, Sánchez F, Quintero A, Usubillaga A (2002) Chemotaxonomic value of essential oil compounds in *citrus* species. ActaHortic 576:49-51.

Kalaycıoğlu Z, Erim FB (2017) Total phenolic contents, antioxidant activities, and bioactive ingredients of juices from pomegranate cultivars worldwide. Food Chem 221:496-507.

Karabiyikli S, Kisla D (2012) Inhibitory effect of sour pomegranate sauces on some green vegetables and kisir. Int J Food Microbiol 155:211-216.

Karabıyıklı Ş, Değirmenci H, Karapınar M (2014) Inhibitory effect of sour orange (*Citrus aurantium*) juice on *Salmonella*Typhimurium and *Listeria monocytogenes*. LWT-Food SciTechnol 55:421-425.

Kim H-E, Lee J-J, Lee M-J, Kim B-S (2019) Analysis of microbiome in raw chicken meat from butcher shops and packaged products in South Korea to detect the potential risk of foodborne illness. Food Res Int 122:517-527.

Kunduhoğlu B, Pilatin S (2004) Nar ekşisivesumakekşisi'nin*Escherichia coli* O157:H7ve*Listeria monocytogenes* üzerine antimikrobiyaletkisininbelirlenmesi (In Turkish). In: Proceedings of the 1. GelenekselGıdalarSempozyumu, Van, Turkey: pp 90-92.

Li Y, Pei X, Zhang X, Wu L, Liu Y, Zhou H, Ma G, Chen Q, Liang H, Yang D (2019) A surveillance of microbiological contamination on raw poultry meat at retail markets in China. Food Control 104:99-104.

Lytou A, Panagou EZ, Nychas G-JE (2016) Development of a predictive model for the growth kinetics of aerobic microbial population on pomegranate marinated chicken breast fillets under isothermal and dynamic temperature conditions. Food Microbiol 55:25-31.

Mehta S, Soni N, Satpathy G, Gupta RK (2014) Evaluation of nutritional, phytochemical, antioxidant and antibacterial activity of dried plum (*Prunusdomestica*). J Pharma Phytochem 3:166-171.

Nasar-Abbas SM, Halkman AK (2004) Antimicrobial effect of water extract of sumac (*Rhuscoriaria* L.) on the growth of some food borne bacteria including pathogens. Int J Food Microbiol 97:63-69.

National Committee for Clinical Laboratory Standards (2003) Clinical Laboratory Standards MIC testing. Performance standards for antimicrobial susceptibility testing (M100-S13), NCCLS, Wayne, PA (2003).

Naz S, Sıddıqı R, Ahmad S, Rasool SA, Sayeed SA (2007) Antibacterial activity directed isolation of compounds from *Punica granatum*. J Food Sci 72:M341-M345.

Orak HH (2009) Evaluation of antioxidant activity, colour and some nutritional characteristics of pomegranate (*Punica granatum* L.) juice and its sour concentrate processed by conventional evaporation. Int J Food SciNutr 60:1-11.

OsimaniA, Aquilanti L, Clementi F (2016) Salmonellosis associated with mass catering: a survey of European Union cases over a 15-year period. Epidemiol Infect 144:3000-3012.

Pérez C, Anesini C (1994) In vitro antibacterial activity of Argentine folk medicinal plants against *Salmonella typhi*. J Ethnopharmacol 44:41-46.

Ponce AG, Fritz R, del Valle C, Roura SI (2003) Antimicrobial activity of essential oils on the native microflora of organic Swiss chard. LWT-Food SciTechnol 36:679-684.

Pradeep BV, Manojbabu MK, Palaniswamy M (2008) Antibacterial activity of *Punica granatum* L. against gastro intestinal tract infection causing organisms. Ethnobotanical Leaflets 12:1085-1089.

Prashanth D, Asha MK, Amit AA (2001) Antibacterial activity of *Punicagranatum*. Fitoterapia 72:171-173.

Quinto EJ, Caro I, Villalobos-Delgado, LH, Mateo J, De-Mateo-Silleras, Redondo-Del-Río MP (2019) Food safety through natural antimicrobials. Antibiotics 8:1-30.

Saad SM, Ibrahim HM, Hassan MA, Eldin SNS (2020) Molecular detection of virulence factors in some food poisoning bacteria isolated

from chicken meat and giblet. BVMJ 38:106-111.
Senter SD, Arnold JW, Chew V (2000) APC values and volatile compounds formed in commercially processed, raw chicken parts during storage at 4 and 13 °C and under simulated temperature abuse conditions. J Sci Food Agric 80:1559-1564.
Shen H, Chen H, Ou Y, Huang T, Chen S, Zhou L, Zhang J, Hu Q, Zhou Y, Ma W (2020) Prevalence, serotypes, and antimicrobial resistance of Salmonella isolates from patients with diarrhea in Shenzhen, China. BMC Microbiol 20:197.
Shyamala Gowri S, Vasantha K (2010) Phytochemical screening and antibacterial activity of *Syzygiumcumini* (L.) (Myrtaceae) leaves extracts. Int J PharmTech Res 2:1569-1573.
Szabo RA, Todd ECD, Jean A (1986) Method to isolate *Escherichia coli* O157:H7 from food. J Food Protect 49:768-772.
Tağı Ş (2010) Nar Suyu Üretim Aşamalarında Antimikrobiyel Aktiviteve Fenolik Madde Miktarındaki Değişimler (In Turkish). Ankara Üniversitesi Bilimsel Araştırma Projesi Kesin Raporu. Ankara, Turkey, Proje Numarası: 08B4343002:37pp.
Tanveer A, Farooq U, Akram K, Hayat Z, Shafi A, Nazar H, Ahmad Z (2015) Pomegranate extracts: A natural preventive measure against spoilage and pathogenic microorganisms. Food Rev Int 31:29-51.
Ting SV, Rouseff RL (1986) Citrus fruits and their products. Analysis-technology. Marcel Dekker, New York, 293 pp.
Uzunlu S, Niranjan K (2017) Laboratory antimicrobial activity of cinnamaldehyde and pomegranate-based polycaprolactone films. J Appl Polym Sci 134:45347.
Var I, Sağlam S, Kamberoğlu MA (2016) Geleneksel Meyve Ekşilerinin Antimikrobiyal Özellikleri (In Turkish). In: Proceedings of the Gıda, Metabolizma & Sağlık: Biyoaktif Bileşenlerve Doğal Katkılar Kongresi, Istanbul, Turkey: pp 41-45.
Vetchapitak T, Misawa N (2019) Current status of Campylobacter food poisoning in Japan. Food Safety 7:61-73.
Voravuthikunchai S, Lortheeranuwat A, Jeeju W, Sririrak T, Phongpaichit S, Supawita T (2004) Effective medicinal plants against enterohaemorrhagic *Escherichia coli* O157:H7. J Ethnopharmacol94:49-54.
Weerakkody NS, Caffin N, Turner MS, Dykes GA (2010) *In vitro* antimicrobial activity of less-utilized spice and herb extracts against selected food-borne bacteria. Food Control 21:1408-1414.
Yapar F (2006) The antibacterial effects of plum sauce, pomegranate sauce and citric acid to chopped and minced meat (In Turkish). ÇukurovaÜniv Fen BilEnstMsC Thesis, Turkey, 49 pp.

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Research article
Ερευνητικό άρθρο

Impact of *Amaranthus hypochondriacus* in nutrition for rabbits on meat quality

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ABSTRACT: Unique properties of *Amaranthus hypochondriacus* have been extensively utilized in the recent time worldwide both in food technologies and husbandry. An intensive growth of *Amaranthus hypochondriacus* plants has been made in Ukraine there and, respectively, production of foods based on amaranth is growing, however no research on its use for feeding rabbits is available. Therefore, the study aimed to review the impact of *Amaranthus hypochondriacus* on slaughter features of rabbits, meat quality including those under storage conditions. The California race rabbits of 60 days age were divided into two groups, 28 animals in each. The rabbits in the study group were fed (by adding to the basal diet) with 20% of amaranth oilcake. The rabbits were slaughtered at age of 120 days. The following key features of meat output and quality performance were determined: pH, moisture content, water-holding capacity, cooking losses, protein and cholesterol content as well as change in pH, microorganisms count, and moisture loss percentage during 9-day storage in chilled state. *Amaranthus hypochondriacus* effect on the rabbit live weight, hot carcass weight, dressing out percentage, percentage ratio of heart, kidney, liver, lung weight to carcass weight, pH, moisture content, water-holding capacity, and meat morphological structure was determined. Reduction in cholesterol level by 15.07% ($p < 0.05$) in the meat of rabbits that were fed with *Amaranthus hypochondriacus* was detected. pH and drip loss percentage were not significantly different among the groups in the shelf-life period. However, it was found out that growth of microorganisms in the meat of rabbits that were fed with *Amaranthus hypochondriacus* was slowed down: microorganisms count was 1.65 and 1.71 ($p < 0.05$) times lower than in the control group on the 6th and 9th days of storage, accordingly. The low-cholesterol and bacteriostatic effects of amaranth oilcake in the rabbit nutrition may significantly increase dietary properties of rabbit meat. Use of amaranth oilcake for rabbit fattening has great potential and further studies including the mechanism of antibacterial effect of *A. hypochondriacus* on the rabbit meat are required

Keywords: amaranth, rabbits, meat quality, cholesterol, microorganisms

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INTRODUCTION

Dietary properties of the rabbit meat are advantageous over that of other animals owing to its higher protein level and lower cholesterol level (Nistor et al., 2013). These rabbit meat properties have been provided both through genetic factors and feeding conditions. Many researchers propose to use variousplant additives through rabbit nutrition or partial substitution offeedstuffs aimed to enhance quality performance of meat, decrease sickness rate and fodder cost (Cardinali et al., 2015;Dalle et al., 2016;Cullere et al., 2016;Kone et al., 2016;Dabbouet al., 2017;Dudaet al., 2018; Hernández-Martínez et al., 2018; Mancini et al., 2018;Fathi et al., 2019).

High potential capabilities of amaranth (*Amaranthus* spp.*)* have been acknowledged by the international scientific community for animal feeding, particularly as an alternative source of protein and fibre (Chhayet al., 2013; Peiretti, 2018;Peiretti et al., 2018). The scientists report on effective inclusion of amaranths: leaf meal, seeds and oilcake (*Amaranthus dubius, Amaranthus hypochondriacus, Amaranthus caudatus*) to the rabbit nutrition (Bamikole, 2000; Chhay et al., 2013; Molina et al., 2015; Molina et al., 2018).

Amaranth (mainly, *Amaranthus hypochondriacus*) is intensively grown and processed in recent years in Ukraine, however there are no studies of its use in rabbit nutrition (Hoptsii et al., 2018). Potential for the efficient use of vegetative mass and waste from the production of amaranth oil in rabbit breeding requires careful study and research. Research findings regarding use of amaranth in rabbit fattening in various countries are slightly controversial and insufficient. Today, we have not enough data on amaranth effect on change of quality features of the meat of rabbits bred on nutrition with amaranth. Extension of meat shelf-life period is also a relevant issue. Therefore, the study was aimed to review the effect of *Amaranthus hypochondriacus* on shelf-life qualities of rabbit meat.

MATERIALS AND METHODS

The study was carried out at the private rabbit farm Olbest LLC where rabbits of Californian race given by **Veselyi Khutorok Private Company, Novomoskovsk** were bred. Keeping, feeding, care of and all other operations with animals were carried out in conformity with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 18 March 1986), General Ethical Principles of Animal Experimentation approved by the First National Congress on Bioethics (Kyiv, 20 September 2001), Article 26 of the Law of Ukraine No. 5456-VI of 16.10.2012 'On Animal Protection Against Cruelty', and EU Directive 86/609/EEC of 24 November 1986.

Animals and housing conditions

Clinically healthy fifty-six rabbits of two months old with average live weight of 1,430±61.3g were random selected and divided into two groups, 28 rabbits each. Veterinary examinations of animals were regularly conducted during the study.The control group (I) included 15 males and 13 females, the experimental group (IIa) - 16 males and 12 females. The rabbits were kept in a rabbit warren separately in individual universal galvanized wire hutches during 2 months before being slaughtered, at average temperatures (19.4^0C±0.07) and relative air humidity (63.5±0.04%), under 10/12-hour cycle of light and dark.

Feeding animals

Watering and feeding were carried out automatically; rabbits had free access to water and fodder. The first (I), control group of rabbits received basal diet without amaranth, the second (IIa) study group received diet with addition of 20% amaranth oilcake (Table 1). Experimental diet was prepared by partial substitution of wheat and sunflower oilcake in control diet with 20% of *A. hypochondriacus* (diet of IIa group). The basal diet consisted of commercial granulated mixed fodder by Domashenko D.I.(individual entrepreneur from Kryvyi Rig) for fattening the young animals (Recipe K-94-22) according to Smith (1966). The amaranth oilcake that was produced by "Amarant" Trading and Production Farm, LLC (Dnipro) by means of cold pressing of *Amaranthus hypochondriacus* seed was used in production of granulated mixed fodder fed to the rabbits of IIa group. Diets for feeding rabbits of both groups consisted of equivalent shares of crude protein and digestible energy. The dry matter, crude protein, crude fat, crude fibre, ash, calcium and phosphorus were analyzed in diets by the procedures ofthe national standard DSTU 7693:2015 (2016).The digestible energy (DE)was calculated according with Axelson's formula modified by Grigorev and Volkov: DE = 0.73 × GE (1 - CF × 1.05), where 0.73 is the exchangeability coefficient, GE is the gross energy per 1 kg of dry matter of the feed, CF is crude fiber, (1 - CF × 1, 05) - coefficient reduction energy

value by crude fiber (Grigorev et al., 1989).During the experiment feed intake were recorded individually on a fortnightly basis. Average daily feed intake (ADFI) were calculated.

Table 1. Ingredients and chemical compositions of rabbit nutrition

Indicators	Groups	
	I	IIa
Ingredients, %		
Lucerne	25.00	25.00
Wheat	54.50	42.40
Sunflower oilcake	17.66	9.96
Amaranth oilcake	-	20.00
Vitamin and mineral pre-mix	2.84	2.64
Chemical composition		
Crude protein,%	15.84	15.85
Crude fibre, %	11.96	10.97
Crude fat, %	3.78	3.50
Digestible energy, MJ/kg	11.30	11.09
Ca, %	0.93	0.71
P, %	0.45	0.46

Dressing out animals and determination of meat output

Animals of 120-day age after 12-hour fasting were slaughtered at the company's slaughterhouse using Sprut Krol-100 equipment in accordance with the National Requirements "Rules for pre-dressing out veterinarian animal inspection and veterinary and sanitary expertise of meat and meat products" (2002). The technological process included: electrical stunning, blood draining, skinning, removal of head and limb distal parts, evisceration, and carcass scraping. After weighing and determination of meat pH, the slaughter products were chilled at the temperature of $+4^0$C, relative air humidity 92% for 24 hours, and then chilled carcasses were weighted. Meat productive carcass features were measured or calculated in accordance with Blasco and Ouhayoun (1996): live weight (LW), hot carcass weight (HCW), chilled carcass weight (CCW), dressing out percentage (DoP), drip loss percentage (DLP),percentage in carcass weight: liver (LvP), kidney (KiP), heart (HeP), lung (LuP).

Determination of meat quality performance

Laboratory tests were made in the testing center of Zaporizhzhia Regional State Laboratory at the State Service for Safety of Foods and Consumer Protection accredited in conformity with ISO/IE 17025: 2006, Accreditation Certificate No. 2H305 of the National Accreditation Agency of Ukraine. Quality performance was determined in *Longissimus dorsi* muscles. Hot carcass pH (15 minutes after dressed out - pH_0) and chilled carcass pH (24 hours - pH_{24}) were measured with portable meat pH-meter with metal pin (Gondo PS-45, China) that was inserted into muscles *Longissimus dorsi* at the level of 5th lumbar vertebra, 5th and 8th thoracic vertebra. Water-producing capacity (WPC) of meat was determined with the method described by Penny (1975) modified with technique by Earl et al. (1996). Ground meat specimens of 3 g weight were wrapped into nylonnet with mesh diameter of 0.5 mm, then placed into a 'basket' of filter paper and inserted into centrifugal test-tubes. Centrifugation was carried out for 15 minutes at 1,400 rpm. Moisture produced of meat was absorbed with filter paper and its amount was determined as weighing difference. Water-holding capacity (WHC) of meat was calculated as difference between 100% and percentage of moisture produced with centrifugation method (WPC). Moisture loss after heat cooking treatment was calculated as difference between fresh meat weight and one after cooking for 1 hour at temperature of 163°C (AMSA, 2015). Meat output after heat treatment was calculated as percentage of cooking loss deducted from 100%. Determination of pH, WPC, moisture loss after heat cooking treatment was made in 3 repetitions. Meat moisture was determined according to DSTU ISO 1442:2005 (2005);protein content - with biuret method (Tortenand Whitaker, 2006); cholesterol with spectrophotometric analysis (Li-Hua, 2019).

Meat freshness shelf-life estimation

After carcasses were cut (Blasco and Ouhayoun, 1996), all hind legs one by one were put into clean plastic containers with absorbing insert, weighed and kept storage for 9 days at temperature of +4^{0}C and 80% relative air humidity. On the 1st, 3rd, 6th and 9th day of storage, 14 containers for each period were opened and pH estimated (pH_{1d}, $pH_{3d,}$ $pH_{6d,}$ pH_{9d}) with "PHS-25C" laboratory pH-meter ("BROM" Private Company, Kyiv), measurement accuracy of up to ± 0.05 (GOST P 51478-99 (ISO 2917-74);drip loss percentage (by difference in weight after leg blotting with filter paper) and microorganisms count in tissue smears of surface muscle layers according toNational Standard GOST 23392-78. Microorganisms count was calculated in 3 tissue smears (25 microscope fields of view in each) from each sample and arithmetic mean value obtained.

Histomorphology of meat

Portions of *Longissimus dorsi* muscles in both rabbit groups were selected for histomorphology. The material was fixed in 10% neutral buffered formalin. Muscle parts were dewatered in ethanol (alcohol) solutions of increasing concentration and compressed in histology paraffin (Goralskyj et al., 2011). Serial histological sections of 3 to 5 microns thick were prepared on a sliding microtome with subsequent coloring with hematoxylin and eosin. Histology preparations were studied with light microscope Leica CX1000 and QWin 3 morphology processing data software.

Statistical analysis

The results were statistically analyzed using the Statistica 10 software. Distribution of the data within groups was evaluated using a Shapiro-Wilk test.All parameters showed a normal distribution. Reliability of the difference between values was verified under T-test (Independent-Samples T-Test). Levene's test was used to test whether variances were homogenous. Data are presented as mean ± standard deviation. The significance degree between two groups was determined to be $P<0.05$.

RESULTS

The inclusion of amaranth in the diet did not significantly change the level of ADPI (I: 237.50±11.47 g and IIa: 243.6±9.09 g, at$P < 0.1$).

Effect of dietary inclusion of amaranth as main feedstuff on certain carcass parameters of Californian rabbits are given in Table 2.

Table 2. Effect of diet with use of amaranth on carcass characteristics of rabbits (mean±standard deviation)

Indicators	Groups		P value
	I	IIa	
Live weight, g	3543.6±296.62	3417.5±212.17	0.07
Hot carcass weight, g	2057.6±203.96	1991.6±181.40	0.21
Chilled carcass weight, g	2007.2±157.62	1959.4±145.46	0.24
Dressing out percentage, %	58.0±1.11	58.28±1.05	0.33
Drip loss percentage, %	2.45±0.46	1.92±0.48	0.06
HeP, %	0.45±0.21	0.46±0.10	0.08
LvP, %	5.15±0.49	5.45±0,54	0.05
LuP, %	0.68±0,05	0.64±0.06	0.06
KiP, %	0.84±0.05	0.87±0.05	0.11

Notes: HeP - percentage in carcass weight heart; LvP - percentage in carcass weight liver; LuP - percentage in carcass weight lung; KiP - percentage in carcass weight kidney

Live weights of rabbits at 120-day age and dressing out percentage were in conformity to standard race values; however, reliable difference between the groups was not established. Neither was established the effect of feeding *Amaranthus hypochondriacus* on HCW, CCW, LvP, KiP, HeP, LuP. Drip loss percentage after chilling the carcasses in both groups did not exceed 2.5% and the carcasses of control rabbits showed slightly increased value (by 0.53%).

The determined quality performance of rabbit meat (Table 3) showed no significant difference between the groups by contents of moisture, protein and pH, however revealed a highly accurate ($p<0.05$) decrease in cholesterol content in result of feeding rabbits with *Amaranthus hypochondriacus*. Water-holding capacity of rabbit meat from IIa group was slightly higher than in control group but the difference was insignificant. No difference in heat treated meat output be-

tween the groups was detected.

Monitoring the changes in rabbit meat freshness during the 9-day shelf-life period (Table 4), data showed that meat pH was increasing with time but by the end of the study the findings did not exceed value of 6.2 and no difference between the experimental and control groups was reported. Drip loss percentage for each three days of shelf-life period varied from 0.62% to 1.27% and had no significant deviation in groups.

Table 3. Qualities of rabbit meat with various nutrition (mean±standard deviation)

Indicators	Groups		P value
	I	IIa	
pH_0	7.13±0.02	7.12±0.02	0.10
pH_{24}	5.95±0.04	5.93±0.03	0.26
Moisture, %	71.87±2.37	72.65±2.10	0.44
Water-producing capacity (WPC), %	33.21±3.26	31.56±2.80	0.24
Water-holding capacity (WHC), %	66.79±2.07	68.44±2.68	0.14
Moisture loss after heat cooking treatment, %	29.27±2.08	28.14±1.90	0.22
Meat output after heat cooking treatment, %	70.73±3.36	71.86±2.56	0.41
Protein, %	20.12±1.19	21.46±1.72	0.06
Cholesterol, mg/100 g	51.64±2.46	36.57±2.28*	<0.001

* - means are significantly different between groups

Table 4. Rabbit meat freshness dynamics (mean±standard deviation)

Indicators	Groups		P value
	I	IIa	
pH_{1d}	5.92±0.10	5.89±0.05	0.05
pH_{3d}	5.98±0.05	5.95±0.10	0.07
pH_{6d}	6.02±0.05	6.01±0.05	0.11
pH_{9d}	6.1±0.16	6.08±0.10	0.08
Moisture loss during shelf-life, % day:			
3	0.89±0.16	0.74±0.20	0.06
6	1.27±0.27	1.08±0.28	0.09
9	0.82±0.27	0.62±0.21	0.05
Microbe count in microscope field of view, day:			
1	2.3±1.92	1.45±1.36	0.16
3	2.24±1.66	3.98±1.98	0.05
6	6.91±1.65	4.2±1.62*	< 0.001
9	14.9±4.59	8.7±2.73*	< 0.001

* - means are significantly different between groups

Microorganisms count on the rabbit meat surface layers in both groups on the 9th day after dressing out increased 6-6.5 times compared to the first day, however, in IIa group, this value was in conformity to the fresh meat standards (<10) and in the control group to the low-quality freshness meat (11-30). The group that received *Amaranthus hypochondriacus* reported microorganisms count in meat to range between 1.65 and 1.71 times lower at the 6th ($p<0.05$) and 9th ($p<0.001$) days of shelf-life, accordingly. Dynamics changes for microorganisms count are shown in meat during the shelf-life (Fig. 1).

Figure 2 shows various tendencies for microbe growth during the shelf-life. While the group with amaranth reportedthe slow reproductionof microorganisms from 1 to 6 day, in the control group this period was 2 times shorter.

Histology of rabbit *Longissimus dorsi* muscle fragments (Fig. 2) established that animals in both groups reported no morphology differences in muscle tissue structure. Animals in I group had more dense located fibers. Nuclei were clearly outlined. Linear fibers prevailed in both animal groups, some were wavy with slight thickening and compression nodes which were

associated with post-mortem changes in muscle tissue. Endomysium, folded in some areas, was clearly visible. Transversal sections showed that muscle fiberswere uneven colored,whichwas due to physical and chemical changes in muscle tissue in the course of maturation.

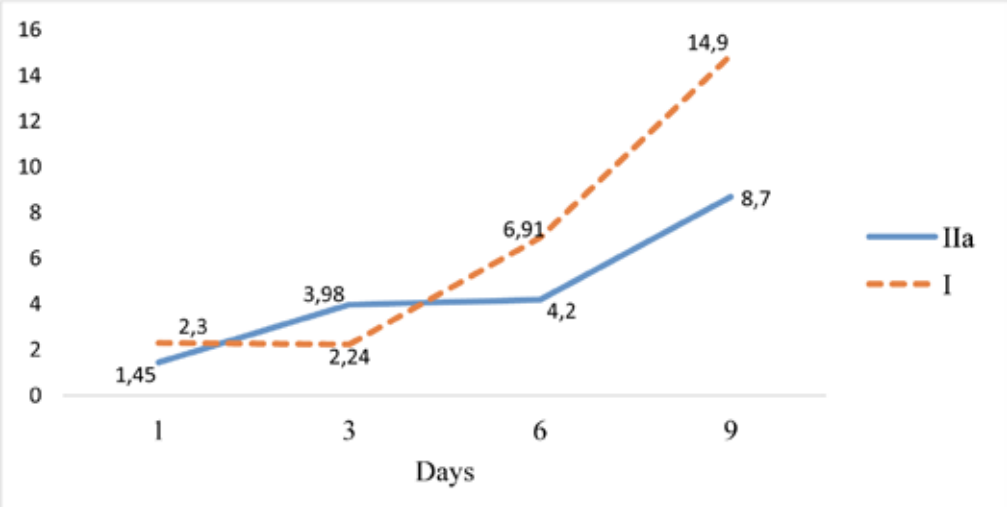
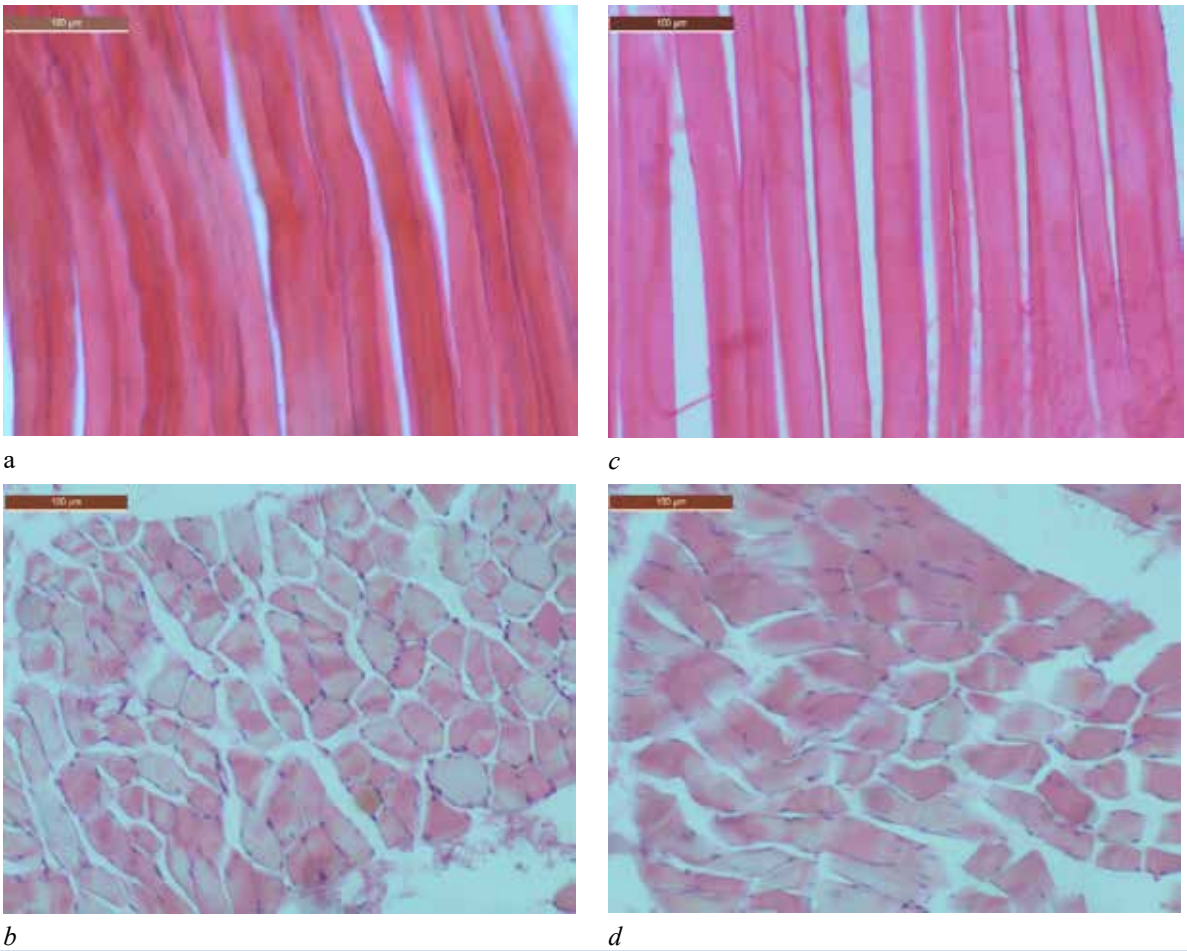


Figure 1. Dynamics of microorganisms count in tissue smears of rabbit meat during shelf-life



a *c*

b *d*

Figure 2. Histology longitude and transverse sections of rabbit muscles *Longissimus dorsi*: *a, b* - I group; *c, d* - IIa group. Haematoxylin and eosin, × 200

DISCUSSION

Adding 20% of *Amaranthus hypochondriacus* oilcake to the rabbit diet did not change meat characteristics of rabbits. Findings of our research also showed no effect of amaranth on the rabbit pre-dressed out live weight, hot carcass weight, dressing out percentage, percentage ratio of heart, kidney, liver, lung weight to carcass weight being coincide with data by Molina et al. (2015) and Mumford et al. (2018) who fed rabbits with sheet amaranth flour *A. dubius.* In contrast, Alfaro et al. (1987) reported an increase in rabbit carcass weight after 15% sheet *Amaranthus hypochondriacus* flour being added to their diet.

The results of our studies and the data of Molina et al. (2018) do not confirm reports of other authors about reducing the weight of rabbits fed with additives of: 20% and 30% barren inflorescences and crude seeds of amaranth (Bamikole et al., 2000); 25%, 50%, 75% of amaranth green mass (Chhay et al., 2013); 30%, 45% and 60% of sheet *Amaranthus hypochondriacus* flour (Alfaro et al., 1987).

Dressing out percentage was almost the same in the groups, 58.0% and 58.28%, accordingly, which is likely explained with genetic factors that have prevailing effect on this value compared to nutrition enriched with plant additives (Daszkiewicz et al., 2012). At chilling of heat carcasses, we reported on a decrease in drip loss percentage by 0.53% in IIa group (with amaranth added) versus I (control) group, however this deviation between the groups was insignificant. In contrast, Molina et al. (2018) determined that dietary addition of amaranth increased moisture loss during chilling of heat rabbit carcasses by 1.21%-1.36%. In both groups of our studies, carcass weight loss after 24-hour chilling were 1.92% and 2.45%,which is in agreement with the findings of Honikel and Hamm (1994), Shevchik et al. (2020) of drip meat losses for 24 hours that may constitute up to 3%.

pH values determined in this study did not change in result by feeding rabbits with amaranth oilcake which is in agreement with data by Molina et al. (2018),Manciniet al. (2018); however, in other studies reported of increase in meat pH for rabbits that were fed with natural plant fodder additives (Cullereet al., 2016; Zepeda-Bastida et al., 2019).

Determined moisture content in rabbit meat of 71.87±0.24 and 72.65±0.98% was slightly lower than average rabbit meat moisture values (73.8%) reported by DalleZotte (2014).In addition, we did not find out the effect of amaranth at the meat levels of moisture and protein. Similar findings were obtained by other researchers who also stated that plant fodder additives had no effect on moisture content (Hernández-Martínez et al., 2018) and protein levels (Kone et al., 2016) in the rabbit meat. In contrast, Molina et al. (2018) found out that protein level in meat increased with decrease in moisture in the groups of rabbits fed with amaranth. Cardinali et al. (2015) reported an increase in moisture and protein levels in rabbit meat as result of addition of oregano and rosemary to the rabbit nutrition.

It is well-known that rabbit meat contains cholesterol level lower than meat of other animals with average values as follows: 47.2-61.2 mg/100 g (Dalle Zotte, 2014); 45-67 mg/100g (Dinh et al., 2011),which is similar to cholesterol levels in our studies (51.64mg/100g) in the meat of control group rabbits that were given basal diet. We have found decrease in cholesterol level in meat by 15.07% ($p<0.05$) as a result of feeding rabbits with amaranth which coincided with statements by Palazzo et al. (2019) who determined cholesterol decrease in rabbit meat by 9.8 and 13% after dietary inclusion of additives with carotenoid pigment and phospholipid complexes. Similarly, it was confirmed by the studies by Plate and Areas (2002) who found out that consumption by rabbits of extruded skimmed amaranth flour reduced the total cholesterol level in blood serum by 50%. Cholesterol lowering action of amaranth is explained by the researches with its phytocomponents as squalen, unsaturated fatty acids, protein, tocotrienols, tocopherols (Caselatoand Amaya-Farfan, 2012, Tang andTsao, 2017).

Water-holding capacity of meat is an important quality that is directly associated with tenderness, juiciness and influences the product output (Qiaofen Cheng and Da-Wen Sun, 2008; Warner, 2014; Lee et al., 2017; Mir Nasiret al., 2017). We did not establish significant changes of feeding amaranth on WHC and meat output after heat treatment which coincided with reports by researches on no effect of plant additives, including amaranth on water-holding capacity of rabbit meat (Alagónet al., 2015; Molina et al., 2018) and boiling losses (Koneet al., 2016; Hernández-Martínez

et al., 2018; Mancini et al., 2018).

No statistically significant difference in meat pH during 9-day shelf-life in between the groups were determined, however we outlined gradual and even increase of pH in both groups: from 5.89 to 6.08 and from 5.92 to 6.1. Increase of pH during storage of rabbit meat in chilled state was explained by Hulot and Ouhayoun (1999) by protein amino acid deamination. On the contrary, pH decrease in the rabbit meat from the 2nd to 8th day of storage was reported by researches as result of ginger powder added to nutrition(Mancini et al., 2018). Drip loss percentage during the meat shelf-life had no significant deviation in groups and varied from 0.62% to 1.27% for 3-day period which coincided with data by Kone et al., 2016, Mancini et al., 2018. Low microorganismscount on the meat surface layers at the beginning of shelf-life indicated that veterinarian and sanitary requirements and technologies for dressing out of rabbits are met (Shevchik et al., 2019). Slow reproduction of microorganisms in the meat of rabbits that were fed with *Amaranthus hypochondriacus* oilcake was reported on the 6th ($p<0.05$) and 9th ($p<0.05$) days of shelf-life which coincided with reports by researches on depression of rabbit meat microflora by adding some plant extracts (Kone et al., 2016), oregano essential oil (Soultos et al., 2009) to nutrition. Emission of 17 phenolic compounds of amaranth leaves and seeds (Karamaćet al., 2019) and revealed abilities of plant polyphenols to delay growth of some microbes (Papucet al., 2017) may explain the bacteriostatic effect of amaranth. In the future, the mechanism of antibacterial action of amaranth components on rabbit meatneeds to be determined in thorough research.

CONCLUSIONS

Research findings have shown that addition of 20% of *Amaranthus hypochondriacus* oilcake to the rabbit diet had no effect on carcass features, pH, moisture content, water-holding capacity, and meat morphological structure. Reduction in cholesterol level by 15.07% ($p < 0.05$) in the meat of rabbits that were fed with amaranth was detected. Such indicators as pH and drip loss percentage did not change significantly in between groups in the shelf-life period. However, microorganisms count in meat of rabbits that were given feed with amaranth was 1.65 and 1.71 ($p < 0.05$) times lower than in the control group on the 6th and 9th days of storage, respectively.

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REFERENCES

Alagón G, Arce O, Serrano P,Rodenas L,Martínez-Paredes E,Cervera C,Pascual JJ,Pascual M. (2015) Effect of feeding diets containing barley, wheat and corn distillers dried grains with solubles on carcass traits and meat quality in growing rabbits. Meat Science 101:56-62.

Alfaro MA, Ramírez R, Martínez A.,Bressani R (1987) Evaluation of different amounts of amaranth meal (vegetative parts) to replace lucerne leaf meal in diets for growing rabbits. ArchivosLatinoamericanos de Nutricion, 37:174-185.

American Meat Science Association (AMSA),(2015) Research Guidelines for Cookery, Sensory Evaluation, and Instrumental Tenderness Measurements of Fresh Meat, 2nd ed.; American Meat Science Association: Savoy, IL, USA.

Bamikole MA, Ezenwa I, Adewumi MK, Omojola AB, Adetimirin VO, Arigbede OM,Orisadeyi SA (2000) Alternative feed resources for formulating concentrate diets of rabbits. 1. Unthreshed grain amaranth seedhead. World Rabbit Sci 8:125-129.

Blasco A,Ouhayoun J (1996) Harmonization of criteria and terminology in rabbit meat research. Revised proposal. World Rabbit Sci 4:93-99.

Cardinali R,Cullere M, Dal Bosco A, Mugnai C, Ruggeri S, Mattioli S,-Castellini C,TrabalzaMarinucci M,DalleZotte A (2015) Oregano, rosemary and vitamin E dietary supplementation in growing rabbits: Effect on growth performance, carcass traits, bone development and meat chemical composition. Livestock Sci 175:83-89.

Caselato V, Amaya-Farfan J (2012) State of Knowledge on Amaranth Grain: A Comprehensive Review. Journal of Food Sci77:93-104.

Chhay T, Pok S, Borin K, Preston TR (2013) Amaranth (*Amaranthus spp.*) as replacement for water spinach *(Ipomoea aquatica*) with or without paddy rice on growth performance of rabbits. Livestock Research for Rural Development 25 (12) from http://www.lrrd.org/lrrd25/1/chha25012.htm

Cullere M, DalleZotte A, Celia C, Renteria-Monterrubio AL, Gerencsér Z, Szendrő Z, Kovács M, Kachlek ML,Matics Z (2016) Effect of Silybummarianum herb on the productive performance, carcass traits and meat quality of growing rabbits. Livestock Sci 194:31-36.

Dabbou, S., Renna, M., Lussiana, C.; Gai, F., Rotolo, L., Kovitvadhi, A., Brugiapaglia, A.; Helal, A. N., Schiavone, A., Zoccarato, I., Gasco, L. (2017). Bilberry pomace in growing rabbit diets: effects on quality traits of hind leg meat. Italian Journal of Animal Science 16:371-379.

DalleZotte A (2014) Rabbit farming for meat purposes. Animal Frontiers 4 (4):62-67.

DalleZotte A, Celia C,SzendroZs(2016)Herbs and spices inclusion as feedstuff or additive in growing rabbit diets and as additive in rabbit meat: a review. Livestock Sci 189:82-90.

DaszkiewiczT, GugołekA, JaniszewskiP, Kubiak D, CzoikM (2012)The effect of intensive and extensive production systems on carcass quality in New Zealand White rabbits. World Rabbit Sci. 20:25-32.

Dinh TTN, Thompson LD, Galyean ML, Brooks JC, Patterson KY, Boylan LM (2011) Cholesterol content and methods for cholesterol determination in meat and poultryCompr. Rev. Food Sci. Food Saf. 10 (5):269-289.

DSTU 7693:2015 (2016). National Standard of Ukraine Compound Feed Raw Materials General technical conditions. Official publication Kyiv SE "UkrNDNC". http://online.budstandart.com/ua/catalog/doc-page?id_doc=62176

DSTU ISO 1442:2005 (2007). Meat and meat products.Method for determination of moisture.Kiev, Holding Ukraine: pp 4-11.

Duda YV, Prus MP, Shevchyk PS, KunevaLV, KoreybaLV (2018) Effect of Amaranthus-based fodder additive on activity of rabbit blood enzymes under eimeriosis. In: 21st International Scientific and Practical Conference "Relevant issues of intensive development of animal breeding", Gorki,Belarus 2:186-189.

Earl LA, King AJ, Fitzpatrick DP, Cooper JE (1996) A modification of method to determine expressible moisture in ground, dark poultry meat. Poultry Sci 75:1433-1436.

Goralskyj LP, Khomich VT, Kononskyi OI (2011) The technology of manufacturing histological preparations. In: Fundamentals of histology technique and morphofunctional research methods under norm and pathology 3rd ed,Polissia, Zhytomyr: pp9-108.

GOST P 51478-99 (ISO 2917-74) Meat and meat products. Reference method for measurement of pH.http://docs.cntd.ru/document/1200028185 [accessed 18 March 2020].

GOST 23392-78. Meat. Methods for chemical and microscopic analysis of meat freshness.Publishing house of standards,Moscow: pp 6-19.

Grigorev N.G., Volkov N.P., Vorobev E.S. (1989). Biological usefulness of feed,Moscow, Agropromizdat: pp 68-82.

Hernández-Martínez CA,Treviño-Cabrera GF, Hernández-Luna CE, Silva-Vázquez R, Hume ME, Gutiérrez-Soto G, Méndez-Zamora G (2018) The effects of hydrolysed sorghum on growth performance and meat quality of rabbits. World Rabbit Sci 26:155-163.

Honikel KO, Hamm R (1994) Measurement of water-holding capacity and juiciness. In: Pearson A.M., Dutson T.R. (eds) Quality Attributes and their Measurement in Meat, Poultry and Fish Products. Advances in Meat Research, vol 9. Springer, Boston, MA.

Hoptsii TI, Voronkov MF, Bobro MA, Miroshnychenko LO, Lymanska SV, Hudym OV, Hudkovska NB, Duda YV (2018) Amaranth: breeding, genetics and cultivation prospects: monograph.NAU,Kharkiv: pp 65-68.

Hulot F,Ouhayoun J (1999) Muscular pH and related traits in rabbits: a review. World Rabbit Sci 7:15-36.

Fathi M,Abdelsalam M, Al-Homidan I,Ebeid T,Shehab-El-Deen M,Abd El-Razik M,Abou-Emera O, Mostafa M (2019) Supplemental effects of eucalyptus*(Eucalyptus camaldulensis)*leaves on growth performance, carcass characteristics, blood biochemistry and immune response of growing rabbits. Annals of AnimSci 19:779-791.

Karamać M, Gai F, Longato E, Meineri G, Janiak MA, Amarowicz R, Peiretti PG (2019) Antioxidant Activity and Phenolic Composition of Amaranth (Amaranthuscaudatus) during Plant Growth. Antioxidants (Basel, Switzerland) 8(6), 173.

Kone AP, Cinq-Mars D, Desjardins Y,Guay F, Gosselin A, Saucier L (2016) Effects of plant extracts and essential oils as feed supplements on quality and microbial traits of rabbit meat. World Rabbit Sci 24:107-119.

Lee, MT, Lai, LP, Lin, WC, Ciou, JY, Chang, SC, Yu, B, & Lee, TT. (2017). Improving Nutrition Utilization and Meat Quality of Broiler Chickens Through Solid-State Fermentation of Agricultural By-Products by Aureobasidium Pullulans. Brazilian Journal of Poultry Science, 19(4), 645-654. https://doi.org/10.1590/1806-9061-2017-0495

Li-Hua Li, DutkiewiczEwelina P, Ying-Chen Huang, Hsin-Bai Zhou, Hsu,Cheng-Chih (2019) Analytical methods for cholesterol quantification. Journal of Food and Drug Analysis; Philadelphia 27 (2): 375-386.

Mancini S,Secci G,Preziuso G,Parisi G,Paci G (2018) Ginger *(Zingiberofficinale* Roscoe) powder as dietary supplementation in rabbit: life performances, carcass characteristics and meat quality. Italian Journal of AnimSci 17:867-872.

Mir NA, Rafiq A, Kumar F, Singh V, Shukla V (2017) Determinants of broiler chicken meat quality and factors affecting them: a review. Journal of Food Science and Technology 54:1-13.

Molina E, González-Redondo P, Moreno-Rojas R, Montero-Quintero K, BrachoB,Sánchez-Urdanetaet A (2015) Effects of diets with *Amaranthusdubius* Mart. ex Thell. on performance and digestibility of growing rabbits. World Rabbit Sci 23 (1):9-18.

Molina E, González-Redondo P, Moreno-Rojas R, Montero-Quintero K,Sánchez-Urdaneta A (2018) Effect of the inclusion of *Amaranthusdubius* in diets on carcass characteristics and meat quality of fattening rabbits. Journal of Applied Animal Research 46:218-223.

Nistor E,Bampidis V,Păcală N,Pentea M, Tozer J,Prundeanu H (2013) Nutrient content of rabbit meat as compared to chicken, beef and pork meat. J Anim. Prod Adv. 3:172-176.DOI: 10.5455/japa.20130411110313

Palazzo M, Schiavitto M, Cinone M,Vizzarri F (2019) Rabbit metabolic response and selected meat quality traits: Evaluation of dietary

PLX®23 and LycoBeads® feed supplement. Journal of Animal Physiology and Animal Nutrition, 103 (1):383-394.

Papuc C, Goran GV,Predescu CN, Nicorescu V, Stefan G (2017) Plant polyphenols as antioxidant and antibacterial agents for shelf-life extension of meat and meat products: Classification, structures, sources, and action mechanisms. Comprehensive Reviews in Food Science and Food Safety 16:1243-1268.

Peiretti PG (2018) Amaranth in animal nutrition: A review. Livestock Research for Rural Development, 30 (88).

Peiretti PG, Meineri G, Longato E,Tassone S (2018) Chemical Composition, In Vitro Digestibility and Fatty Acid Profile of *Amaranthuscaudatus*. Herbage During its Growth Cycle. Animal Nutrition and Feed Technology, 18:107-116.

Penny IF(1975) Using a centrifugation method to measure drops of Longissimus dorsi pork slices before and after freezing and thawing. J. Sci. Food Agric. 26:1593-1602.

Plate AYA,Areas JAG (2002) Cholesterol-lowering effect of extruded amaranth (Amaranthuscaudatus L.) in hypercholesterolemic rabbits. Food Chem 76(1):1-6.

Rules for pre-dressing out veterinarian animal inspection and veterinary and sanitary expertise of meat and meat products (2002). https://zakon.rada.gov.ua/laws/show/z0524-02 [accessed 18 March 2020].

Qiaofen Cheng, Da-Wen Sun (2008) Factors Affecting the Water Holding Capacity of Red Meat Products: A Review of Recent Research Advances, Critical Reviews in Food Science and Nutrition 48(2):137-159.

Shevchik RS, DudaYV, Kuneva LV (2019) Effect of veterinary and sanitary and technological conditions of slaughter on microbiological indicators of rabbit meat. In: 18th All-Ukrainian Scientific and Practical Conference of Young Scholars, Lviv, Ukraine 21(3):168.

Shevchik, R., Duda, Y., Gavrilina, O., Kuneva, L. (2020). Comparative assessment of the quality of rabbit meat, which was obtained in the conditions of a slaughter enterprise and backyard. Scientific Messenger of LNU of Veterinary Medicine and Biotechnologies. Series: Veterinary Sciences, 22(97): 162-168.

Smith SE (1966) Nutrient Requirements of Rabbits; The National Academies Press: Washington, DC, USA.

Soultos N, Tzikas Z, Christaki E, Papageorgiou K, Steris V (2009) The effect of dietary oregano essential oil on microbial growth of rabbit carcasses during refrigerated storage. Meat Sci 81:474-478.

Tang Y, Tsao R (2017) Phytochemicals in quinoa and amaranth grains and their antioxidant, anti-inflammatory, and potential health beneficial effects: a review. MolNutr Food Res 61(7).

Torten J, Whitaker J (2006) Evaluation of the Biuret and Dye-Binding Methods for Protein Determination in Meats. Journal of Food Sci 29:168-174.

Warner R (2014) Measurements of Water-holding Capacity and Color: Objective and Subjective. doi: 10.1016/B978-0-12-384731-7.00210-5

Zepeda-Bastida A, Martínez MA, Simental SS (2019) Carcass and meat quality of rabbits fed Tithoniatubaeformis weed. RevistaBrasileira de Zootecnia, 48. https://doi.org/10.1590/rbz4820190074

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Research article
Ερευνητικό άρθρο

Prevalence of *Salmonella* spp. in layer and broiler farms in Palestine in 2018, with special emphasis on *Salmonella enterica* serovar Enteritidis

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ABSTRACT: *Salmonella* spp. are one of a major public health concerns worldwide, as well as it is the most frequently isolated foodborne pathogen. Human illness with *Salmonella* spp. is often due to the consumption of contaminated food of animal origin such as eggs or their products and poultry meat. The prevalence of this pathogen in egg-laying poultry farms and in broiler farms is an important public health risk factor. *Salmonella enterica* serovar Enteritidis has been the major cause of foodborne salmonellosis in humans. Data on the prevalence of *Salmonella* spp. in the Palestinian territories' poultry flocks is lacking. The objective of this study was to investigate the prevalence of *Salmonella* spp. in local layer and broiler flocks, and to find out the rate of *S.* Enteritidis among the isolated samples.

A total of 1180 cloacal swabs were collected from several layer and broiler farms from different locations in the West Bank, Palestine. Identification of *Salmonella* spp. was carried out using conventional and serological methods. Molecular methods using Polymerase Chain Reaction was used for confirmation of *Salmonella* spp., and to detect the presence of *S.* Enteritidis among the isolated *Salmonella* spp.

Results of the current study showed that, the rate of *Salmonella* spp. in the sample tested from layer and broiler farms was 10.7% (65/608) and 4.7% (27/572), respectively. In addition, the prevalence rate of *S.* Enteritidis among other *Salmonella* spp. was 0.0% and 14.8% for layer and broiler farms, respectively.

It is highly recommended that further studies should be conducted, including high number of samples with serotyping and molecular characterization of the positive samples.

Keywords: *Salmonella*, *S.* Enteritidis, foodborne pathogens, PCR, poultry, Palestine.

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INTRODUCTION

Foodborne illnesses including salmonellosis have become a serious public health problem in many countries in the recent decade. Salmonellosis is the second major cause of foodborne disease worldwide and may lead to episodes of hospitalization and death (Jeníková et al., 2000; Scallan et al., 2011; EFSA 2019). Salmonellae in humans can cause typhoid fever resulting from bacteremia, and gastroenteritis resulting from foodborne infection (Boseret et al., 2013; Seifi et al., 2019).

Although *Salmonella* spp. cause three million deaths annually worldwide (Mohammadzadeh et al., 2018), non-typhoidal *Salmonella* spp. are believed to cause 93.8 million infected cases of acute gastroenteritis and 155,000 deaths yearly (Eng et al., 2015; Nouichi et al., 2018). Salmonellosis comprises a big common health load and represents considerable costs in many countries. It can cause a variety of illnesses in several hosts whereas in others can exist as asymptomatic disease (Herrero-Fresno and Olsen 2018).

Salmonella spp. exist commonly in domestic animals, including poultry, and can be isolated from wild animals, including birds, creepers, and rodents. *Salmonella* spp. are isolated more often from poultry and poultry products rather than from any other food animals. The majority of salmonellosis cases were associated with poultry meat and eggs consumption (EFSA 2019). The eggs may be contaminated on the outer surface of the shell and internally (Schoeni et al., 1995; Radkowski, 2002; Messens et al., 2006). Internal contamination can be the result of penetration of bacteria through the egg shell or by direct contamination of the egg content before oviposition, caused by infection of the reproductive organs. Poultry, turkey and chicken in particular are extremely colonized with *Salmonella* spp. without remarkable symptoms at incipient production scale. The existence of *Salmonella* spp. in healthy poultry is considered a major risk factor, transporting the infection from poultry meat and table eggs to humans (Antunes et al., 2016). Poultry can be infected mainly with *Salmonella enterica* serovar Enteritidis, Typhimurium and Heidelberg, which are all of worldwide distribution, wide host range and they are of major economic and public health significance (Mengistu et al., 2011; EFSA 2019). The most common *Salmonella* serotypes, from broiler flocks in Europe were Enteritidis, Infantis, Mbandaka, Typhimurium and Hadar (van de Giessen et al., 2006). In the United States, during the surveillance for foodborne outbreak 2009-2010, *S.* Enteritidis was the most common serotype with 76 outbreaks out of 225 confirmed *Salmonella* outbreaks (Ekaterinadou et al., 2015).

S. Enteritidis has been the major cause of foodborne salmonellosis in humans over the last 20 years, during which contaminated poultry meat and eggs were the most important transporter of the infection (Ekateriniadou et al., 2015; Legesse, 2017). It has a unique ability to contaminate eggs without causing discernible illness in the infected birds. Epidemiological investigations in Hungary, the United Kingdom, the United States, Austria, Germany and many other countries, confirmed that several outbreaks of salmonellosis were associated with the consumption of contaminated eggs, which are the food most associated with increased illness in humans (Guard-Petter, 2001; Much et al., 2009; Li et al., 2017). The transmission of *S.* Enteritidis infections to humans by contaminated eggs has been a prominent food safety issue throughout much of the world for more than a decade.

Infected hens with *S.* Enteritidis have been found to carry the organism in the reproductive tract as well as in the ceca (Poppe et al., 1992). It seems that the Enteritidis serotype has an intrinsic characteristic that allows an epidemiological association with chicken eggs that are not yet defined (Gantois et al., 2009). *S.* Enteritidis has been considered as an important pathogen that infects not only poultry meat and eggs, but also fresh meat and its products, milk, fruits and vegetables causing salmonellosis or gastroenteritis manifested by fulminant diarrhea with low grade fever, septicemia, nausea and vomiting in affected humans. Numerous and complex environmental influences on *Salmonella* spp. persistence and transmission are exerted by management practices and housing facilities used in commercial egg and meat production (Gast et al., 2017).

Poultry production, either layers or broilers, form an important agricultural industry in Palestine. Poultry farming is primarily limited to small private companies owned by individuals and not limited to big companies as in many neighboring countries. Therefore, the large number of farms distributed throughout the country makes controlling of health status of the flocks by the Ministry of Agriculture extremely difficult.

According to the Annual Reports of Health published by the Ministry of Health in Palestine (Abed

Al-Daym, 2019), many cases of foodborne illness were reported between 2009 and 2017 (Table 1). The number of cases of foodborne illness caused by *Salmonella* spp. has also been monitored and reported annually (Table 1). The number of *Salmonella* cases reported ranged from 406 in 2009 to 64 in 2017. This reduction in the cases of foodborne illness and *Salmonella* may be attributed to the measures introduced by the governmental and non-governmental institutions to limit and control foodborne illness and *Salmonella*.

Table 1. Foodborne illnesses in Palestine between 2009 and 2017, and the cases of Salmonellosis for the same period

Year	Foodborne illnesses		Salmonellosis	
	n	Rate/100K	n	Rate/100K
2009	598	15.2	406	10.32
2010	442	11.6	319	8.3
2011	288	6.9	227	5.5
2012	320	7.3	196	4.5
2014	221	4.9	54	1.2
2015	156	3.33	20	0.42
2017	132	3	64	1.41

n: number of cases.
Rate/100K: Rate per 100,000 population

Data on the prevalence of *Salmonella* spp. in poultry and broiler farms in Palestine is lacking. Therefore, the objective of this study was to investigate the prevalence of *Salmonella* spp. in general and *S.* Enteritidis in particular among local layer and broiler flocks in the West Bank, Palestine.

MATERIALS AND METHODS

Study Area

Samples were collected from four governorates in northern part of the West Bank in Palestine. These governorates are Tulkarm, Nablus, Qalqylia and Jenin (Figure 1).

Depending on the Report of the Palestinian Central Bureau of Statistics (PCBS 2017), the total area of these governorates is about 1,600 square kilometers, which include about 28.3% of the total land area in the West Bank. The population of these governorates in 2018 was estimated to be about one million inhabitants, representing more than 35% of the Palestinian population in West Bank. The number of animals and mixed holdings in these West Bank-governorates was 10,563 representing about 41.1% of the total animals and mixed holdings in the West Bank during the agricultural year.

Fig. 1. Palestinian Governorates in the West Bank

Sample collection

Samples were collected randomly between January and November 2018, from layers and broilers farms in the selected study area in the northern part of the West Bank. Samples were obtained from the clo-

acae using sterile swabs. Then the swabs were placed individually in sterile tubes containing 10 ml of buffered peptone water (Oxoid, CM0509). The swabs were transported to the laboratory on the same day and incubated at 37 ºC for 24 hours.

A total of 608 and 572 samples were collected from 63 layer farms and 69 broiler farms respectively. 7-11 random samples were taken from each farm. The age of the chickens that were sampled was 25-35 weeks and 4-6 weeks for layers and broilers respectively. Sample collection was distributed to governorates as shown in table 2.

Table 2. Geographical distribution of layer and broiler farms and samples to governorates

Governorates	Number of farms		Number of sample (%)	
	Layers	Broilers	Layers	Broilers
Tulkarm	25	20	215 (35.4)	194 (33.9)
Nablus	18	16	193 (31.7)	165 (28.8)
Qalqylia	8	15	80 (13.2)	85 (14.9)
Jenin	12	18	120 (19.7)	128 (22.4)
Total	63	69	608 (100)	572 (100)

Isolation and identification of *Salmonella* spp.

For the isolation and detection of *Salmonella* spp., the method described in the Bacteriological Analytical Manual (BAM) (Food and Drug Administration 1995) and Jinu et al. (2014), was used with little modifications. Samples were cultivated in non-selective pre-enrichment media (Buffered Peptone Water (BPW) medium (Oxoid, CM0509)) and incubated at 37 ºC for 24 hours. 1 ml of the result sample was cultured in Tetrathionate broth (Müller-Kauffman) at 37 ºC for 24 hours. 100 µl was then streaked on *Salmonella Shigella* agar (SS agar, Oxoid, CM0533) and incubated at 37 ºC for 24 hours. 3-5 typical black colonies were selected from the SS agar, streaked into the surface of nutrient agar dishes and incubated at 37 ºC for 24 hours.

Biochemical confirmation

The suspected *Salmonella* isolates were subjected to biochemical testing for identification. The isolates were analyzed for the biochemical characteristics according to the standard protocol described in the laboratory manual for the isolation and identification of foodborne pathogens (Agarwal et al., 2003). The biochemical characterization included Triple Sugar Iron test (TSI), Urease test and Sulfide, Indole and Motility test (SIM). All the cultures that were negative for Lactose/Sucrose, positive for Glucose and produce H_2S, negative urease test, motile and indole negative, were kept for serological confirmation.

Serological confirmation

The serological confirmation used is genus-specific, to confirm the *Salmonella* isolates using specific antisera (Biorad). The agglutination test was carried out on a glass slide. One drop from specific antiserum was mixed with one drop from suspected *Salmonella* broth culture or 0.9% sterile saline suspected *Salmonella* suspension. Any agglutination after two minutes for both the somatic "O" and flagella "H" antisera was considered positive reaction for the tested *Salmonella* spp. The Live attenuated vaccine for *S.* Enteritidis (Biovac Company) was used as a quality control in all of the experiments of serological confirmation.

DNA extraction

Genome of *Salmonella* spp. was prepared for PCR as mentioned by De Medici et al. (2003) with little modifications. A loopful of bacterial cells was scraped off an overnight nutrient agar plate, washed with 0.5 ml sterile distilled H O, centrifuged at 10,000 X *g* for 5 min, the supernatant was discarded and the pellet was re-suspended in another 0.5 ml of sterile double distilled H O, and boiled for 10 min. The cells were incubated on ice for 8 min. The debris was pelleted by centrifugation at 10,000 X *g* for 5 min. The DNA concentration was determined using spectrophotometer. The DNA samples were stored at -20 ºC.

Determination of DNA concentration and purity

The extracted DNA was quantified by using the Nano drop measuring the absorbance at 260 and 280 nm (Abed Al-Daym, 2019). In addition, the integrity and purity of the extracted DNA was also evaluated by electrophoresis. 1% agarose gel containing ethidium bromide was used. The extracted DNA samples

from *Salmonella* isolates were loaded with a loading dye on the gel, electrophoresed for 60 minutes, viewed under the UV light and photographed using the Gel Documentation System (Biorad, CA, USA).

PCR test

PCR was carried out using two sets of primers, one as described previously (Fratamico, 2003), to confirm *Salmonella* isolates. Forward INVAF primer (5'-CGG TGG TTT TAA GCG TAC TCT T-3') and reverse INVAR primer (5'-CGA ATA TGC TCC ACA AGG TTA-3') were used to amplify a portion of the *invA* gene of *Salmonella* spp. Each PCR reaction mix (25 µl) was performed using 12.5 µl of PCR master mix (promega), 0.2 µM of each primer, and 100-125 ng of DNA template. The cycling conditions were: initial denaturation at 94 ºC for 2 min; followed by 35 cycles of denaturation at 94 ºC for 20 s, annealing at 57 ºC for 1 min and extension at 72 ºC for 1 min; followed by a single final extension step at 72 ºC for 10 min. The PCR products were then detected by agarose gel electrophoresis on 2% (w/v) agarose gel to determine the size of amplified fragments after staining with a final concentration 0.5 µg/ml of ethidium bromide dye. The size of the amplicon (796-bp) was determined by comparing them with a 100-bp DNA ladder.

The other set of primers was described previously (Wang and Yeh, 2002), to identify *S.* Enteritidis. The primer set IE1L forward primer (5'-AGTGC-CATACTTTTAATGAC-3') and IE1R reverse primer (5'-ACTATGTCGATACGGTGGG-3') were used to amplify the *IE* gene sequence of *S.* Enteritidis.

Each PCR reaction mix (40 µl) was performed using 30 µl PCR buffer containing 4 µl 10x PCR buffer, each dNTP at a concentration of 0.25 mmol, 50 pmol of each PCR primer, and 100-125 ng of the DNA template. Distilled water was added to a final volume of 40 µl. After heating at 95ºC for 10 min, 2.5 units Taq DNA polymerase in 10 µl 1x PCR buffer were added and the mixture subjected to PCR. A drop of mineral oil was added on the top of the reaction mixture to prevent solvent evaporation.

The cycling conditions were: initial denaturation at 94 ºC for 2 min; followed by 35 cycles of denaturation at 94 ºC for 40 sec, annealing at 55 ºC for 50 sec and extension at 72 ºC for 50 sec; followed by a single final extension step at 72 ºC for 2 min. The PCR products were then detected by agarose gel electrophoresis on 2% (w/v) agarose gel to determine the size of amplified fragments after staining with a final concentration 0.5 µg/ml of ethidium bromide dye. The size of the amplicon (316-bp) was determined by comparing them with a 100-bp DNA ladder.

Live attenuated vaccine for *S.* Enteritidis (Biovac Company) was used as a positive control, and *E. coli* ATCC25922 was used as negative control. DNA amplification was performed using thermal cycler (Mastercycler Personal, Eppendorf).

RESULTS

156 samples (13.2%) among the 1,180 tested samples (608 and 572 samples from layers and broilers respectively), showed black colonies on SS agar. Out of these samples, just 106 samples (67.9%) were biochemically confirmed to be *Salmonella* spp. After serological testing just 92 were confirmed as *Salmonella* spp. positive samples.

Using PCR assay, all the suspected isolates either from layer or broiler farms, which were confirmed with serology, were positive for *Salmonella* spp. (Figure 2). The prevalence of *Salmonella* isolates among the tested samples from layer and broiler farms were 10.7% (65/608) and 4.7% (27/572), respectively. All *Salmonella* isolates recovered from layer farms were negative for *S.* Enteritidis. On the other hand, the rate of *S.* Enteritidis among *Salmonella* isolates according to PCR assay (Figure 3), recovered from broiler farms was 14.8% (4/27). Data about the distribution of the *Salmonella* spp. and *S.* Enteritidis isolates from layer and broiler farms according to the governorates are presented in tables 3 and 4.

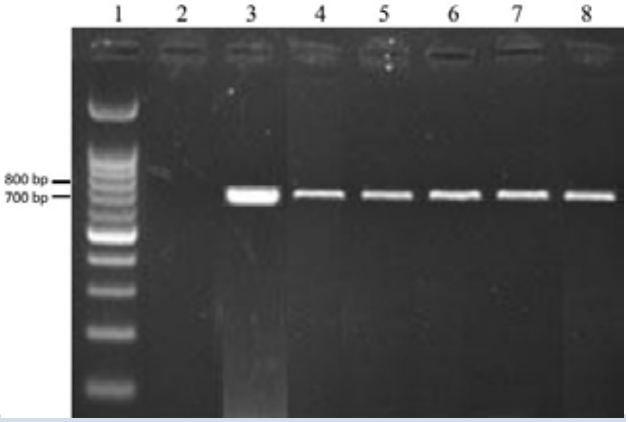

Fig. 2. Gel electrophoresis of PCR amplification product of *invA* gene of *Salmonella* spp.: lane 1: 100-bp ladder, lane 2: Negative control, lane 3: Positive control, lanes 4-8: Positive *Salmonella* isolates

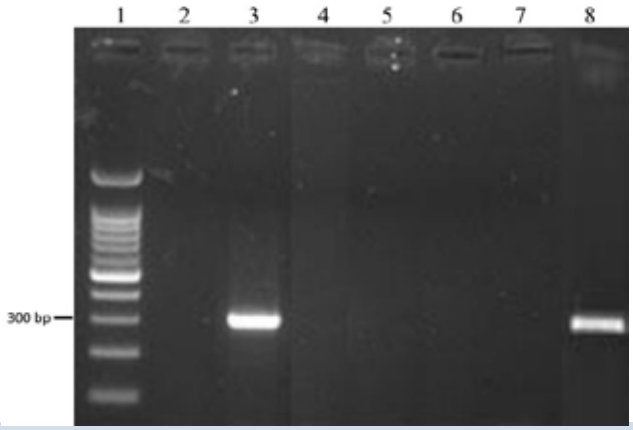

Fig. 3. Gel electrophoresis of the PCR amplification product of the *IE* gene sequence of *S.* Enteritidis: lane 1: 100-bp ladder, lane 2: Negative control, lane 3: Positive control. lanes 4-7: Negative *S.* Enteritidis samples, lane 8: Positive *S.* Enteritidis isolate

Table 3. Distribution of the positive samples of *Salmonella* spp. and ***S.*** Enteritidis among layer farms in northern part of the West Bank, Palestine

Governorate	Number of samples	Number of positive *Salmonella* samples (%)	Number of *S.* Enteritidis isolates among all *Salmonella* isolates (%)
Tulkarm	215	30 (14.0)	0.0 (0.0)
Nablus	193	13 (6.7)	0.0 (0.0)
Qalqylia	80	4 (5.0)	0.0 (0.0)
Jenin	120	18 (15.0)	0.0 (0.0)
Total	608	65 (10.7)	0.0 (0.0)

Table 4. Distribution of the positive samples of *Salmonella* and *S.* Enteritidis among broiler farms in northern part of the West Bank, Palestine

Governorate	Number of samples	Number of positive *Salmonella* samples	Number of *S.* Enteritidis isolates among all *Salmonella* isolates (%)
Tulkarm	194	12 (6.2)	2 (16.7)
Nablus	165	2 (1.2)	0.0 (0.0)
Qalqylia	85	5 (5.9)	1 (20)
Jenin	128	8 (6.2)	1 (12.5)
Total	572	27 (4.7)	4 (14.8)

DISCUSSION

Eggs, egg products and broiler meat, still remain an important source of human infection and accounted for more than 50% of strong-evidence salmonellosis foodborne outbreaks (EFSA 2019). Salmonellosis is of major concern in many countries worldwide for causing public health problems. In addition, it causes major economic losses despite improved hygienic processing of poultry and products (Suresh et al., 2011).

The results of the current study showed that, the rate of *Salmonella* spp. in the sample tested from layer and broiler farms was 10.7% (65/608) and 4.7% (27/572), respectively. A study conducted in Gaza strip, Palestine, showed that 13.3% of fresh chicken, 10% of fresh minced meat, 6.7% of frozen minced meat, 3.3% of fresh meat and 3.3% of frozen meat were contaminated with *Salmonella* (Elmanama et al., 2013). The frequency of isolated *Salmonella* spp. from feces of diarrheal cases in Gaza strip, Palestine was between 1.8 to 4.0% (Abu Elamreen et al., 2008; Al Jarousha et al., 2011). The prevalence of *Salmonella* in different poultry and meat food products in Hebron, the main district at the southern of Palestine, was 3.2%, 3.2%, and 4.9% in frozen chicken, schnitzel and turkey, respectively (Issa et al., 2017).

The occurrence rate of *Salmonella* spp. varies among different studies. In total, 0.37% and 7.15% of the tested table eggs and broiler meat, respectively, in the EU were *Salmonella* positive (EFSA 2019). A study conducted in Egypt to investigate the presence of *Salmonella* in eggs, showed that the rate of *Salmonella* was about 4.0% (3/75) (Awny et al., 2018). Comparison between conventional isolation methods of *Salmonella* in food, with the more sensitive and accurate molecular techniques (PCR) was done (Malkawi, 2003). The results showed that the rate of *Salmonella* was 81.0% (172/212) by the conventional method as compared to the more sensitive and specific PCR method of 87.0% (185/212). In the current study, conventional isolation and biochemical methods were complemented with serological and molecular methods to identify *Salmonella* in the samples tested. Further studies are required to get an accurate profile of *Salmonella* spp. infecting poultry. This will contribute in implementing guidelines to better handling and processing of poultry meat, eggs and their products to prevent or reduce salmonellosis.

From other methods used for DNA extraction, the boiling method was chosen as it is often sufficient to achieve good DNA extraction and PCR amplification results (De Medici et al., 2003; Hill et al., 2015). Selecting an appropriate method for DNA extraction takes into account certain factors, such as time, cost and toxicity of the chemicals employed (Chapela et al., 2007). The boiling method is one of the simplest protocols, and largely used for genome extraction from microorganisms. It is almost restricted to the lysis step, which is less laborious and inexpensive. It provides reasonable amounts of DNA that are usually enough to be used in PCR-based methodologies. In addition, the DNA extracted using this method gave better results for the PCR amplification used in this work (Wang and Yeh, 2002), and it showed better results than DNA extraction by the phenol-chloroform method. The major drawbacks are the low DNA yield and purity. However, most amplification techniques are robust enough to be unaffected by inhibitors, and require very low amounts of target DNA (Barbosa et al., 2016).

On the other hand, the prevalence rate of *S.* Enteritidis among other *Salmonella* spp. was 0.0% and 14.8% for layer and broiler farms, respectively. There is a variation in occurrence of *S.* Enteritidis among other *Salmonella* spp. in previously published reports. Jaradat et al. (2014), investigated the presence of *Salmonella* in seven outbreaks in Jordan. Confirmation of the results with PCR showed that, out of the 200 isolates presumptively identified with conventional methods, only 180 isolates were true *Salmonella* spp. The presence of *S.* Enteritidis among the *Salmonella* isolates was 25% (45/180). The occurrence of *S.* Enteritidis and *S.* Typhimurium in raw chicken meat in Egypt was 2.0% and 3.0%, respectively (Tarabees et al., 2017). *S.* Enteritidis has been the most frequently isolated and predominant serovar from the oviduct of layer chicken, and it has tropism to the ovary of the layer chicken poultry (Gantois et al., 2008). The enhanced ability of the *S.* Enteritidis among other *Salmonella* serovars to survive in and transmit through eggs has been contributed to special genes that are necessary for *S.* Enteritidis to persist in egg albumen and may be useful for generating safer live for it (Clavijo et al., 2006). Despite all this, *S.* Enteritidis was not recovered from samples taken from the layer farms in this study. A possible cause for this is that the frequency and duration of fecal shedding of *S.* Enteritidis may vary depending on many factors. Following experimental oral infection with large doses of *S.* Enteritidis, a small percentage of hens continued shedding the pathogen in their feces for weeks (Gast et al., 2017). Both the frequency and duration of fecal shedding by orally infected hens are directly related to the *S.* Enteritidis exposure dose (Gast et al., 2011). Commercial laying hens are likely exposed to relatively low doses of salmonellae from environmental sources or via horizontal contact transmission, generally resulting in infrequent infection and shedding (Esaki et al., 2013). In addition, the sustained participation of egg producers in comprehensive *S.* Enteritidis flock testing and risk reduction programs has recently been linked to decreased incidences of contamination and human illness in several nations (Gast et al., 2017). In fact, in Europe it is assumed that the observed reduction in salmonellosis cases (32% between 2008 and 2012) is mainly due to successful *Salmonella* control measures (involving surveillance, biosecurity and vaccination) implemented in poultry/egg production (Antunes et al., 2016), although the presence of *Salmonella* spp. or *S.* Enteritidis in layer farms or broiler farms, even at low incidence has important implications.

Although serotyping is important in characterization of *Salmonella* isolates and it is highly needed for surveillance, source tracking, and outbreak detection (Yoshida et al., 2016), this work aimed to the detection of *Salmonella* spp. and *S.* Enteritidis, which is

the most common cause of foodborne salmonellosis worldwide, with simple, inexpensive, and effective conventional, serological and molecular methods. In addition, there were many obstacles to perform serotyping, including the absence of a reference lab in the Palestinian territories, the difficulty of sending the samples to the nearest reference lab in the area and the very high cost that couldn't be afforded or covered by any governmental or non-governmental fund.

With all this taken into account, it is important that control programs for *Salmonella* spp. have to be implemented in all stages of the food production chain, starting from animal farms. It is recommended to establish a permanent program and continuous monitoring for surveillance of *Salmonella* in Palestine. Further studies should be conducted, including a high number of samples with serotyping and molecular characterization of the positive samples.

CONCLUSIONS

This study provides important epidemiological information on *Salmonella* contamination status in layer and broiler farms in the northern part of Palestine. The prevalence of *Salmonella* in layers and broilers in the northern part of Palestine is still relatively high. So the reported decrease in the cases of salmonellosis in humans cannot be attributed only to the measures and sanitary regulations taken by the Veterinary and Health Institutions, but it could be also attributed to an improvement in the consumer hygienic practices, mainly related to safe handling and heat treatment of food products that are the main cause of salmonellosis and to more attention by the physician and the lab work for isolation, identification and treatment.

The percentage of *S.* Enteritidis among the confirmed cases of *Salmonella* was very low. Particular emphasis on detection of other *Salmonella* serotypes, which might be involved in the infection, is recommended.

Conventional isolation and biochemical methods used for *Salmonella* isolation and identification together with a PCR-based method can eliminate any false positive cases that may arise using conventional methods.

CONFLICT OF INTEREST

None declared by the authors.

REFERENCES

Abed Al-Daym M (2019) Determination of the rate of *Salmonella enteritidis* in layer farms by polymerase chain reaction. MSC Thesis. Faculty of Pharmacy Nursing and Health Professions, Birzeit University, Palestine.

Abu Elamreen FH, Sharif FA, Elshikh Deeb J (2008) Isolation and antibiotic susceptibility of *Salmonella* and *Shigella* strains isolated from children in Gaza, Palestine from 1999 to 2006. Journal of Gastroenterology and Hepatology 23(8): e330-333.

Agarwal RK, Bhilegaonkar KN, Singh DK, Kumar A, Rathore RS (2003) Laboratory Manual for the Isolation and Identification of Foodborne Pathogens. Indian Veterinary Research Institute, Izatnagar, Bareilly, India: pp 35-37.

Al Jarousha AMK, El Jarou MA, El Qouqa IA (2011) Bacterial Enteropathogens and Risk Factors Associated with Childhood Diarrhea. Indian J Pediatr 78: 165-170.

Antunes P, Mourão J, Campos J, Peixe L (2016) Salmonellosis: the role of poultry meat. Clin Microbiol Infect 22: 110-121.

Awny C, Amr AA, Abo El-Makarem HS (2018) Microbial Hazards Associated with Consumption of Table Eggs. AJVS 58(1): 139-146.

Barbosa C, Nogueira S, Gadanho M, Chaves S (2016) DNA extraction: Finding the most suitable method. In Molecular microbial diagnostic methods: Pathways to implementation for the food and water industries. Elsevier Inc., USA: pp 135-154.

Boseret G, Losson B, Mainil JG, Thiry E, Saegerman C (2013) Zoonoses in pet birds: review and perspectives. Vet Res. 44: 36.

Chapela MJ, Sotelo CG, Pérez-Martín, RI, Pardo M, Pérez-Villareal B, Gilardi P, Riese J (2007). Comparison of DNA extraction methods from muscle of canned tuna for species identification. Food Control 18(10): 1211-1215.

Clavijo RI, Loui C, Andersen GL, Riley LW, Lu S (2006) Identification of Genes Associated with Survival of *Salmonella enterica* Serovar Enteritidis in Chicken Egg Albumen. Appl Environ Microbiol 72(2): 1055-1064.

De Medici D, Croci L, Delibato E, Di Pasquale S, Filetici E, Toti L (2003) Evaluation of DNA Extraction Methods for Use in Combination with SYBR Green I Real-Time PCR to Detect *Salmonella enterica* Serotype Enteritidis in Poultry. Appl Environ Microbiol 69(6): 3456-3461.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2019. The European Union One Health 2018 Zoonoses Report. EFSA Journal 2019 17(12): 5926, doi: 10.2903/j.efsa.2019.5926.

Ekateriniadou LV, Boukouvala E, Papi RM, Zdragas A, Giantzi V, Kyriakidis DA (2015) Antibiotic resistance and distribution of SodCI, sopE, sefA genes among *Salmonella* enteric serotype Enteritidis isolates from poultry. J HELLENIC VET MED SOC 66(2): 70-79.

Elmanama AA, El kahlout KAE, Elnakhalaa SR (2013) Occurrence of *Salmonella* in Fresh and Frozen Meat in the Gaza Strip. Journal of Purity, Utility Reaction and Environment 2(6): 142-152.

Eng Sk, Pusparajah P, Ab Mutalib NS, Ser HL, Chan KG, Lee LH (2015) *Salmonella*: A review on pathogenesis, epidemiology and antibiotic resistance. Frontiers in Life Science 8 (3): 284-293.

Esaki H, Shimura K, Yamazaki Y, Eguchi M, Nakamura M (2013) National surveillance of *Salmonella* Enteritidis in commercial eggs in Japan. Epidemiol Infect 141: 941-3.

Food and Drug Administration (1995) Bacteriological Analytical Manual, 8th edn. Arlington, VA, USA: Association of Analytical Chemists.

Fratamico PM (2003) Comparison of culture, polymerase chain reaction (PCR), TaqMan *Salmonella*, and Transia Card *Salmonella* assays for detection of *Salmonella* spp. in naturally-contaminated ground chicken, ground turkey, and ground beef. Molecular and Cellular Probes 17: 215-221.

Gantois I, Ducatelle R, Pasmans F, Haesebrouck F, Van Immerseel F (2008) *Salmonella enterica* Serovar Enteritidis Genes Induced during Oviduct Colonization and Egg Contamination in Laying Hens. Appl Environ Microbiol 74(21): 6616-6622.

Gantois, I, Ducatelle R, Pasmans F, Haesebrouck F, Gast R, Humphrey T, Van Immerseel F (2009) Mechanisms of egg contamination by *Salmonella* Enteritidis: Review article". FEMS Microbial Rev 33: 718-738.

Gast RK, Guraya R, Holt PS (2011) Frequency and persistence of fecal shedding following exposure of laying hens to different oral doses of *Salmonella* Enteritidis. Int J Poult Sci 10:750-756.

Gast R, Guraya R, Jones D, Anderson K, Karcher D (2017) Frequency and Duration of Fecal shedding of *Salmonella enteritidis* by experimentally infected laying hens housed in enriched colony cages at Different stocking Densities. Frontiers in Veterinary Science 4(47): 1-7.

Guard-Petter J (2001) The chicken, the egg and *Salmonella enteritidis*. Environmental Microbiology 3(7): 421-430.

Herrero-Fresno A, Olsen JE (2018) *Salmonella* Typhimurium metabolism affects virulence in the host - A mini-review. Food Microbiol 71: 98-110.

Hill VR, Narayanan J, Gallen RR, Ferdinand KL, Cromeans T, Vinjé J (2015) Development of a Nucleic Acid Extraction Procedure for Simultaneous Recovery of DNA and RNA from Diverse Microbes in Water. *Pathogens* 4: 335-354.

Issa Y, Abu-Rayyan A, Hemidat S (2017) Prevalence of *Salmonella* in different poultry and meat food products in Hebron district: a prevalence study. The Lancet 390: S33.

Jaradat ZW, Abedel Hafiz L, Ababneh MM, Ababneh QO, Al Mousa W, Al-Nabulsi A, Osaili TM, Holley R (2014) Comparative analysis of virulence and resistance profiles of *Salmonella* Enteritidis isolates from poultry meat and foodborne outbreaks in northern Jordan". Virulence 5(5): 601-610.

Jeníková G, Pazlarová J, Demnerová K (2000) Detection of *Salmonella* in food samples by the combination of immunomagnetic separation and PCR assay. Int Microbiol 3(4): 225-229.

Jinu M, Agarwal RK, Sailo B, Wani MA, Kumar A, Dhama K, Singh MK (2014) Comparison of PCR and Conventional Cultural Method for Detection of *Salmonella* from Poultry Blood and Faeces. Asian Journal of Animal and Veterinary Advances 9(11): 690-701.

Legesse D (2017) A Review on *Salmonella enteritidis*: Transovarial Transmission Mechanism in Laying Chickens. College of agriculture and Veterinary Medicine, Jimma University, Ethiopia. https://www.grin.com/document/383938.

Li X, Liu L, Li Q, Xu G, Zheng J (2017) *Salmonella Enteritidis* in Layer Farms of Different Sizes Located in Northern China: On-Farm Sampling and Detection by the PCR Method. Brazilian Journal of Poultry Science 19(3): 377-386.

Malkawi HI (2003) Molecular identification of *Salmonella* isolates from poultry and meat products in Irbid City, Jordan. World Journal of Microbiology and Biotechnology 19: 455- 459.

Mengistu H, Rathore R, Dhama k, Agarwal R (2011) Isolation, Identification and Polymerase Chain Reaction (PCR) Detection of *Salmonella* Species from Field Materials of Poultry Origin. International Journal of Microbiological Research 2(2): 135-142.

Messens W, Grijspeerdt K, Herman L (2006) Eggshell penetration of hen's eggs by *Salmonella enterica* serovar Enteritidis upon various storage conditions. British Poultry Science 47(5): 554-560.

Mohammadzadeh A, Ashrafi Tamail I, Koochakzadeh A, Ghoddusi A, Mahmoodi P (2018) Detection and Molecular Identification of *Salmonella* Strains Isolated from an Industrial Ostriches farm. J HELLENIC VET MED SOC 68(4): 507-512.

Much P, Pichler J, Kasper S, Lassnig H, Kornschober C, Buchner A (2009) A foodborne outbreak of *Salmonella* Enteritidis phage type 6 in Austria, 2008. Wiener Klinische Wochenschrift 121(3-4): 132-136.

Nouichi S, Ouatouat R, Can H, Mezali L, Belkader C, Ouar-Korichi M, Bertrand S, Cantekin Z, Hamdi T (2018) Prevalence and antimicrobial resistance of *Salmonella* isolated from bovine and ovine samples in slaughterhouses of Algiers, Algeria. J HELLENIC VET MED SOC 69(1): 863-872.

PCBS (Palestinian Central Bureau of Statistics), 2017 Estimated Population in Palestine Mid-Year by Governorate, 1997-2021: http://www.pcbs.

gov.ps/Portals/_Rainbow/Documents/% D8%A7%D9%84%D9%85 %D8%AD%D8%A7%D9% 81%D8%B8%D8%A7%D8%AA%20 %D8%A7%D9%86%D8%AC% D9%84%D9%8A%D8%B2%D9% 8A%2097-2017.html.

Poppe, C, Johnson R, Forsberg C, Irwin R (1992) *Salmonella enteritidis* and other *Salmonella* in Laying Hens and Eggs from Flocks with *Salmonella* in their Environment. Can J Vet Res 56: 226-232.

Radkowski M (2002) Effect of moisture and temperature on survival of *Salmonella enteritidis* on shell eggs. Arch. Geflugelk 66 (3): 119-123.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM (2011) Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis 17(1): 7-15.

Schoeni JL, Glass KA, McDermott JL, Wong AC (1995) Growth and penetration of *Salmonella enteritidis*, *Salmonella heidelberg* and *Salmonella typhimurium* in eggs. Int J Food Microbiol 24(3): 385-96.

Seifi S, Khoshbakht R, Azizpour A (2019) Occurrence of *Campylobacter*, *Salmonella*, and *Arcobacter* in pet birds of northern Iran. J HELLENIC VET MED SOC 70(4): 1771-1776.

Suresh T, Hatha AAM, Harsha HT, Lakshmanaperumalsamy P (2011) Prevalence and distribution of *Salmonella* serotypes in marketed broiler chickens and processing environment in Coimbatore City of southern India. Food Research International 44(3): 823-825.

Tarabees R, Elsayed MSA, Shawish R, Basiouni S, Shehata AA (2017) Isolation and characterization of *Salmonella enteritidis* and *Salmonella typhimurium* from chicken meat in Egypt. J. Infect Dev Ctries 11(4): 314-319.

Van de Giessen AW, Bouwknegt M, Dam-Deisz WD, van Pelt W, Wannet WJ, Visser G (2006) Surveillance of *Salmonella* spp. and *Campylobacter* spp. in poultry production flocks in The Netherlands. Epidemiol Infect 134: 1266-75

Wang SJ, Yeh DB (2002) Designing of polymerase chain reaction primers for the detection of *Salmonella enteritidis* in foods and faecal samples. Letters in Applied Microbiology 34: 422-427.

Yoshida C, Gurnik S, Ahmad A, Blimkie T, Murphy TA, Kropinski AM, Nash JHE (2016) Evaluation of Molecular Methods for Identification of Salmonella Serovars. J Clin Microbiol 54(8): 1992-1998.

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Research article
Ερευνητικό άρθρο

Investigation of omentin-1 and metabolic parameters in periparturient cows

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ABSTRACT: We aimed to determine the level of omentin-1 hormone and other in periparturient period of dairy cows. It was also aimed to determine whether there is a correlation between omentin-1, glucose, Non-esterified fatty acids (NEFA), triglycerides (TG) and β-hydroxybutyrate (BHB). Blood samples were taken a month before parturition (PreP30), two weeks before parturition (PreP14), at parturition (P), two weeks after parturition (PostP14) and a month after parturition (PostP30). Concentrations of serum omentin-1 at P, serum glucose at P, PostP14, and PostP30, serum NEFA at P, serum TG PreP30 and PreP14, and serum BHB at P were statistically significantly higher than at other times. A positive correlation was observed between omentin-1 and glucose, NEFA and BHB, glucose and NEFA and BHB, and NEFA and BHB. A negative correlation was found between TG and omentin-1, glucose, NEFA and BHB. In conclusion, findings of the present study shows that omentin-1 may play an important role in the periparturient period. A positive correlation of omentin-1 with energy fuels NEFA, glucose, and BHB suggests that omentin-1 plays a role in energy metabolism like other adipokines. The fact that omentin-1 levels increase during delivery, when the fetus needs energy most, supports this hypothesis.

Keywords: Periparturient period, Omentin-1, NEFA, BHB

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INTRODUCTION

The "periparturient period" or "transition period" in dairy cows is defined in three phases: before, at, and post parturition (Ocal et al., 2015) including 2-4 weeks before parturition and the 3-4 weeks after parturition. The period before parturition is called the dry period and the period after parturition is called the lactation period (Moore et al., 2015). The periparturient period in cows is a critical time in which many metabolic and infectious diseases occur, the immune system is influenced, and physiological disturbances appear (Mendonça et al., 2014). Thereis an important increase in the need for nutrients in cows in the last phase of pregnancy and at the start of lactation after parturition. In the periparturient period, a negative energy balance occurs due to insufficient feed consumption as a result of the increasing nutritional needs of the offspring before parturition and the onset of lactation (Raboisson et al., 2014; Jaakson et al., 2018; Suzuki et al., 2018). Adipokines released by the adipose tissue include many substances such as omentin, leptin, adiponectin, resistin, tumor necrosis factor-α, interleukin-6, visfatin, and retinol-binding protein (Tan et al., 2015). Omentin-1 was first defined in 2001 as intelectin-1 and then named "omentin" in 2004 since it is expressed at a high level in the omentum (Sittichoroon et al., 2014). It was also defined as intestinal lactoferrin receptor and intelectin-1 in intestinal paneth cells in 2005, and named endothelial lectinas a result of being located in endothelial cells as well (Tan et al., 2015). Protein sequence analyses demonstrated that omentin-1 mRNA codes a protein of 313 amino acids with a peptide amino terminal that is highly hydrophobic. The portion that has 296 amino acid secretion functions is obtained following this edge as a result of the division between the 17th and 18th amino acids (Boron et al., 2015; Tan et al., 2015; Elsaid et al., 2018). The omentin-1 hormone, which was first found expressed in heart, lungs, ovary, small bowel, as well as placenta, muscle and kidneys at a lower rate, was later determined to be expressed in fat tissue too (Yang et al., 2006; Ohashi et al., 2014; Elsaid et al., 2018). Omentin has two homolog isoforms, omentin-1 and omentin-2. Omentin-1 is the most encountered isoform in the circulatory system (Pan et al., 2010; Kafalidis et al.,2013; Antonio de Luis et al., 2018). The amino acid sequences of omentin-1 and omentin-2 are 83% similar (Tan et al.,2015). Omentin-1 is an adipokine at a density of 33-40 kDa with 8 exon and 7 intron regions (Tohidi et al., 2012; Shen et al., 2016; Antonio de Luis et al., 2018). Omentin-1, with the UniProt code of Q8WWAQ and GenBank expression number of AY549722, has been studied more than omentin-2 (Tan et al., 2015). Omentin-1 is an anti-inflammatory adipokine. Since it increases the insulin transduction signal, omentin-1 plays an important role in increasing insulin sensitivity and glucose metabolism in local omental adipose tissue and modulating paracrine and endocrine factors (Boron et al.,2015; Elsaid et al., 2018). Glucose, a substantial energy source for animals, meets the energy needs of the organism and is used in the synthesis of milk compounds. Lactose synthesis is increased with parturition, thus more glucose is required by the body (Larsen and Kristensen, 2013). Non-esterified fatty acids (NEFA) are the main component of triglycerides (TG) and are used as an energy source by many tissues (LeBlanc et al., 2005). Because of the formation of a negative energy balance, fat reserves pass into the blood as NEFA in dairy cows during early lactation and contribute to the energy requirements. NEFA are used or stored as very low-density lipoproteins in the liver, or they are esterified to TG. Metabolic problems arise in dairy cows in cases where NEFA and β-hydroxybutyrate (BHB) increase to extreme levels. In the transition period, measurement of NEFA and BHB can be used to determine a negative energy balance or ketosis index (Barletta et al., 2017). In light of this knowledge we aimed to determine the level of omentin-1 hormone and other blood metabolites in periparturient period of dairy cows. It was also aimed to determine whether there is a correlation between omentin-1, glucose, NEFA, TG and BHB.

MATERIALS AND METHODS

Experimental animals and animal management

This study was conducted at Kafkas University, Faculty of Veterinary Medicine, Education and Research Farm. Ethical approval was obtained (KAÜ-HADYEK/2017-078) before starting the study. A total of 15 multiparous Brown Swiss and Simmental dairy cows, average 2-4 years of age, were usedin the study. These 15 animals were moved to a closed area of concrete floor space 20 days before starting the study. The animals were fed with pelled dairy feed (feedingredient: raw protein 18%, raw cellulose 14%, raw oil 2,5%, raw ash 9%; feed raw materials: corn, barley, wheat, cottonseed meal, soy meal, sunflower seed meal, wheat bran, vetch, molasses, calcium carbonate, sodium chloride). Feed and water were provided *ad libitum* toanimals regularly every morning starting at 7 am. To find the periparturient period of the 15 cows,

the study was conducted between September 2017 and February 2018. The animals started parturition between November and December. After parturition cows were milked and the calves fed this milk.

Blood sampling

Blood samples (a total of 75 samples at 5 timepoints) were collected before feeding between 6-7 am every morning from the tail vein into non-anticoagulant tubes a month before parturition (PreP30), two weeks before parturition (PreP14), at parturition (P), two weeks after parturition (PostP14), and a month after parturition (PostP30). Serum samples obtained by centrifuging the blood at 3000 rpm at 4°C for 15 minutes were stored at -20°C with aprotinin (SIGMA, A1153) added. When all measurements were complete, ELISA and other colorimetric analyses were performed in duplicateusing the BIO-TEK EPOCH, GEN 5 software.

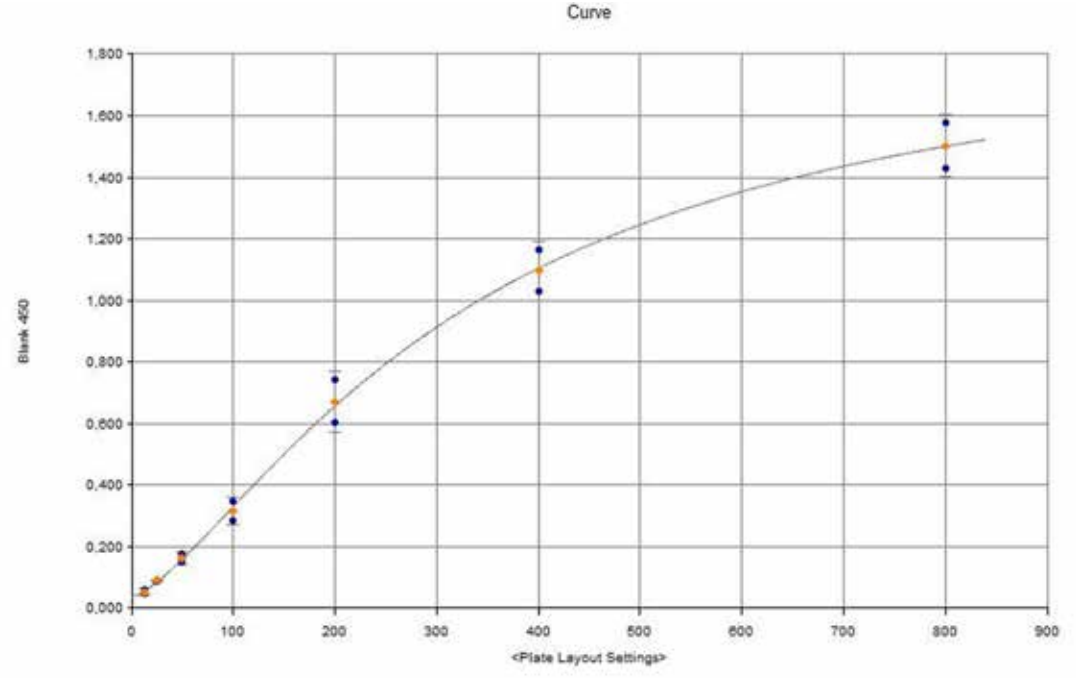
Biochemical analysis

Serum omentin-1 levels were measured using the commercial ELISA kits (SunRed, China, specific for Bovine, Catalogue No.: 201-04-0280). To determine the omentin-1 levels in the samples, double-antibody sandwich enzyme-linked immunosorbent analysis was used. The serum standard of omentin-1 is showed in graphic 1. The sensitivity of the kit was 2.358 ng/ml, and the assay range was 2.5-750 ng/ml. The intra-assay coefficient of variation was <10%, and the inter-assay coefficient of variation was <12%.

Serum glucose and TG levels were determined witha BIOLABO colorimetric measurement kit (BIOLABO, France).

Serum NEFA levels were measured with an EFFA-100 colorimetric EnzyChrom™ Free Fatty Acid Assay Kit (BioAssay Systems, Hayward, CA, USA).

Serum BHB levels were established with a colorimetric enzymatic measurement kit (Cayman Chemical, Ann Arbor, MI, USA).



Graph 1. Standard graph for omentin. The standard graph was generated based on the formula Y=(A-D)/(1+(X/C)^B)+D. The findings were as follows: (A:0.038, B:1.41, C:331, D:1.92) and R^2:1

Statistical analyses

In this study of cows in their periparturient period, the SPSS Windows 18.0 packaged software was used for the statistical analyses of data obtained from the studied parameters. Repeated measures were used in determining the differences between the PreP30, PreP14, P, PostP14, and PostP30 periods for omentin-1, glucose, NEFA, TG, and BHB. For identifying the relationship between parameters, a first normality test was executed, followed by Pearson's correlation analysis. Results are providedas mean±standard error (X±SEM). $P<0.05$ was accepted as statistically significant.

RESULTS

In this study of cows in their periparturient period, the serum omentin-1 level was higher (P<0.001) in P than in PreP30, PostP14, and PostP30 periods (Figure 1). The serum glucose level was higher (P<0.001) in PreP30 than P and PostP14 periods and in PreP14 thanP, PostP14, and PostP30 periods (Figure 2). The serum NEFA level was higher (P<0.001) in P than in PreP14 and PostP30 periods (Figure 3). The serum TG level was higher (P<0.001) in PreP30 thanP, PostP14, and PostP30 periods and in PreP14 than P and PostP14 periods (Figure 4). The serum BHB level was higher (P<0.001) in P than PreP30 and PostP30 periods (Figure 5).

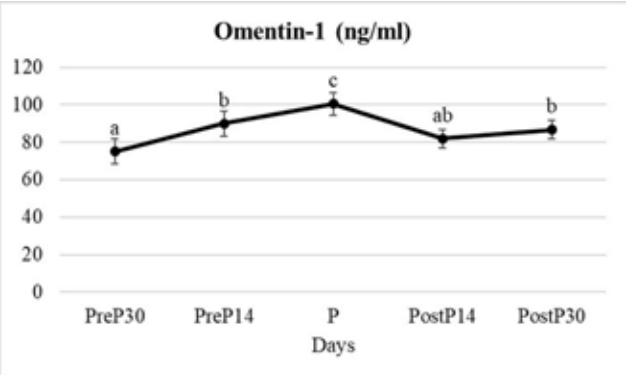


Figure 1. The change in omentin-1 concentration according to periparturient sampling periods (abc: present statistical differences between groups)

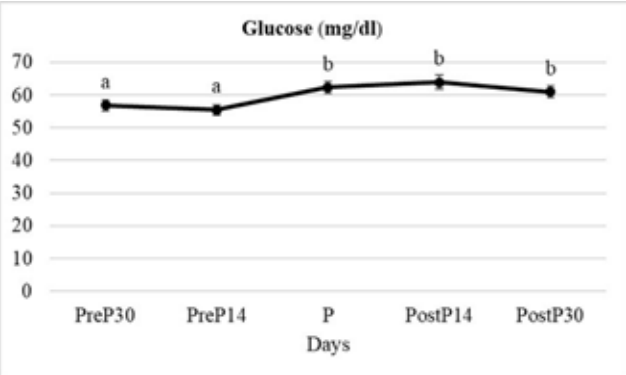


Figure 2. The change in glucose concentration according to periparturient sampling periods(abc: present statistical differences between groups)

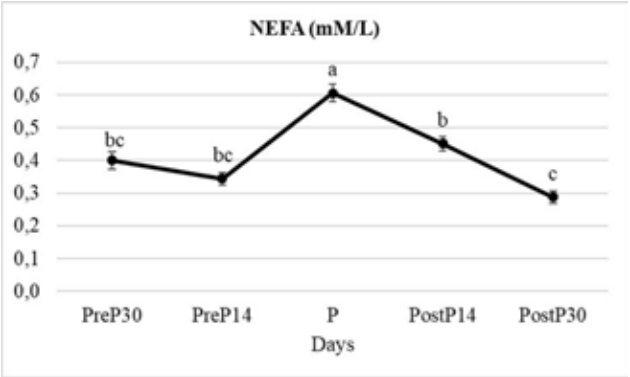


Figure 3. The change in NEFA concentration according to periparturient sampling periods (abc: present statistical differences between groups)

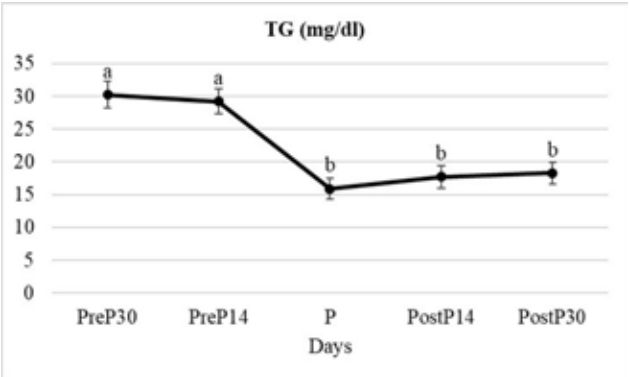


Figure 4. The change in TG concentration according to periparturient sampling periods (abc: present statistical differences between groups)

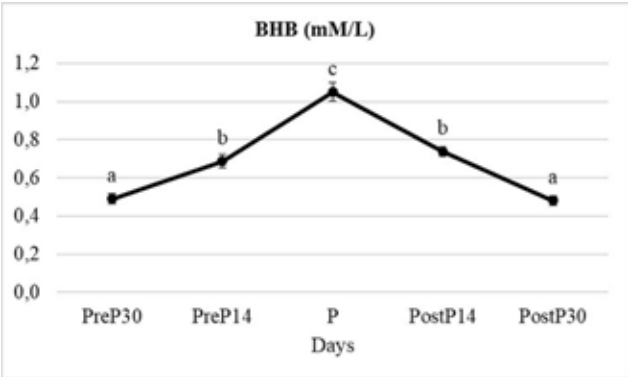


Figure 5. The change in BHB concentration according to periparturient sampling periods (abc: present statistical differences between groups)

In the correlation tests, a positive correlation was found between serum omentin-1 and serum glucose (r^2=0.768), serum NEFA (r^2=0.155), and serum BHB (r^2=0.431). A negative correlation was found between serum omentin-1 and serum TG (r^2=-0.253). A positive correlation was found between serum glucose,

serum NEFA (r^2=0.149), and serum BHB (r^2=0.217). A negative correlation was found between serum glucose and serum TG (r^2=-0.391) and a negative correlation was found between serum NEFA and serum TG (r^2=-0.203). A positive correlation was found between serum NEFA and serum BHB (r^2=0.722). Additionally, the serum TG level was negatively correlated with serum BHB (r^2=-0.260) (Table 1).

Table 1. The correlation of omentin-1, glucose, NEFA, TG and BHB concentrations according to periparturient sampling periods

		Omentin-1	Glucose	NEFA	TG
Glucose	r	0.768			
	P	0.01			
NEFA	r	0.155	0.149		
	P	NS	NS		
TG	r	-0.253	-0.391	-0.203	
	P	0.05	0.01	NS	
BHB	r	0.431	0.217	0.722	-0.260
	P	0.01	NS	0.01	NS

DISCUSSION

In cows, the periparturient period is associated with many metabolic failures related to energy metabolism. In the present study, it has been revealed that omentin-1 was studied for the first time in the periparturient period of cows based on the literature research. Therefore, we had to discuss our findings on omentin-1 with other species.It was reported that, serum omentin-1 levels were found to be lower in rats on the 21st day of pregnancy compared to levels in non-pregnant rats. In the same study, omentin-1 levels, measured in women during the three trimesters (early, mid, and late pregnancy), were observed to be significantly lower in mid and late pregnancy compared to the levels in non-pregnant women. Similarly, in pregnant rats and women, omentin-1 levels were low by the end of pregnancy (Garces et al., 2015). On the contrary, in the present study conducted on cows, omentin-1 levels started to increase in the serum on the 14th day before parturition and the level was found to be significantly high during parturition (Figure 1), decreasing again by the 14th day after parturition. In another study, omentin-1 was determined to be synthesized in fetal or maternal tissues and the concentration was discovered to have a positive correlation between these two tissues. Thus, the adipocytokines are considered to be carried by way of transplacental passage. Additionally, omentin-1 concentrations were found to be significantly high in umbilical serum samples. As a result ofhigh concentrations found in the fetus, omentin-1 is claimed to encourage fetal growth (Briana et al., 2011). Omentin-1 should be expected to increase in pregnancy since it is particularly synthesized in fetal and maternal tissues. Moreover, an increase in omentin-1 synthesis may be expected with the growing fetus as a result ofits increasing glucose needs, which is closely related to energy metabolism. Therefore, an increased amount of omentin-1 in the serum near the end of parturition may be expected.

While serum glucose level was significantly low at the prenatal 30th and 14th days in the present study,it significantly increased at parturition and on the postnatal 14th and 30th days (Figure 2). These findings display similarities with the control group that did not receive any treatment in the study of Markantonatos and Varga (2017), conducted on cows during their transition periods. However, studies determining plasma glucose concentrations in cows in their transition periods are not completely consistent. Some studies state that glucose concentrations do not change in the transition period (Asl et al., 2011; Weber et al., 2016; Jaakson et al., 2018), while others studies indicate that glucose concentrations are low before parturition and high after parturition (Markantonatos and Varga, 2017), and otherstate glucose concentrations are high before parturition and low after parturition (De Koster et al., 2015; Bicalho et al., 2017; Zarrin et al., 2017; Salin et al., 2017). Increasing glucose demands during lactation for lactose synthesis and consequently increasing milk synthesis are claimed to cause plasma glucose concentrations to decrease in cows in early lactation (Zarrin et al., 2017). Nonetheless, blood glucose levels are thought to increase as a result of the glucose required to be synthesized from glucose precursors together with milk synthesis. Zachut et al. (2013) reported prepartum insulin levels to be significantly higher than postpartum levels which could explain the reason why blood glucose levels would be higher in the postpartum period. Zachut et al. (2013) examined mRNA levels of gluconeogenesis enzymes in the liver and ascertained them to be higher in the postpartum period compared to the prepartum period. Higher gluconeogenesis and lower insulin levels in the postpartum period can explain the glucose increase in postpartum periods compared to prepartum periods in the present study. Omentin-1 increases glucose uptake and decreases insulin resistance. High glucose levels and high omentin-1 levels are claimed to be related in gestational diabetes. Additionally, the

upregulation of omentin-1 expression in gestational diabetes is thought to form a new mechanism of placental glucose homeostasis in pregnancy (Mast et al., 2012). A negative correlation has been found between plasma glucose levels and omentin-1, indicating that omentin-1 insufficiency may contribute to the development of insulin resistance and type 2 diabetes mellitus (Pan et al., 2010). Conversely, a significantly positive correlation ($P<0.01$) in the present study was found between serum glucose and omentin-1 levels (Table 1). Since the energy metabolism of ruminants and monogastrics are different, it is believed that further studies are necessary to exam the relationship between omentin-1 and glucose in the serum of cows. In the present study, while the serum TG concentration was high before parturition, it decreased at the moment of parturition similar to the findings of Schuermann et al. (2019). In our study, it remained low until the 30th day (Figure 4). Similarly, Schuermann et al. (2019) found it to remain low until the pre-breeding period (4th week after parturition). In human studies, generally, a negative correlation is found between serum omentin-1 levels and serum TG levels similar to our findings(Yan et al., 2011; Garces et al., 2015; Korany et al., 2018).

In cows, ketone bodies and especially BHB are known to cause subclinical and clinical ketosis, suppress feed intake, and havea negative impact on reproduction (Duffield et al., 2009; Ospina et al., 2010; Zarrin et al., 2013; Raboisson et al., 2014; Zarrin et al., 2017). While NEFA and BHB levels were low before parturition in our study similar to other studies (Weber et al., 2015; McCarthy et al., 2015), they reached their highest levels at parturition (Figs. 3 and 5). Nevertheless, different results have been obtained in different studies, such as similar BHB concentrations in the two weeks before parturition and two weeks after parturition (Duffield et al., 2009; Ospina et al., 2010; Zarrin et al., 2013; Raboisson et al., 2014; Zarrin et al., 2017), BHB values peaking between the first and third weeks after parturition (Zhang et al., 2016), high NEFA amounts in cows before parturition (De Koster et al., 2015), and serum NEFA amounts decreasing after parturition (Hausmann et al., 2017). McCarthy et al. (2015) found a negative correlation between NEFA and BHB levels in cows studied during the transition period. They attributed this result to the carbon source necessary for BHB synthesis provided from other compounds such as lactate and ketogenic amino acids, instead of NEFA. However, a significant positive correlation ($P<0.01$) between serum NEFA and BHB was found in our study (Table 1). NEFA molecules transform into BHB through Acetyl-coA as their levels increase in blood and they are degraded to be used as energy. Because of this mechanism, a positive correlation between them is natural.In the present study, astatistically significant relationship between-NEFA levels andTG, glucose, and omentin-1 levels could not be determined. While asignificant relationship between BHB levels and TG and glucose did not exist, a significant positive correlation ($P<0.01$) was found between omentin-1 and BHB levels (Table 1).

CONCLUSION

In conclusion, findings of the present study shows that omentin-1 may play an important role in the periparturient period. A positive correlation of omentin-1 with energy fuels NEFA, glucose, and BHB suggests that omentin-1 plays a role in energy metabolism like other adipokies. The fact that omentin-1 levels increase during delivery, when the fetus needs energy most, supports this hypothesis.

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CONFLICT OF INTEREST

All authors declare that there is no potential conflict of interest.

REFERENCES

Antonio de Luis D, Izaola O, Primo D,Aller R(2018) Modifications of serum levels of omentin-1 and other cardiovascular risk factors following weight loss secondary to a Mediterranean hypocaloric diet. Clinical Nutrition37: 2280-2283.

Asl AN, Nazifi S, Ghasrodashti AR, Olyaee A(2011)Prevalence of subclinical ketosis in dairy cattle in the Southwestern Iran and detection of cutoff point for NEFA and glucose concentrations for diagnosis of subclinical ketosis.Preventive Veterinary Medicine100: 38-43.

Barletta RV, MaturanaFilho M, Carvalho PD, Del Valle TA, Netto AS, Rennó FP, Mingoti RD, Gandra JR, Mourão GB, Fricke PM, Sartori R, Madureira EH, Wiltbank MC(2017) Association of changes among body condition score during the transition period with NEFA and BHBA concentrations, milk production, fertility, and health of Holstein cows. Theriogenology 104: 30-36.

Bicalho ML, Marques EC, Gilbert RO, Bicalho RC(2017)The association of plasma glucose, BHBA, and NEFA with postpartum uterine diseases, fertility, and milk production of Holstein dairy cows. Theriogenology 88: 270-282.

Boron D,CzernyB, Bartkowiak-WieczorekJ, Sieron D, Wolski H(2015) Omentin polymorphism and its relations to bone mineral density in women. Archives of Medical Research 46: 173-180.

Briana DD, Boutsikou M, Baka S, Gourgiotis D, Marmarinos A, Liosi S, Hassiakos D, Malamitsi-Puchner A(2011) Omentin-1 and vaspin are present in the fetus and neonate, and perinatal concentrations are similar in normal and growth-restricted pregnancies. Metabolism-Clinical and Experimental 60: 486-490.

De Koster J, Hostens M, Van Eetvelde M, Hermans K, Moerman S, Bogaert H, Depreester E, van den Broeck W, Opsomer G(2015) Insulin response of the glucose and fatty acid metabolism in dry dairy cows across a range of body condition scores. Journal of Dairy Science98: 4580-4592.

Duffield TF, Lissemore KD, McBride BW,Leslie KE(2009) Impact of hyperketonemia in early lactation dairy cows on health and production. Journal of Dairy Science92: 571-580.

Elsaid NH, Sadik NA, Ahmed NR, Fayez SE, El-Ghaffar Mohammed NA(2018) Serum omentin-1 levels in type 2 diabetic obese women in relation to glycemic control, insulin resistance and metabolic parameters. Journal of Clinical and Translational Endocrinology 13: 14-19.

Garces MF, Ruiz-Linares CE, Vallejo SA, Peralta JJ, Sanchez E, Ortiz-Rovira A, Curtidor Y, Parra MO, Leal LG, Alzate JP, Acosta BJ, Dieguez C, Nogueiras R, Caminos JE(2015) Maternal serum omentin-1 profile is similar in humans and in the rat animal model. Cytokine 75: 136-141.

Hausmann J, Deiner C, Immig I, Pieper R, Starke A, Aschenbach JR(2017) Effects of combined supplementation with plant bioactive lipid compounds and biotin on ruminal fermentation, body condition and energy metabolism in transition dairy cows. Animal Feed Science and Technology 225: 27-37.

Jaakson H, Karis P, Ling K, Ilves-Luht A, Samarütel J, Henno M, Joudu I, Waldmann A, Reimann E, Parn P, Bruckmaier RM, Gross JJ, Kaart T, Kass M, Ots M(2018) Adipose tissue insulin receptor and glucose transporter 4 expression, and blood glucose and insulin responses during glucose tolerance tests in transition Holstein cows with different body condition. Journal of Dairy Science 101: 752-766.

Kafalidis G,Boutsikou T, Briana DD, Boutsikou M, Marmarinos A, BakaS, Hassiakos D, Gourgiotis D, Malamitsi-Puchner A(2013)Adipokinesvaspin and omentin-1 are up-regulated in large for gestational age infants at term. Cytokine 62: 70-74.

Korany MA, Sonbol A, Elgouhary SM(2018) Omentin-1 and diabetic retinopathy in type 2 diabetic patients. Alexandria Journal of Medicine 54: 323-326.

Larsen M,Kristensen NB(2013) Precursors for liver gluconeogenesis in periparturient dairy cows. Animal 7: 1640-1650.

LeBlanc SJ, Leslie KE, Duffield TF(2005)Metabolic predictors of displaced abomasum in dairy cattle. Journal of Dairy Science88: 159-170.

Markantonatos X, Varga GA(2017) Effects of monensin on glucose metabolism in transition dairy cows. Journal of Dairy Science 100: 9020-9035.

Mast S, Pearce C, Epstein R, Playforth K, Lain K, Pearson K(2012)Omentin as a marker of abnormal glucose metabolism in pregnancy. American Journal of Obstetrics and Gynecology 1: 111.

McCarthy MM, Mann S, Nydam DV, Overton TR, McArt JA(2015)Short communication: Concentrations of nonesterified fatty acids and β-hydroxybutyrate in dairy cows are not well correlated during the transition period. Journal of Dairy Science 98:6284-6290.

Mendonça LG,Abade CC, da Silva EM, Litherland NB, Hansen LB, Hansen WP, Chebel, RC(2014)Comparison of peripartum metabolic status and postpartum health of Holstein and Montbéliarde-sired crossbred dairy cows. Journal of Dairy Science 97: 805-818.

Moore SA,Laportaj J, Crenshow TD, Hernandez LL(2015) Patterns of circulating serotonin and related metabolites in multiparous dairy cows in the peripartum period. Journal of Dairy Science 98: 3754-3765.

Ocal H,Rigvanlı A, Kalkan C, Doğan H(2015) Care of mother and calf in peripartum period in dairy cows. Turkey clinics Journal Veterinary Science Obstetrics and Gynecology-Special Topics 1: pp. 42-60.

Ohashi K, Shibata R, Murohara T, Ouchi N(2014) Role of anti-inflammatory adipokines in obesity-related diseases.Trends in Endocrinology and Metabolism25: 348-355.

Ospina PA, Nydam DV, Stokol T, Overton TR(2010) Evaluation of nonesterified fatty acids and β-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. Journal of Dairy Science 93: 546-554.

Pan HY,Guo L, Li Q(2010) Changes of serum omentin-1 levels in normal subjects and in patients with impaired glucose regulation and with newly diagnosed and untreated type 2 diabetes. Diabetes Research and Clinical Practice 88: 29-33.

Raboisson D,Mounie M, Maigne E(2014) Diseases, reproductive performance, and changes in milk production associated with subclinical ketosis in dairy cows: A meta-analysis and review. Journal of Dairy Science 97: 7547-7563.

Salin S,Vanhatalo A, Elo K, Taponen J, Boston RC, Kokkonen T(2017) Effects of dietary energy allowance and decline in dry matter intake during the dry period on responses to glucose and insulin in transition dairy cows. Journal of Dairy Science 100: 5266-5280.

Schuermann Y, Welsford GE, Nitschmann E, Wykes L, Duggavathi R(2019) Association between pre-breeding metabolic profiles and reproductive performance in heifers and lactating dairy cows. Theriogenology 131: 79-88.

Shen XD, Zhang L, Che H, Zhang YY, Yang C, Zhou J, Liang CZ(2016) Circulating levels of adipocytokine omentin-1 in patients with renal cell cancer. Cytokine 77: 50-55.

Sitticharoon C, Nway NC, Chatree S, Churintaraphan M, Boonpuan P, Maikaew P(2014)Interactions between adiponectin, visfatin, and omentin in subcutaneous and visceral adipose tissues and serum, and correlations with clinical and peripheral metabolic factors. Peptides62: 164-175.

Suzuki B, Sugiyama T, Yoshida C, Nakao T(2018) Short communication: Temporal changes in the skin morphology of dairy cows during the periparturient period. Journal of Dairy Science 101: 6616-6621.

Tan YL, Zheng XL, Tang CK(2015)The protective functions of omentin in cardiovascular diseases.ClinicaChimicaActa448: 98-106.

Tohidi M, Akbarzadeh S, Larijani B, Kalantarhormozi M, Ostovar A, Assadi M, Vahdat K, Farrokhnia M, Sanjdideh Z, Amirinejad R, Nabipour I (2012)Omentin-1, visfatin and adiponectin levels in relation to bone mineral density in Iranian postmenopausal women. Bone51: 876-881.

Yan P, Liu D, Long M, Ren Y, Pang J,Li R(2011) Changes of Serum Omentin Levels and Relationship between Omentin and Adiponectin Concentrations in Type 2 Diabetes Mellitus. Experimental and Clinical Endocrinology and Diabetes119: 257-263.

Yang RZ, Lee MJ, Hu H, Pray J, Wu HB, Hansen BC, Shuldiner AR, FriedSK, McLenithan JC, Gong DW(2006)Identification of omentin as a novel depot-specific adipokine in human adipose tissue: possible role in modulating insulin action. American Journal of Physiology-Endocrinology and Metabolism 290: 1253-1261.

Zachut M, Honig H, StriemS, Zick Y, Boura-Halfon S, Moallem U(2013) Periparturient dairy cows do not exhibit hepatic insulin resistance, yet adipose-specific insulin resistance occurs in cows prone to high weight loss. Journal of Dairy Science 96: 5656-5669.

Zarrin M, De Matteis L, Vernay MC, Wellnitz O, Van Dorland HA, Bruckmaier RM(2013) Long-term elevation β-hydroxybutyrate in dairy cows through infusion: Effects on feed intake, milk production, and metabolism. Journal of Dairy Science 96: 2960-2972.

Zarrin M, Grossen-Rösti L, Bruckmaier RM, Gross JJ(2017)Elevation of blood β-hydroxybutyrate concentration affects glucose metabolism in dairy cows before and after parturition. Journal of Dairy Science 100: 2323-2333.

ZhangG, Hailemariam D, Dervishi E, Goldansaz SA, Deng Q, Dunn SM,Ametaj BN(2016)Dairy cows affected by ketosis show alterations in innate immunity and lipid and carbohydrate metabolism during the dry off period and postpartum.Research in Veterinary Science107: 246-256.

Weber C, Losand B, Tuchscherer A, Rehbock F, Blum E, Yang W, Bruckmaier RM, Sanftleben P, Hammon HM(2015) Effects of dry period length on milk production, body condition, metabolites, and hepatic glucose metabolism in dairy cows. Journal of Dairy Science 98: 1772-1785.

Weber C, Schaff CT, Kautzsch U, Börner S, Erdmann S, Görs S, Röntgen M, Sauerwein H, Bruckmaier RM, Metges CC, Kuhla B, Hammon HM(2016)Insulin-dependent glucose metabolism in dairy cows with variable fat mobilization around calving. Journal of Dairy Science 99: 6665-6679.

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Research article
Ερευνητικό άρθρο

Detection of Mycotoxigenic Fungi and Mycotoxins in Poultry Feed of Balochistan Pakistan

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ABSTRACT: Mycotoxins are secondary toxic metabolites of fungi, commonly associated to agriculture commodities. Aspergillus, Fusarium, Penicillium and Claviceps genera are the most common mycotoxigenic fungi and the most important types of mycotoxin are Aflatoxins, Zearalenone, Ochratoxin, Fumonisins and Trichothecenes Mycotoxins can be carcinogenic, cytotoxic, mutagenic, teratogenic, neurotoxic, nephrotoxic, estrogenic and immunosuppressant. This study was conducted to examine the presence of mycotoxigenic fungi and mycotoxins in poultry feed used by the poultry farmers of Balochistan. Samples (n=100) were analyzed and found contaminated with fungi such as Aspergillus, Mucor, Rhizopus and Fusarium genera, amongst them Aspergillus species was observed highly prevalent (89%). Aflatoxin production in culture plates were initially screened with the help of ammonia hydroxide vapor test, and subsequently analyzed with the help of AgraStrip®. The Aflatoxins (77%) and Fumonisins (92%) were found in direct examination of feed samples. Toxins presence was also confirmed with the help of ELISA using AgraQuant® kits. Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) were used for characterization and quantification of aflatoxins. It was concluded that the feed samples were abundantly contaminated with aflatoxins and fumonisins and the fungi responsible for their production. This contaminated feed is one of the reasons for economic lossesfor poultry farmers of the region.

***Keywords*:** Aspergillus; Aflatoxins; Fumonisin; Chromatography; ELISA

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INTRODUCTION

Mycotoxins are structurally diverse low molecular weight secondary fungal metabolites produced mostly by the fungal species of Penicillium, Fusarium, Aspergillus, Alternaria and Claviceps (Flores-Flores *et al.,* 2018). Exposure of humans to mycotoxins occurs through the consumption of contaminated agriculture products (rice, cereals, vegetables etc.) or through the intake of contaminated animal products (milk, eggs, etc.) (Capriottiet al., 2012). Contamination of food and feed by fungi and their mycotoxins can occur at every step of the food supply chain from agricultural production, storage and processing (Khan et al., 2020). Several environmental factors including storage time, temperature, pH, oxygen, moisture and humidity promote the growth of fungi and subsequently the production of mycotoxin (Kana et al., 2013).

Around the world, it is estimated that about 25% of the crops are contaminated with mycotoxins (FAO, 2002). More than 480 mycotoxins are currently known, and aflatoxins are of the greatest importance. Among the reported mycotoxins, the most economically important are Aflatoxins, Fumonisins, Ochratoxin A, Trichothecenes, Zearalenone, and Citrinin (Jajić et al., 2018; Shi et al. 2018).

Metabolic function and activity of mycotoxins has not been reported yet (da Rocha et al., 2014). Though mycotoxins have not been extensively studied in Pakistan, their presence in poultry feed is reported by some researchers (Rashid et al., 2012). Mycotoxins such as aflatoxins are also regularly detected in poultry feed from countries around the globe, such as Brazil, Nigeria, Colombia, Malaysia, Saudi Arabia, and India, (Anjum et al., 2012).

As poultry feed can contain toxigenic fungi, it is therefore important to regularly monitor feed for their presence,to ensure food safety and prevent its spread to the human food chain. This study was designed to investigate the presence of mycotoxigenic fungi and mycotoxins in poultry feed used by the poultry farmers of Balochistan.

Balochistan is largely consist of arid and semi-arid lands, with a human population scattered in rural areas, dependent mostly on livestock and poultry farming (Mirza et al., 2009). Our study is focused on small scale poultry farming, mostly practiced in the province due to limited facilities and economic constrains.

MATERIALS AND METHODS

Sample Collection

Poultry feed samples were collected (n=100) from different poultry farms of Quetta, a region of Balochistan. Feed samples were stored in sterile polythene bags and transported to the Food Microbiology and Bioprocess Technology Laboratory, Department of the Microbiology University of Balochistan, Quetta. The samples were divided into two parts each for mycotoxigenic fungal culture isolation and mycotoxins detection.

Isolation and identification of fungi

For each poultry feed sample, 10-fold serial dilution of one-gram feed was performed by adding it to 10mLof sterile distilled water. One ml from each dilution was spread over Sabouraud Dextrose Agar (SDA) supplemented with Chloramphenicol. The Petri plates were incubated at room temperature (25±1 ^{0}C) for 3-7 days. After incubation isolated colonies of fungi were sub-culturedon Potato Dextrose Agar (PDA).The preliminary identification was performed by the assessment of morphological, cultural and microscopic characteristics (Ali et al., 2018).

Mycotoxigenic potential determination

For the determination of mycotoxigenic potential of the fungi isolates, a specific culture media Yeast Extract Agar (YEA)wasused by sub-culturing therepresentative fungal colonies of the isolates from PDA over YEA and incubated at room temperature for 7 days (Shekhar et al., 2017).

Ammonia vapor test for mycotoxin

Concentrated solution of ammonium hydroxide (1-2 drops) were dropped over the surface of *A. flavus* grown on YEA and left for 10–15 minutes.The appearance of pink or redcolor in the culture medium showed the production of mycotoxin (Shekhar et al., 2017).

Detection of mycotoxins production by Agra strip and Thin Layer Chromatography

Fungal culture (45 days) on Potato Dextrose Broth (PDB) was mixed with extractant 10 mLChloroform: Acetone (85:15 v/v) and incubated at room temperature for 15-20 minutes with vigorous stirring for 5 minutes.The extracts were then filtered with Whatman No.1 filter paper and the filtrates were evaporated. The residues were resuspended in 500µL of methanol and aseptically filtered.The filtrates were

then collected for aflatoxin strip test. Part of the fungal culture extracts were processed for TLC by spotting it on TLC plates along with aflatoxin standards. Plate was placed in chromatographic tank using Toluene and Acetone as mobile phase (1:1). The plates were observed in UV lamp at 365 nm wavelengthafter completion of the process (Yazdani et al., 2010).

Fourier Transform Infrared Spectroscopy

Fungal culture (45 days) on PDB was mixed with extractant 10 mL chloroform: acetone (85:15 v/v) and incubated at room temperature (25°C) for 15-20 minutes with vigorous stirring for 5 minutes (Yazdani et al., 2010).The extracts were filtered through Whatman No.1 filter paper and the filtrates were evaporated. The residues were resuspended in 500µL of methanol and filtered aseptically for FTIR analysis. The samples were then process for FTIR in the range of 3500-500 $cm^{-}1$. The peak wasstudied for the mycotoxin presence.

Detection of Aflatoxins in Feed by Agra Strip test

The 2nd part of the feed sampleswas directly used for mycotoxin presence, where Aflatoxin test kits AgraStrip® was used having 4ppb Cut-offvalue. The extraction procedure was performed according to manufactures protocol. Briefly, 10g of ground samples, 60mL of ethanol and distilled water were added to the flask and covered entirely. The Samples were extracted at ratio of 1:2 and mixed vigorously for 1 minute using vortex. Extracts were filtered by Whatman filter paper No.1. andused for aflatoxin detection. An amount 50µL diluent assay was added into themicro-well of aflatoxins detection kit. Sample 50µL was mixed into a well through sterile pipette. Aflatoxin 4ppb strip was added into well and left for 5minutes and results were observed subsequently.

Detection of Fumonisin from Feed by AgraStrip test

Manufactures protocol was followed for extraction. In brief, each sample, 10g of ground sample was added in whirl-pak filter bagand buffer bag was added followed by the addition of 30mL distilled water was added and mixedfor 5 minutes.Then 1mL of buffering agent and 50µL sample was added in yellow tube and mixed vigorously. The dip strip was placed in the well and incubated for 3 minutes at 40-45 ^{0}C Bands were observed after 3 minutes of incubation.

Detection of mycotoxins infeed by Enzyme Linked Immunosorbent Assay (ELISA)

A competitive ELISA was performed with the help of Aflatoxin and Fumonisin ELISA test kit AgraQuant® as per manufacturerinstructions.In brief, 5g of ground poultry feed sample was extracted in 2 mL of methanol (70%). Mixed vigorously with vortex stirrer for 5 minutes and filtered by Whatman filter paper No. 1. The extracts were diluted with distilled water in 1:1 ration for aflatoxins and 1:14 for fumonisins. 50µL of the diluted filtrate was used. An amount 200µL of conjugate and 100µL of each standard were added in dilution wells. Then contents werethoroughlymixed and 100µL of it was transferred tothe well coated with antibody and then well were incubated for 10 minutes at room temperature. The incubated contents were washed out with distilled water 5 times and 100µL of substrate was added to wells coated with antibodies and incubated againat room temperature for 5 minutes. 100µL of stop solution to each well was added and mixed slightly andthe absorbance measured by using Microplate reader at 450 nm.

Detection of aflatoxins in feed by TLC and HPLC

Feed samples were processed by thin-layer chromatography. Briefly, for each feed sample 10g of ground, 90mL chloroform 10mL distilled water and 2g sodium chloride was added in the flask. Contents were shaken at room temperature on an orbital shaker for 5 minutes. The coated TLC plate was spotted with Aflatoxin standard on the baseline along withspotted samples. TLC plate wasthen placed in a TLC chamber filled with solvent mixture of Toluene: Acetone (1:1).The TLC plate was removed when the solvent front raised, and thesolvent finishing line was recorded. Plate was dried in the air and visualized under UV lamp at 364 nm wavelength. Spots withblue fluorescence were encircled and RF value was measured and matched against the standard (Rafique et al., 2018).

Aflatoxins in feed samples were quantified by HPLC according to the procedure defined by Alvarado-Hernándezet al.,(2016) against reference aflatoxins standards. HPLC was performed using C8 column with injection volume of 100µL, acetonitrile; methanol and water (20:20:60 v/v) as mobile phase at a flow rate of 0.8 mL per minute.

RESULTS

The fungal isolates were morphologically identified based on its cultural characteristic and microscopic morphology. It was found that almost all poultry feed samples were contaminated with at least

one fungus, where as Aspergillus spp., Mucor spp., Rhizopus spp., and Fusarium spp. were abundantly found. Among these genera the highestfrequency was recorded for Aspergillus genus (Table 1). In the present study, Aspergillus flavus showed the highest prevalence rate (46%), followed by*Aspergillus niger* (22%), *Aspergillus parasiticus* (21%), Mucor (6%), Rhizopus (4%) and *Fusarium proliferatum* (1%). Aflatoxigenic properties of the fungal isolates were preliminary confirmedwith the help of ammonium vapors test in YEA medium (Figure1).Production of mycotoxins (Aflatoxins and Fumonisins) were furtherconfirmed with the help of AgraStrip® for total Aflatoxin and Fumonisins and ELISA. Direct examinationof feed samples for mycotoxins by AgraStrip and ELISA revealed that the majority of the feed samples was contaminated with Aflatoxins (77%) and Fumonisins (92%).

Table 1. Prevalence of different fungi in feed samples

Fungal Genera	Growth (%)
Aspergillus flavus	46%
Aspergillus niger	22%
Aspergillus parasiticus	21%
Mucor spp.	6%
Rhizopus spp.	4%
Fusarium proliferatum	1%

The quantitative analysis of mycotoxins through ELISA revealed that the aflatoxin quantities in different samples vary between 0-40 PPB while that of Fumonisin between 0-500 PPB. The concentration values of Aflatoxins and Fumonisins in feed samples were determined from a calibration curve prepared by using known concentrations.Aflatoxin produced byfungal isolatesin culture media and extracted from feed samples was also confirmed with Thin Layer Chromatography. Blue/green fluorescence bands under UV light confirmed the presence of aflatoxins.

The FTIR spectroscopy studied in the wavelength range of 3500–500 cm−1for the toxins present showed different peaks for functional groups indication confirming the presence of Aflatoxins and Fumonisin. The FTIR peaks (spectra) were observed at frequency of 3280.10 cm−1 correspond for N-H and O-H stretching, 2969 cm−1 for CH_2 asymmetric stretch composed of lipids with a little contribution of proteins, carbohydrates and nucleic acids, 1737 cm−1 for Ester C=O, 1651 cm−1 for C=O, 1365 cm−1 for CH3, 1228-1216 cm−1 for PO_2asymmetric stretching mainly composed of nucleic acids with little contribution from phospholipids and for PO_2 symmetric stretching nucleic acids and phospholipids stretch of C-O glycogen 1044 cm−1 were observed.

The HPLC analysis of selected samples confirmed the presence of aflatoxins and the aflatoxins B1 and B2 in the samples were quantified as 35.94 µg and 2.53 µg respectively (Figure 2).

Figure1. (A) Shows aflatoxigenic nature under UV light at 365 nm,(B)Ammonia vapor

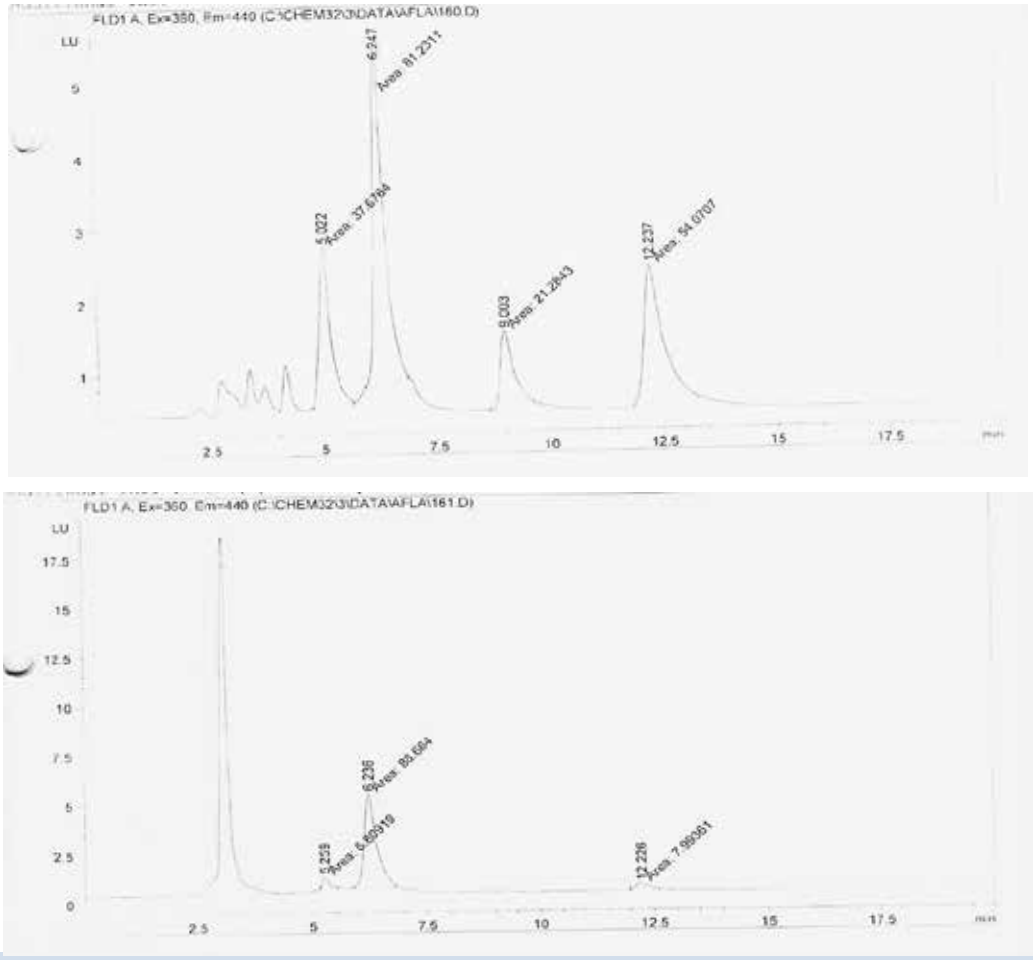

Figure2. Aflatoxins chromatogram of HPLC detection,(A) Standard (B) Sample

DISCUSSION

Occurrence of fungi in poultry feed is not only affecting the organoleptic properties of feed but some of them are lethal toxin producers (Greco et al., 2014). In our study, 100% of the feed samples were found contaminated with fungal genera. Similar results were reported in other regions, such as Brazil (Rosa et al., 2006), Argentina (Dalcero et al., 1997), Nigeria (Osho et al., 2007) and in Pakistan (Sana et al., 2019). Our study showed a prevalent *A. flavus* (46%) contamination, a finding that is in compliance with the study conducted in Saudi Arabia by Gherbawy et al. (2019) that reported a prevalence of 59% *A. flavus* in poultry feed Rafique et al. (2018) reported 34% *A.flavus*in Pakistan, lower than our study. We found 22% *A.niger*, which follow the study conducted by Raju et al.(2018) in Kerala India. Compared to our results regarding *A. parasiticus* (21%), Ahmed et al. (2017) reported 51% *A.parasiticus* presence in their study conducted in Iraq. In our study the presence of Mucor (6%), Rhizopus (4%) and *Fusarium proliferatum* (1%) were reported, which are followingthe figures reported by Pacinet al.,(2003) from Ecuador. The *A. flavus* was found the most dominant fungi,most likely due to its ability to tolerateharsh environment and high temperature (Battilaniet al., 2003).

Aflatoxins are mostly produced by Aspergillus species include*A.parasiticus* and *A.flavus*. Improper storage conditions in poultry feed lead to the production of Aflatoxins. Similarly to our study, Yazdani et al. (2010) performed ammonia vapor test foraflatoxigenic potential determination in their study.Aflatoxins and fumonisins showed 77% and 92% contamination confirmed by AgraStrip® and ELISA AgraQuant®.The ELISA and HPLC are equally sensitive and recommended for the detection of mycotoxins (Beyene et al., 2019).

In our study the mycotoxins production was also analyzed by using FTIR,the results were comparable to that of Kos et al. (2016) and Sieger et al. (2017). Chromatographic techniques such as TLC and HPLC were performed for the mycotoxin detection in our study. Aflatoxins were identified and quantified by HPLC which showed that the AFB1 quantity was

35.94µg/g and that of AFB2 was 2.53µg/g, lower from that found by Saleemi et al. (2012). The HPLC is now widely used technique for identification and quantification of Aflatoxin (Wacoo et al., 2014).

It was concluded from this study that majority of poultry feeds in Balochistan are contaminated with Aflatoxin and Fumonisin. This contamination is usually linked to the uses of raw materials, environmental conditions and other sources, such as storage and transportation practices. Precautions and limitation of fungal growth at different stages of feed preparation can limit the contamination.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Ahmed DA, Al-Khafaji NJ, Ahmed LT(2017) Isolation and molecular identification of Aspergillus spp. collected from different sources of animals feed. Int J Curr Microbiol App Sci*6*(6):1792-1797.

Ali FS, Akbar A,Prasongsuk S, Permpornsakul P, Yanwisetpakdee B, Lotrakul P, Punnapayak H, Asrar M, Ali I(2018) Penicillium Imranianum, A new species from the man-made solar saltern of Phetchaburi province, Thailand. Pak J Bot 50(5):2055-2058.

Alvarado-Hernández JR, Carvajal-Moreno M, Rojo-Callejas F, Ruiz-Velasco S(2016) Aflatoxins in natural peanuts (*Arachishypogaea* L.) of Mexico: Validation of the biochemical methods for extraction and quantification. J Plant BiochemPhysiol 4:1000168.

Anjum MA, Khan SH, Sahota AW, Sardar R(2012) Assessment of aflatoxin B1 in commercial poultry feed and feed ingredients. J Anim Plant Sci 21: 117-120.

Battilani P, Pietri A, Bertuzzi T, Languasco L, Giorni P,Kozakiewicz Z(2003) Occurrence of ochratoxin A–producing fungi in grapes grown in Italy. J Food Prot 66(4):633-636.

BeyeneAM, Du XE, Schrunk D, Steve E,Rumbeiha KW(2019) High-performance liquid chromatography and Enzyme-Linked Immunosorbent Assay techniques for detection and quantification of aflatoxin B1 in feed samples: a comparative study. BMC Res Notes 12: 492. https://doi.org/10.1186/s13104-019-4538-z

Capriotti AL, Caruso G, Cavaliere C, Foglia P, Samperi R,LaganàA(2012) Multiclass mycotoxin analysis in food, environmental and biological matrices with chromatography/ mass spectrometry. Mass Spectr Rev 31(4):466-503.

Dalcero A, Magnoli C, Chiacchiera S, Palacios G, Reynoso M(1997)Mycoflora and incidence of aflatoxin B1, zearalenone and deoxynivalenol in poultry feeds in Argentina. Mycopathologia 137(3):179-184.

da Rocha EBM, Freire FCO, Maia FEF, Guedes FIF, Rondina D (2014). Mycotoxins and their effects on human and animal health. Food Cont 36(1):159-165. DOI: http://doi.org/10.1016/j.foodcont.2013.08.021

FAO (2002) The State of Food Insecurity in the World (SOFI), Food and Agriculture Organization of the United Nations. http://www.fao.org/docrep/005/y7352e/y7352e0 0.htm.

Flores-Flores ME, González-Peñas E(2018) Analysis of mycotoxins in Spanish milk. J Dairy Sci 101:113–117

Gherbawy YA, Shebany YM,Alharthy H (2019) Aspergilli and Their Aflatoxins Contamination of Poultry and Animal Feedstuff Samples in Western Region of Saudi Arabia. Sains Malaysiana48(4):765-771.

Greco MV, Franchi ML, Rico Golba SL, Pardo AG, Pose GN(2014) Mycotoxins and mycotoxigenic fungi in poultry feed for food-producing animals. Sci World J 2014.

Jajić I, Glamočić D, Krstović S, PolovinskiHorvatović M (2018) Aflatoxin M1 occurrence in Serbian milk and its impact on legislative. J Hellenic Vet Med Soc 69(4):1283-1290.

KanaRK, Gnonlonfin BGJ, Harvey J, Wainaina J, Wanjuki I, SkiltonRA, Teguia A(2013) Mycobiota and toxigenicity profile of Aspergillus flavus recovered from food and poultry feed mixtures in cameroon. J Ani Poult Sci *2*(4):98-107.

Khan GI, Akbar A, Anwar M, Shafee M, Sadia H, Razzaq A, Rehman FU (2020) Eco-friendly bacterial biodegradation of mycotoxins. Pure Appl Biol 9(2):1708-1717. http://dx.doi.org/10.19045/bspab.2020.90181

Kos G, Markus S, David M, Celine Z, Michael S, Tuba O, Boris M, Rudolf K(2016) A novel chemometric classification for FTIR spectra of mycotoxin-contaminated maize and peanuts at regulatory limits. Food AdditCont: Part A33:10:1596-1607. DOI: 10.1080/19440049.2016.1217567

Mirza SN, Athar M, Qayyum M (2009) Eff ect of Drought on Rangeland Productivity and Animal Performance in Dryland Region of Balochistan, Pakistan. Agricul Conspectus Sci 74(2):105-109

Osho IB, Awoniyi TAM, Adebayo AI(2007) Mycological investigation of compounded poultry feeds used in poultry farms in southwest Nigeria. Afri J Biotech *6*(15).

Pacin AM, Gonzalez HHL, Etcheverry M, Resnik SL, Vivas L,Espin S(2003) Fungi associated with food and feed commodities from Ecuador. Mycopathologia 156(2):87-92.

Rafique A, Javed MT, Ali SS(2018) Contamination of commercial poultry feeds and feed ingredients with Aspergillus flavus and aflatoxin B1 in Punjab Pakistan. J Ani Plant Sci *28*(4):1021-1026

Raju S, PadamadanCJ,Shenpagam NH(2016) Mycotoxin production by fungi isolated from commercially prepared livestock feed in Kerala. Int J Appl Res*2*(5):154-159.

Rashid N, Bajwa A, Rafeeq M, Khan MA, Ahmad Z, Tariq MM, Wadood A, Abbas F(2012) Prevalence of aflatoxin B1 in finished commercial broiler feed from west central Pakistan. J Anim Plant Sci 22: 6-10.

Rosa CDR, Ribeiro JMM, Fraga MJ, Gatti M, Cavaglieri LR, Magnoli CE, Lopes CWG(2006) Mycoflora of poultry feeds and ochratoxin-producing ability of isolated Aspergillus and Penicillium species. Vet Microbiol 113(1-2):89-96.

Saleemi MK, Khan MZ, Khan A, Javed I, Hasan ZU, Hameed MR, Mehmood MA (2012) Occurrence of toxigenic fungi in maize and maize-gluten meal from Pakistan. PhytopathologiaMediterranea 51(1).

Sana S, Anjum AA, Yaqub T, Nasir M, Ali MA, Abbas M(2019) Molecular approaches for characterization of aflatoxin producing Aspergillus flavus isolates from poultry feed. Pak Vet J 39(2):169-174.

Shekhar M, Singh N, Dutta R, Kumar S, Mahajan V (2017) Comparative study of qualitative and quantitative methods to determine toxicity level of Aspergillus flavus isolates in maize. PLoS ONE 12(12): e0189760. https://doi.org/10.1371/journal. pone.0189760

Shi H, ShengliL, Yueyu B, Luciana LP, Yaogeng L,Peiqiang Y (2018) Mycotoxin contamination of food and feed in china: occurrence, detection techniques, toxicological effects and advances in mitigation technologies. Food Cont 91, 202–215.

Sieger, Gregor MK, Michael S,Matthia G, Rudolf K, Boris M(2017) Portable Infrared Laser Spectroscopy for On-site Mycotoxin Analysis. Sci Rep 7:44028. doi: 10.1038/srep44028.

Yazdani D, Zainal AM, Tan YH,Kamaruzaman S(2010) Evaluation of the detection techniques of toxigenic Aspergillus isolates. Afri J Biotech 9(45):7654-7659.

Wacoo AP, Wendiro D, Vuzi PC, Hawumba JF (2014) Methods for detection of aflatoxins in agricultural food crops. J Appl Chem 2014.

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Research article
Ερευνητικό άρθρο

Evaluation of Milk Yield and Milk Composition of Honamlı Goats

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ABSTRACT: The aim of this study was to determine the milk yield and milk composition (total solids, fat, protein and lactose), freezing point depressionand somatic cell counts of Honamlı goat during second lactationin Turkey. The animal material of the study consisted of a total of 30 goat. Total milk yield was detected by using Fleischmann's method. Milk composition was analyzed by Bentley 150, and somatic cells were counted by Bentley Somacount FC. One-way ANOVA was used to determine the effect of lactation stages on milk yield and milk composition. In the study, mean lactation period and lactation milk yield of Honamlı goat were detected as 202.4 days, and 92.6 kg, respectively. The percentages of fat, protein, lactose, total solid, freezing point depression, and somatic cell counts /mL of milk were 2.4%, 4.2%, 5.1%, 12.7%, -0.57 °C, 82.8 and 2.9%, 4.2%, 4.7%, 12.7%, -0.59 °C, 483 on the 60th and 120thlactation day respectively. Total solids, fat, and protein values significantly increased especially towards the end of lactation ($P < 0.05$). Lactose value decreased slowly from the beginning to the end of lactation. The somatic cell counts increased in milk particularly at the end of lactation ($P < 0.05$). The freezing point depression remained stable throughout lactation. It was thought that results of study were important representing the second lactation milk production of Honamlı goats that is one of the native goat breeds of Turkey.

Keywords: Honamlı goat, milk composition, second lactation

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INTRODUCTION

Milk is one of the most important food including protein, fatty acid, vitamins, and minerals and also it is contained all the necessary energy and nutrients for growth and development (Pereira, 2014). The biological value of milk is determined by the content of bioactive components that offer health benefits (Reklewska et al. 2005, Wong et al. 2006). Its high nutritive value can be attributed to also its unique chemical composition which supports optimal digestion and absorption (Miciński et al., 2013). Like it's composition, the somatic cells count (SCC)determined in milk is of high importance for milk products of hygienic, sanitary, dietetic, nutritious, and gastronomic quality in the dairy industries (Jiménez-Granado et al., 2014; Karagiannis et al., 2018).

The freezing point depression (FPD) is one of the most important indicator of the quality of milk, which is influenced by the composition of cow and goat milk (Genčurová et al., 2008; Kędzierska-Matysek et al., 2011). All components of milk is affected by a number of factors such as breed, parity and lactation stage, milk yield, milking time, feed quality and amount, occurrence of subclinical mastitis, nutritional deficiencies, thermal stress, seasonal influences, and presence of CO_2 in milk (Janštová et al., 2007).

There has been an obvious increase in the demand for goat milk and products in developed and developing countries due to its nutritive and dietetic value (Bernecka, 2011; Akbas et al., 2019).*Caprine* milk contains averagely 12.2% total solids, which is composed of 3.5% protein, 3.8% fat, 4.1% lactose, and 0.8% ash (Park, 2016). While fat content of goat milk across breeds ranges from 2.45 to 7.76% (Jenness, 1980;Haenlein andCaccese, 1984); one of the important components of milk, Lactose make up 44% of the total carbohydrates in goat milk and between 4.1% and 4.8% of the weight of the whole milk (Park et al., 2007; Raynal-Ljutovac et al., 2008). In addition, the unique composition of goat milk, combined with its nutritional value, is related to the release of protein fragments which are more digestible than bovine milk, during digestion or technological processing, which are able to perform specific biological activities (Park et al., 2007; Ceballos et al., 2009). The quality and composition of goat milk is affected several factors that contains genetic factors, environmental conditions, goat farming practices, age, period of lactation, milking type, frequency and period of milking, feeding practices, and udder health situation (Raynal-Ljutovac et al., 2008; Bolacalı and Küçük, 2012; Park, 2016).

Honamlı goats which are named after the Honamlı nomads are generally reared on the Taurus Mountains considered as one of a native goat breed in Mediterranean region in Turkey. Honamlı goat is a multipurpose breed, but usually mentioned for its big body and meat production. Milk production and reproduction traits have also significant meaning in very limited flocks (Saatcı and Elmaz, 2017). They are officially registered as an orginal goat breed in the year of 2015 (Official Gazette of Turkish Republic, 2015).The more in-depth knowledge about Honamlı goat milk composition and properties is needed.

Although there are many studies on changes of major components in goat milk during lactation, only little is known about milk yield, composition, freezing point depression and somatic cell count of Honamlı goat milk.

The aim of this study was to determine milk yield, milk composition (total solids, fat, protein, and lactose content), freezing point depressionandsomatic cell count of Turkish local breed Honamlı goats during the second lactation.The results of the present study are expected to help characterizing the Honamlı goat breed.

MATERIALS AND METHODS

Sampling and Analytical Procedures

In this study were used 30 Honamlı goats in second lactation at the Research and Training Farm of the Faculty of Veterinary Medicine of Burdur Mehmet Akif Ersoy University in Turkey.

The goats were grazed on highland pasture and maquis area including mostly kermes oak (Quercus coccifera) in formations from cultures of green olive tree (Phillyrea latifolia), black pine (Pinus nigra), Calabrian pine (Pinus brutia), and cedar (Cedrus) during spring and summer and they kept out for minimum 8 hours in a day from early in the morning till noon. In addition to grazing and browsing, goats were kept in a barn during winter and fed with 200 g/day concentrate feed (16% crude protein and 2500 kcal metabolisable energy per kg dry matter). Kids continued to suck their mothers until 5 months of age.

Milk samples were provided during the morning and evening milking once a month. Honamlı goats were milked by hand on 30th, 60th, 90th, 120th, 150th,

180th and 210th day of lactation. The California Mastitis Test was applied to the goats milk. However, it was not determined mastitis cases during this study.

Measuring of the quantity of milk was determined using a graduated cylinder. Total milk yield (TMYL) is estimated by the centring date method, also known as Fleischmann's method (Maria and Gabina, 1992; Ruiz et al., 2000), which is currently used by the selection program. The general expression of the Fleischmann's method is:

$$TMYL = y_1 t_1 + \sum_{i=2}^{k} (y_i + y_{i+1})/2(t_{i+1} - t_i) + y_{k+1} * 15$$

Where TMY is total milk yield; y1 is yield at first milk record, tl is interval between lambing and first recording; yi is yield of the record i and ti is interval between the record i and the record (i+1), (i = 1, … k), and 15 = assumed number of days between the last recording and the dry-off.

Milk samples were transported to laboratories by using cool boxes without preservatives at temperatures not exceeding 6°C. All milk samples were analysed using the Bentley 150 (Bentley Analytical Instruments, USA) to determine the milk composition, and FPD. The SCC in goat milk samples were counted by Bentley Somacount FC (Bentley Analytical Instruments, USA).

This study was approved by Burdur Mehmet Akif University, Local Ethics Commission of Experimental Animals (6.9.2012, meeting number:1, resolution number:6).

Statistical Analysis

All statistical analysis were carried out using Minitab 16.1 statistical package (Minitab, 2011). An intense descriptive statistical analysis was applied to the data. Student-t test was employed for the differences between morning and evening milk yields. In addition, One-way ANOVA was used to determine the effect of lactation stages on milk yield and milk composition (total solids, fat, protein, and lactose content), FPD, and SCC. Tukey analysis was used to control for the significance of differences between subgroups.

RESULTS

Table 1 shows the mean lactation milk yield (kg), lactation time (days), and daily milk yield (kg) of milk samples for Honamlı goats. Table 2 shows mean milk yield (g) in different lactation stage. Mean lactation period of Honamlı goats was 202.4 days. In this study, the mean lactation milk yield of Honamlı goats was 92.6 kg. The daily milk yield of Honamlı goats was 0.453 kg (Table 1). While the highest peak of total milk yield was 610 g, and 678 g on 60th and 90th days, respectively; the mean milk yield was decreased towards ends of the lactation (Table 2).It was found to be statistically significant differences among the measurement days of lactation stages ($P < 0.05$).

Figure 1. A Honamlı goat and kids from the research flock

Figure 2. Does of the Honamlı goat, the research flock

Table 1. Mean lactation milk yield of the Honamlı goats

Parameters	n	Mean ± SE
LactationMilkYield (kg)	30	92.6±7.23
Lactation time (days)	30	202.4 ±3.12
Daily milkyield (kg)	30	0.453 ± 0.03

n: Number of theHonamlı goats; SE: Standard Error

Table 2. Mean milk yield (g) in different lactation stage (30 - 210thdays) of Honamlı goats $(\bar{x} \pm S_{\bar{x}})$

MilkingPeriod	n	30th day	n	60th day	n	90th day	n	120th day	n	150th day	n	180th day	n	210thday	P
Morning	30	$238.3^{c} \pm 17.4$	30	$375.0^{a} \pm 26.6$	30	$326.7^{b} \pm 27.9$	30	$324.7^{b} \pm 33.1$	30	$184.7^{c} \pm 16.6$	24	$145.8^{d} \pm 18.0$	7	$257.1^{c} \pm 77.5$	*
Evening	30	$190.0^{b} \pm 14.1$	30	$235.0^{b} \pm 18.3$	30	$351.7^{a} \pm 25.7$	30	$144.0^{c} \pm 19.5$	30	$68.3^{d} \pm 8.21$	24	$275.0^{b} \pm 36.7$	7	$142.9^{c} \pm 40.0$	*
Total	30	$428.3^{b} \pm 30.7$	30	$610.0^{a} \pm 41.6$	30	$678.3^{a} \pm 47.2$	30	$468.0^{b} \pm 47.3$	30	$253.0^{c} \pm 20.8$	24	$420.8^{b} \pm 51.3$	7	$400.0^{b} \pm 115$	*
P		*		*		-		*		*		*		*	

P values at the end of the each row indicate the statistical differences according to measurement days.
P values at end of the each columns how the statistical differences between morning and evening milk in each measurement days.
[a,b,c,d]: Mean with different superscripts (only for rows) are statistically different. *:P<0.05

Table 3a. Milk composition, FPD, and SSC in different lactation stage (30 - 120thdays) of Honamlı goats $(\bar{x} \pm S_{\bar{x}})$

Parameters	n	30th day			60th day			90th day			120th day			
		Morning	Evening	Mean	Morning	Evening	Mean	Morning	Evening	Mean	Morning	Evening	Mean	P
Fat (%)	30	2.2 ± 0.30	3.8 ± 0.28	$3.0^{a} \pm 0.27$	1.5 ± 0.17	3.2 ± 0.19	$2.4^{b} \pm 0.17$	1.8 ± 0.12	1.5 ± 0.10	$1.7^{b} \pm 0.10$	2.4 ± 0.26	3.4 ± 0.16	$2.9^{a} \pm 0.19$	*
Protein (%)	30	4.6 ± 0.09	4.5 ± 0.09	$4.6^{a} \pm 0.09$	4.2 ± 0.06	4.2 ± 0.05	$4.2^{b} \pm 0.05$	4.2 ± 0.06	4.1 ± 0.06	$4.1^{b} \pm 0.06$	4.3 ± 0.06	4.1 ± 0.06	$4.2^{b} \pm 0.06$	*
Lactose (%)	30	5.1 ± 0.03	4.9 ± 0.03	5.0 ± 0.03	5.2 ± 0.03	5.0 ± 0.02	5.1 ± 0.02	5.0 ± 0.03	5.0 ± 0.02	5.0 ± 0.02	4.8 ± 0.02	4.6 ± 0.03	4.7 ± 0.02	-
Total Solids (%)	30	12.0 ± 0.37	14.1 ± 0.34	$13.4^{a} \pm 0.34$	11.9 ± 0.21	13.5 ± 0.22	$12.7^{a} \pm 0.21$	11.9 ± 0.15	11.5 ± 0.15	$11.7^{b} \pm 0.15$	12.5 ± 0.31	12.9 ± 0.22	$12.7^{a} \pm 0.25$	*
FPD (-°C)	30	0.58 ± 0.01	0.57 ± 0.01	0.57 ± 0.01	0.57 ± 0.01	0.56 ± 0.01	0.57 ± 0.01	0.58 ± 0.01	0.57 ± 0.01	0.57 ± 0.01	0.59 ± 0.01	0.59 ± 0.01	0.59 ± 0.01	-
SCC ($x10^3$cell/ml)	30	115.7 ± 32.20	175.7 ± 66.00	$145.7^{b} \pm 41.20$	53.8 ± 16.00	111.8 ± 25.30	$82.8^{c} \pm 19.50$	92.3 ± 47.90	138.8 ± 75.40	$113.1^{b} \pm 61.40$	331.0 ± 105	634.0 ± 155	$483.0^{a} \pm 124$	*

FPD:Freezing point depression, SCC: Somatic cell count, [a,b,c]: Mean with different superscripts (only for rows) are statistically different.
*:P<0.05 -:Non-significant (P>0.05)

Table 3b. Milkcomposition, FDP, and SSC in differentlactationstage (150 - 210thdays) of Honamlıgoats $(\bar{x} \pm S_{\bar{x}})$

Parameters	n	150th day			180th day			210th day			
		Morning	Evening	Mean	Morning	Evening	Mean	Morning	Evening	Mean	P
Fat (%)	30	3.0 ± 0.21	3.3 ± 0.17	$3.2^{b} \pm 0.17$	4.4 ± 0.26	3.6 ± 0.21	$4.0^{b} \pm 0.22$	5.9 ± 0.85	8.1 ± 0.84	$6.6^{a} \pm 0.71$	*
Protein (%)	30	4.2 ± 0.06	3.9 ± 0.08	$4.0^{c} \pm 0.07$	5.4 ± 0.16	5.2 ± 0.13	$5.3^{b} \pm 0.12$	9.0 ± 0.51	8.7 ± 0.57	$8.8^{a} \pm 0.43$	*
Lactose (%)	30	4.7 ± 0.02	4.7 ± 0.02	$4.7^{a} \pm 0.02$	4.4 ± 0.15	4.5 ± 0.03	$4.4^{a} \pm 0.07$	3.0 ± 0.36	3.4 ± 0.31	$3.2^{b} \pm 0.25$	*
Total Solids (%)	30	12.9 ± 0.26	12.7 ± 0.25	$12.8^{c} \pm 0.23$	14.9 ± 0.35	14.2 ± 0.31	$14.6^{b} \pm 0.31$	16.9 ± 1.02	20.3 ± 0.106	$18.6^{a} \pm 0.90$	*
FPD (-°C)	30	0.59 ± 0.01	0.59 ± 0.01	0.59 ± 0.01	0.60 ± 0.01	0.59 ± 0.01	0.59 ± 0.01	0.61 ± 0.01	0.62 ± 0.01	0.61 ± 0.01	-
SCC ($x10^3$ cell/ml)	30	337.0 ± 125.0	527.0 ± 119.0	$432.0^{b} \pm 118.0$	939.0 ± 232.0	728.0 ± 160.0	$834.0^{a} \pm 176.0$	816.0 ± 315.0	977.0 ± 417.0	$896.0^{a} \pm 339.0$	*

FPD:Freezing point depression, SCC: Somatic cell count, [a,b,c]: Mean with different superscripts (only for rows) are statistically different
*:P<0.05 -:Non-significant (P>0.05)

Table 3a and Table 3bpresent the milk composition (total solids, fat, protein, lactose), FPD, and SCC in different periods of lactation in this study. As seen from tables, the mean total solids percentage of lactation was 13.4%, 12.7%, 11.7%, 12.7%, 12.8%, 14.6%, and 18.6% on 30th, 60th, 90th, 120th, 150th, 180th, and 210th days, respectively. In the present study, it was found to be statistically significant differences among lactation stages (P <0.05).

The mean fat percentage of lactation was 3.0%, 2.4%, 1.7%, 2.9%, 3.2%, 4.0% and 6.6% on 30th, 60th, 90th, 120th, 150th, 180th and 210th days, respectively (Table 3a and 3b). The fat content decreased during the first three months, and then it started to rise on the 150th day and reached the peak on 210th day.

The mean protein percentages of lactation were 4.6%, 4.2%, 4.1%,4.2%; 4.0%, 5.3%, and 8.8% on 30th, 60th, 90th, 120th, 150th, 180th, and 210th days, respectively according to Tables 3a and 3b. Additionaly, it was found to be statistically significant differences among lactation stages (P <0.05).

In the present study, the mean lactose percentage of lactation was 5.0%, 5.1%, 5.0%, 4.7%, 4.7%,

4.4%, and 3.2% on 30th, 60th, 90th, 120th, 150th, 180th, and 210th days, respectively (Table 3a and 3b). Although the lactose value was high between the 30th and the 90th days, it started to decrease on the 120th day, and remained at the rate of 3.2% on the 210th day of lactation.

The calculated average values of freezing points of raw goat milk ranged from -0.570°C to -0.610°C in the course of lactation (Table 3a and 3b). In this study, it was not found to be statistically significantdifferences between lactation stages (P >0.05).

In the present study, it was determined that SCC was 145.7 x 10^3 cell/ml, 82.8 x 10^3 cell/ml, 113.1 x 10^3 cell/ml, 483.0 x 10^3 cell/ml, 432.0 x 10^3 cell/ml, 834.0 x 10^3 cell/ml, and 896.0 x 10^3 cell/ml on 30th, 60th, 90th, 120th, 150th, 180th, and 210th days, respectively (Table 3a and 3b). In this study, it was found to be significantly different among lactation stages (P <0.05).

DISCUSSION

In the present study, mean lactation period of Honamlı goats was detected as 202.4 days. Whilethis value was lower than the mean lactation periodobserved for goats by Bolacalı and Küçük (2012), and Králíčková et al. (2013); but higher than the mean lactation perioddetermined for goats by some researchers (Tuncel and Okuyan, 1985; Forik, 1995; Sengonca et al., 2003; Simşek et al., 2006; Ata, 2007; El-Tarabany et al., 2016). When compared with values in the literature, lactation period ofHonamlı goats may have been associated with genotype and environmental factors such as manegement and feeding regime.

The mean lactation milk yield of Honamlı goats was 92.6 kg. In Turkey, the lactation milk yield of Turkish Hair goats, which is one of the native breeds reared under the same conditions with Honamlı goat, was reported to between 50-90 kg by some researchers (Tuncel and Okuyan, 1985; Cengiz and Yener, 1993; Forik, 1995; Sengonca et al., 2003; Simsek et al., 2006; Ata, 2007). While, the low milk yield might be associated with effecting by the deteriorating quality of the pastures; it was also seen that lactation milk yields of Honamlı show similaritywith native goat breeds of Turkey because of same management procedures of goats.

In the present study, the daily milk yield of Honamlı goats was 0.453 kg and it was decreased towards ends the lactation (P<0.05). While this value was compatible with the other studies (Sengonca et al; 2003; Ata, 2007) related to native goats breeds of Turkey; Simsek et al. (2006) found higher values(0.900 kg.) than the present study.Additionally, there were variability of daily milk yield reports for different goat breeds for numerous authors (Mestawet et al., 2012; Králíčková et al., 2013; El-Tarabany et al. 2016; Idamokoro et al., 2017). The milk yield value of Honamlı goat might be associated with genotype and especially inadequacy of nutritional imbalance because of extensive rearing system. Similarly, Soryal et al. (2004) reported significant pasture quality effects for milk production.

In the present study, the mean total solids percentage increased slowly from 90th day of lactation to 210th day of lactation. Due to this fact, it is believed that it was affected by the decreasing daily milk yield as a negative correlation between these traits. Similarly, Králíčková et al. (2013) and El-Tarabany et al. (2016) reported increasing of total solids value at the end of lactation. However, Mestawet et al. (2012) found that total solids were significantly higher at the beginning and the end of lactation.

In this study, while the fat content which was the most variable compared to the other parameters decreased during the first three months and then it started to rise, statistically significant differences (P<0.05) were found for fat contents among lactation stages. Similary to this, Brendehaug and Abrahamsen (1986) reported that fat content decreased over the first 4 months of lactation and increased during the pasture period. Contrary to this, Šlyžius et al. (2017) and Idamokore et al. (2017) reported that the highest milk fat content was determined during the early stages of lactation. On the other hand, there were some reports related to variations of fat content (Pridalová et al., 2008; Strzałkowska et al., 2010; Králíčková et al., 2013; Klir et al., 2015). When the results of this study were compared with previous studies, it can be said that they were lower than the values reported by some researchers (Klir et al., 2015; Kučević et al., 2016; Idamokoro et al., 2017). Fat composition of Honamlı goat milk may be associated with feeding regime, because it's a fact that fat and protein content of milk is influenced mostly by feeding (Toledo-Alonzo, 2003; Goetsch et al., 2011). Additonally, İt was reported that factors such as breed, parity, stage of lactation, and flock had an effect on quantity of goat milk fat (Šlyžius et al., 2017).

In the present study, although protein content changed over the whole period of lactation, an increase in this value was recorded only at the end

of lactation.Similarytothis,Králíčková et al. (2013) reported an increase in protein content value only during last periods of lactation. Kuchtík et al. (2015) detected that total protein was relatively high during early lactation (2.9%), decreased as lactation peaked (2.7%), and increased towards end of lactation (3.7%). Contrary to present study, El-Tarabany et al. (2016) reported non-significant differences for protein percentages (3.6%) at different stages of lactation. In the present study, the protein content of milk was also higher than most of the reported results by other researchers (Kuchtík et al., 2015; El-Tarabany et al., 2016). Additionall, Raynal-Ljutovac et al.(2008) reported that goat milk contains higher levels of protein ranging from 2.6 g/l to 4.1 g/l. The higher content of the milk components along with high protein content is an indication for the presence of higher potential for cheese production (Guo et al., 2001; Soryal et al., 2004;Fekadu et al., 2005). Because of the higher protein content, Honamlı goat milk is more advantageous for making types of cheese.

In this study, it was not found to be statistically significant differences ($P > 0.05$) among lactation stages for the period between the 30th and the 90th days for lactose value of Honamlı goats.The lactose content of Honamlı goats were similar to the results reported by Olechnowicz and Sobek (2008), Pridalová et al. (2008), Strzałkowska et al. (2010), and El-Tarabany et al. (2016). Contrary to this, Ibnelbachyr et al. (2015) mentioned that lactose content was the lowest in the early lactation stage (4.62%) and highest in the middle lactation (5.70%). Kuchtík et al. (2015)determined that the lactose content increased from 3.83 to 4.58%during lactation.In the present study, decreasing lactose concentration might be associated with a decreased freezing point in the present study.

While the calculated average values of freezing points of raw goat milk ranged from -0.570°C to -0.610°C in the present study. Janštová et al., 2007 reported compatible findings with the present study. While Park et al. (2007) reported that the freezing point for goat milk ranged between -0.540 and -0.570°C; Strzałkowska et al. (2009) determined that freezing point for the goat milk ranged between -0.609°C, -0.596°C and -0.625°C for three subsequent lactation stages, respectively. In the present study, there were not statistically significant differences between measuring periods ($P >0.05$).Contrary to this, Sousa et al. (1993) determined that there was a significant difference ($P < 0.05$) between the freezing points of goat milk collected in the morning and evening.Milk with the lowest freezing point in the study, was also characterised by the highest concentrations of protein and fat and therefore had the largest total solids content.

The SCC of Honamlı goats which is considered in quality and hygiene standards of milk was changeable in the measurement periods. While SCC of Honamlı goat milk on the 60th and 90th lactation days was $82.8x10^3$ cell/ml and $113.1x10^3$ cell/ml, respectively, daily milk yield of Honamlı goat on the 60th and 90th lactation days was 610 g and 678.3 g, respectively. While SCC was lower compared to milk of other goat breeds (Pizarro Borges et al., 2004;Pridalová et al., 2009;Martini et al., 2010; Králíčková et al., 2013); Paape et al. (2007) reported that the SCC mean in milk of healthy goats ranged from 270 x 10^3 to 2.000 x 10^3 cell/ml.In the present study, together with the increase of SCC, a decrease occurredin the daily milk yield. High SCCs in goat milk appear to be natural, particularly in the later stages of lactation. As lactation progresses, SCC increases and milk production decreases (Zeng and Escobar, 1995). In the European Union, the legal limit for cows is 400.0 x 10^3 cells/ml, but there is no legal limit for goat or sheep milk (EC, 1992). In the regulation issued by the Republic of Turkey on this subject, there is no legal limit for SCC in goats and also for SCC variation in Honamlı goats in Turkey (TFC, 2011). High SCCs in Honamlı goat milk appear to be natural, particularly at the end of theof lactation.

CONCLUSION

Milk yield of Honamlı goats was determined to be similar to that of other native goat breeds of Turkey. It was thought that the results of the present study would contribute to determination of the SCC of goat milk in the legal regulations and acceptable goat milk FPD standards in Turkey. Additionally, the findings would provide an important database for future studies in order to encourage goat farming and the consumption of dairy products from goat's milk in Turkey.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Akbas AA, Elmaz O, Sarı M, Saatcı M (2019). Assesment of some udder and teat traits of Honamlı goats in terms of dairy characters. J Res Vet Med 38(2):57-64.

Ata M (2007) Milk yield of Hairy goats in Kahramanmaraş. Master's thesis, University of KahramanmaraşSutcu Imam, Institute of Natural and Applied Sciences, Department of Animal Science,Turkey.

Bernecka H (2011) Health-promoting properties of goat milk. Med Weter 67:507-511.

Bolacalı M, Küçük M (2012) Fertility and milk production characteristics of Saanen goats raised in Mus region.KafkasUniv Vet FakDerg 18:351-358.

Brendehaug J, Abrahamsen RK (1986) Chemical composition of milk from a herd of Norwegian goats. J DairySci 53: 211-221.

Ceballos LS, Morales ER, Adarve GT, Castro JD, Martinez LP, Sanz-Sampelayo MR (2009) Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. J Food Compost Anal 22:322-329.

Cengiz F, Yener SM (1993) Sheep and goat production in highland Turkey. Drylan pasture and forage legume network news. In: International Center of Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria:pp 34.

European Council (EC) (1992) Council Directive 92/46/EEC of 16 June 1992 laying down the health rules for the production and placing on the market of raw milk, heat-treated milk and milk-based products, Official Journal of the European 268:1-34.

El-Tarabany MS, El-Tarabany AA,Roushdy EM (2016) Impact of lactation stage on milk composition and blood biochemical and haematological parameters of dairy Baladi goats.Saudi J BiolSci25:1632-1638.

Fekadu B, Soryal K, Zeng S, Van Hekken D, Bah B, Villaquiran M (2005) Changes in goat milk composition during lactation and their effect on yield and quality of hard and semi-hard cheeses.Small Rumin Res59:55-63.

Forik A (1995) A study on the determination of some characteristics related to milk, progeny and hair yields of hair goats raised in village conditions. Master's thesis, University of Uludag, Institute of Natural and Applied Sciences, Department of Animal Science, Turkey.

Genčurová V, Hanuš O, Vyletělová M, Landová H, Jedelská R (2008) The relationships between goat and cow milk freezing point, milk composition and properties. SciAgricBohem39:324-328.

Goetsch AL, Zeng SS, Gipson TA (2011) Factors affecting goat milk production and quality. Small Rumin Res 101:55-63.

Guo MR, Dixon PH, Park YW, Gilmore JA, Kindstedt PS (2001) Seasonal changes in the chemical composition of commingled goat milk. J DairySci84:79-83.

Haenlein GFW,Caccese R (1984) Goat milk versus cow milk. In: Extension Goat Handbook. USDA Publications, Washington.

Ibnelbachyr M, Boujenane I, Chikhi A, Noutfia Y (2015) Effect of some non-genetic factors on milk yield and composition of Draa indigenous goats under an intensive system of three kiddings in 2 years. Trop Anim HealthProd47:727-733.

Idamokoro EM, Muchenje V,Masika PJ (2017) Yield and milk composition at different stages of lactation from a small herd of Nguni, Boer, and Non-Descript Goats raised in an extensive production system. Sustainability 9:1000.

Janštová B, Dračková M, Navrátilová P, Hadra L, Vorlová L (2007) Freezing point of raw and heat-treated goat milk. Czech J AnimSci 52:394-398.

Jenness R (1980) Composition and characteristics of goat milk: review 1968-1979. J DairySci63:1605-1630.

Jiménez-Granado R, Sánchez-Rodríguez M, Arce C, Rodríguez-Estévez V (2014) Factors affecting somatic cell count in dairy goats: a review. Span J Agric Res 12:133-150.

Karagiannis I, Brozos C, Kiossis E, Boscos C, Fthenakis G, Panousis N (2018) Associations of periparturient β-hydroxybutyricacidandnon-esterifiedfattyacidsblood serum concentrationswithmilkyield, milkcompositionandmilksomaticcellscount of intensivelymanagedChios-dairyewes. J HellenicVetMedSoc 69(4):1195-1204.

Kędzierska-Matysek M, Litwińczuk Z, Florek M,Barłowska J (2011) The effects of breed and other factors on the composition and freezing point of cow's milk in Poland. Int J Dairy Technol 64:336-342.

KlirŽ, Potočnik K, Antunović Z, Novoselec J, Barać Z, Mulc D,Kompan D (2015) Milk production traits from alpine breed of goats in Croatia and Slovenia. Bulg J AgricSci 21: 1064-1068.

KrálíčkováŠ, Kuchtík J, Filipčík R, Lužová T, Šustová K (2013) Effect of chosen factors on milk yield, basic composition and somatic cell count of organic milk of Brown Short-Haired goats. ActaUnivAgric Silvic Mendel Brun 61:99-105.

Kučević D, Pihler I, Plavšić M,Vuković T (2016) The composition of goat milk in different types of farmings. BiotechnolAnimHusb 32:403-412.

Kuchtík J, KrálíčkováŠ, Zapletal D, Węglarzy K,Šustová K, Skrzyżala I (2015) Changes in physico-chemical characteristics, somatic cell count and fatty acid profile of Brown Short-Haired goat milk during lactation. Anim SciPapRep33:71-83.

Maria G, Gabina D (1992) Simplification of milk recording scheme in Latxa milking sheep. Livest Prod Sci31:313-320.

Martini M, Salari F, Altomonte I, Rignanese D, Chessa S, Gigliotti C,Caroli A (2010) The Garfagnina goat: A zootechnical overview of a local dairy population. J DairySci93:4659-4667.

Mestawet TA, Girma A, Adnoy T, Devold TG, Narvhus JA,Vegarud GE (2012) Milk production, compostion and variation at different lactation stages of four goat breeds in Ethiopia.Small Rumin Res 105:176-181.

Miciński J, Zwierzchowski G, Kowalski IM, Szarek J (2013) Health-promoting properties of selected milk components. J Elem 18(1):165-186.

MinitabInc (2011) Minitab 16.1.1 for Windows. Minitab Inc., State College, PA, USA.

Official Gazette of Turkish Republic (2015) Communique on the registration of national animal breeds and lines, Turkey: National Official Gazette No: 2015/43, No: 29535.

Olechnowicz J, Sobek Z (2008) Factors of variation influencing production level, SCC and basic milk composition in dairy goats. J Anim FeedSci17:41-49

Paape MJ, Wiggans GR, Bannerman DD, Thomas DL, Sanders AH, Contreras A, Moroni P,Miller RH (2007) Monitoring goat and sheep milk somatic cell counts. Small Rumin Res 68:114-125.

Park YW, Juárez M, Ramos M, Haenlein GFW (2007) Physico-chemical characteristics of goat and sheep milk. Small RuminRes68:88-113.

Park YW (2016) Production and composition of milk are affected by multivariate factors. J Adv Dairy Res 4:131.

Pereira PC (2014) Milk nutritional composition and its role in human health. Nutrition 30:619-27.

Pizarro Borges CH, Cordeiro PRC,Bresslau S (2004) Seasonal variation of goat milk composition and somatic cell count in Southeastern Brazil. In: Internationall Symposium The Future of The Sheep and Goat Dairy Sectors, Zaragoza, Spain.

Pridalová H, Janštová B, Cupáková S, Dracková M, Navrátilová P, Vorlová L (2009) Somatic cell count in goat milk. Folia Vet 53:101-105.

Pridalová H, Janštová B, Dracková M, Navrátilová P, Vorlová L (2008) Safety and quality of milk products from goat's farm in Czech Republic.ActaSciPolTechnolAliment7:13-20.

Raynal-Ljutovac K, Lagriffoul G, Paccard P, Guillet I,Chillard Y (2008) Composition of goat and sheep milk products: an update.Small Rumin Res 79:57-72.

Reklewska B, Bernatowicz E, Reklewski Z, Kuczyñska B, Zdziarski K, Sakowski T, S£oniewski K (2005) Functional components of milk produced by Polish Black and White, Polish Red and Simmental cows. El J Pol AgriUniverAnimHusb 8(3).

Ruiz R, Oregui LM,Herrero M (2000) Comparison of models for describing the lactation curve of Latxa sheep and an analysis of factors affecting milk yield. J Dairy Sci 83:2709-2719.

Saatcı M, Elmaz Ö (2017) Honamlı, Newly registered special goat breed of Turkey. In: Sustainable Goat Production in Adverse Environments: Volume II. Springer, Cham; pp 131-146.

Sengonca M, Taşkın T,Koşum N (2003) Simultaneous comparison of various production traits of Saanen x Hair crossbred and pure Hair goats. Turk J Vet Anim Sci27:1319-1325.

Simşek ÜG, Bayraktar M,Gürses M (2006) ÇiftlikkoşullarındaKılkeçilerine ait bazıverimözelliklerininaraştırılması. FıratÜnivSağlıkBilDerg 20:221-227.

Šlyžius E, Šlyžienė B,Lindžiūtė V (2017) Factors affecting goat milk fat yield. ŽemėsŪkioMokslai24:91-100.

Soryal KA, Zeng SS, Min BR, Hart SP, Beyene FA (2004) Effect of feeding systems on composition of goat milk and yield of Domiati cheese. Small Rumin Res54:121-129.

Sousa JT de, Dias MJ, Tanezini CA, D'Alessandro WT, Oliveira BC de, Melo Rocha J de, Santos Pontes I dos (1993) Freezing point depression of raw goat's milk from the region of Goiania, Brazil. Asian-Australas J AnimSci 6:555-559.

Strzałkowska N, Jóźwik A, Bagnicka E, Krzyżewski J, Horbańczuk K, Pyzel B, Horbańczuk JO (2009) Chemical composition, physical traits and fatty acid profile of goat milk as related to the stage of lactation. AnimSci Pap Rep 27:311-320.

Strzałkowska N, Jóźwik A, Bagnicka E, Krzyżewski J, Horbańczuk K, Pyzel B, SłoniewskaD, Horbańczuk JO (2010) The concentration of free fatty acids in goat milk as related to the stage of lactation, age and somatic cell count. AnimSci Pap Rep 28:389-395.

Toledo-Alonzo P (2003) Studies of raw milk from sustainable organic production systems. Licentiate thesis, Swedish University of Agricultural Sciences, Sweden.

Tuncel E, Okuyan R (1985) Present status and future of goat husbandary in Turkey. comission on sheep and goat production. In: 35th Annual Meeting of the European Assocation for Animal Production, Halhidiki, Greece.

Turkish Food Codex (TFC) (2011) Regulation of specialhygienerulesforanimalfood. Turkey: NationalOfficialGazette No: 28155.

Wong WMJ, De Souza R, Kendall CWC, Emam A, Jenkins DJA (2006) Colonic health: fermentation and short chain fatty acids. J ClinGastroenterol 40:235 243.

Zeng SS, Escobar EN (1995) Effect of parity and milk production on somatic cell count, standard plate count and composition of goat milk. Small Rumin Res 17:269-274.

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Research article
Ερευνητικό άρθρο

Sarcoptes scabiei dermatitis in adult sheep: an immunohistochemical study of 34 chronic cases with extensive lesions

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ABSTRACT: Ovine sarcoptic mange is a contagious ectoparasitic skin disease, seen in many countries with sheep production. Although several studies concerning dermatopathology have been published, the local cutaneous immune response to *Sarcoptes scabiei* has not been studied by immunohistochemistry. The present study aims to evaluate immunohistochemically the adaptive cellular immune response in chronic natural cases with extensive gross lesions. Facial and foot skin biopsies of 32 ewes and 2 rams were obtained, and moreover from the scrotal scabietic lesions of the 2 rams. Each biopsy was bisected and processed for paraffin and cryostat sections. Mites were not observed in the vast majority of skin histology sections. Epidermal hyperplasia and chronic inflammation were the main histopathologic features. The dermal inflammatory infiltrate was mixed, dominated by eosinophils and lymphocytes equally. Tissue sections immunostained with a panel of monoclonal antibodies showed among lymphocytes an almost exclusively T-cell population (CD3+), while CD79a + cells were sparse. T-helper cells (CD4+) were predominant versus T-cytotoxic cells (CD8+) in 4:1 to 5:1 ratios. The mixed inflammatory infiltrate combined with the immunohistochemical findings suggest both a type-I and type-IV hypersensitivity reactions during the chronic course of the disease. Moreover, all these chronic cases in adult sheep are recorded into the hypersensitivity form of sarcoptic mange ("classical or ordinary" scabies) and no cases of the hyperkeratotic form of the disease ("Norwegian or crusted" scabies) were found.

Keywords: adult sheep; *Sarcoptes scabiei;* extensive lesions; immunohistochemistry; adaptive cellular immune response

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INTRODUCTION

Sarcoptic mange or scabies is a parasitic skin disease of humans, domestic and wild mammals caused by *Sarcoptes scabiei* mite. It is characterized by a complex immunopathology with innate and adaptive immune mechanisms (Pence et al., 2002; Bhat et al., 2017). In general, the distribution and severity of the cutaneous lesions as well as the disease outcome vary among host species or among individuals of the same species, due to regional differences in the cutaneous microarchitecture and lipids, the different level of immune response and/or different stages of the disease (Salvadori et al., 2016; Arlian and Morgan, 2017; Bhat et al., 2017). According to histopathological features and cellular immune response in dermatopathological lesions, two main forms of the disease have been described in human and many animal species: a) the hypersensitivity form, and b) the hyperkeratotic form (Pence and Ueckermann, 2002; Bhat et al., 2017; Mauldin and Peters-Kennedy, 2016).

The hypersensitivity form of sarcoptic mange (also known as "classical or ordinary" scabies) is an intensely pruritic dermatitis, typically seen in almost all infected human and animals (Bhat et al., 2017). No or very few mites have been observed in skin sections along with a mixed inflammatory infiltrate consisting of eosinophils and T-lymphocytes. Macrophages, neutrophils, mast cells, as well as a few B-cells and plasmocytes have also been observed (Pence and Ueckermann, 2002; Mauldin and Peters-Kennedy, 2016). In humans, dogs and pigs CD4+ cells have been demonstrated as the most prevalent T-lymphocyte subpopulation in inflammatory skin lesions compared to CD8+ cells (Arlian et al., 1997; Gallardo et al., 2002; Liu et al., 2014). This effective cell-mediated immune response limits the spread of mites, preventing overwhelming infestation and widespread lesions in human or animal hosts (Salvadori et al., 2016; Arlian and Morgan, 2017; Bhat et al., 2017).

The hyperkeratotic form of sarcoptic mange (also known as "Norwegian or crusted scabies") has been reported both in human and veterinary literature. As far as domestic mammals, it has been diagnosed in pigs, dogs and cats (Goyena et al., 2013; Mauldin and Peters-Kennedy 2016). Some cases have been reported in wild mammals as well (Pence and Ueckermann, 2002). Norwegian scabies tends to occur in undernourished or immune-compromised individuals (Pence and Ueckermann, 2002). A high number of mites are seen in skin sections with the dermal inflammatory infiltrate dominated by lymphocytes than eosinophils (Pence and Ueckermann, 2002; Mauldin and Peters-Kennedy, 2016).

Immunohistochemistry applied in skin biopsies obtained from human and pig with Norwegian scabies, has shown a higher number of CD8+ T cells compared to CD4+ cells, and absence of B cells in the dermis (Gallardo et al., 2002; Walton et al., 2008; Liu et al., 2014). The impaired cell-mediated immunity allows *Sarcoptes scabiei* mites to multiply in extremely high numbers within widespread lesions (Arlian and Morgan, 2017; Bhat et al., 2017).

Ovine sarcoptic mange is a disease, prevalent in many Mediterranean and Middle East countries (Fthenakis et al., 2000; Hidalgo-Arguello et al., 2001; Rahbari et al., 2009). *Sarcoptes scabiei* tends to affect the non-woolly body regions (Abu-Samra et al., 1981; Rahbari et al., 2009). The infestation usually begins near the mouth (lips, nostrils) and spreads to the ear pinnae, head, legs and other non-woolly areas, such as scrotum, mammary gland and perineum. Skin lesions include pustules, alopecia, severe scaling and thick crusts, as well as fissures and less frequently excoriations due to intense pruritus (Hidalgo-Arguello et al., 2001; Rahbari et al., 2009; Rodriguez-Cadenas et al., 2010). Generalized lesions have only been observed in the more hairy desert sheep of the Sudan (Abu-Samra et al., 1981). Sarcoptic mange may affect both lambs and adult sheep leading to growth retardation, reduced milk yield and adverse reproductive effects (Fthenakis et al., 2000; Fthenakis et al., 2001).

There has been so far no immunohistochemical study on ovine sarcoptic mange and its immunopathology. Thus, the aim of this study was to evaluate the adaptive cellular immune response in chronic cases of ovine scabies.

MATERIALS AND METHODS

Animals & study design

A total of 44 sheep were included in the study. Thirty four (34) adult sheep (32 crossbreed Karagouniko ewes and 2 Karagouniko rams), aged 1.5 to 5 years old, with chronic dermatitis due to *Sarcoptes scabiei* used. In addition, 10 clinical healthy crossbreed Karagouniko ewes used as controls.

All affected animals (Figures 1, 2) were selected from 5 naturally infested flocks in the Prefecture of Thessaly, Greece. Inclusion criteria included the pres-

ence of *Sarcoptes scabiei* mites in skin scrapings, at least 3 months duration of skin disease and no use of acaricide treatment (either topical or systemic) over the past 6 months.

Figure 1. An ewe with severe chronic lesions all over the head extending to ventral part of the upper neck. The main features are alopecia and skin thickening with deep cracks. The arrow indicates the biopsy site

Figure 2. A ram with cutaneous chronic lesions of sarcoptic mange, affecting the feet as well as the prepuce and scrotum. The arrow indicates the biopsy site

The study was completed in 2 phases: a) the 27 cases with sarcoptic mange and the 10 healthy sheep (controls) were selected, examined and evaluated during a doctoral thesis study; b) the next 7 cases with extensive lesions of chronic sarcoptic mange were selected and examined during a 6 month post-doctoral survey.

Tissue Samples

Two (2) skin punch biopsies (8mm), from the face and front feet of affected animals and controls were obtained under local anesthesia with lidocaine. In addition, a biopsy was also obtained from the scabietic scrotum of the two affected rams. Each biopsy was bisected, with one half fixed in 10% buffered formalin for 24 hours, dehydrated in a graded series of ethanol and xylenes, and embedded in paraffin. The other half was put in optimal cutting temperature compound (Tissue-Tek O.C.T compound, Polysciences Inc, USA), immersed in isopentane, cooled to its freezing point in liquid nitrogen and stored at -80^{o}C, until cryosectioning.

A submandibular lymph node of a clinically healthy lamb, sampled within 30 minutes after slaughter in a local slaughterhouse and treated as described above, served as positive control during immunohistochemistry.

Histopathology

The paraffin embedded skin biopsies were sectioned at 5-μm and tissue sections were stained with hematoxylin-eosin (H-E) according to a standard protocol.

Immunohistochemistry (IHC)

Immunohistochemical staining was applied on paraffin and cryostat sections, using a panel of monoclonal antibodies (mouse anti-sheep or mouse anti-human with known cross reactivity to sheep): CD3 as pan T-cell marker; CD4 for helper T-lymphocytes; CD8 for cytotoxic T-lymphocytes; CD79a for both B-lymphocytes and plasmocytes, according to Perez et al., 2005; Gulbahar et al., 2006; Vismarra et al., 2015.

Specific technical details of monoclonal antibodies used are presented in Table-1.

i) Paraffin sections

Immunostaining for CD3 and CD79a was carried out in paraffin-embedded 5-μm tissue sections, which were placed in a 60^{0}C oven for 30 minutes and rehydrated by sequential immersion in xylene, graded concentrations of ethanol, and distilled water.

Antigen retrieval for CD3 antibody was performed by heating the sections in Trilogy solution (Cell Marque) for 20min at 850W microwave oven (the Trilogy solution was preheated for 5 min at 450W, before mmersing the sections). Antigen retrieval for CD79a antibody was performed by heating the sections in Target Retrieval Solution-pH 9 (DAKO) for 20min at 850W microwave oven (the Target Retriev-

al Solution-pH 9 was preheated for 5 min at 450W, before mmersing the sections). Sections were washed with distilled water and washing buffer (Tris-buffered NaCl Solution with Tween 20 (TBST), pH 7.6). Endogenous peroxidase activity was blocked by 3% hydrogen peroxide (H_2O_2) for 10 minutes at room temperature. Subsequently, the sections were washed with buffer and incubated with the primary antibodies at room temperature (Table-1). Then, the sections were washed with the buffer and incubated with the Envision polymer (EnVision™ Detection system, K5007, Dako) for 45 minutes. The signal was developed in 3,3'-diaminobenzidine (DAB) solution for 5 minutes and finally counterstained with Mayer's hematoxylin. After detection, sections were dehydrated with graded ethanol and xylenes, and were finally coverslipped.

ii) Cryostat sections

Immunostaining for CD4 and CD8 was carried out in 5-μm cryostat sections, air-dried at room temperature for 20 minutes. The sections were washed with buffer and incubated with the primary antibodies at room temperature (Table-1). Following incubation with the primary antibody, the steps described above were followed without any other modification.

For all immunohistochemical reactions, histological sections of a healthy lamb lymphnode, treated as described above, were used as positive and negative controls. Each primary antibody was replaced by non-immunogenic mouse serum (Mouse Gamma Globulin, 015-000-002; Dianova, Germany) as control for nonspecific binding of the secondary antibody.

Table 1. Technical details of the monoclonal antibodies used

antigen	*clone*	*Isotype*	*specificity*	*source*	*sections*	*dilution*	*incubation time*
CD3	F7.2.38	IgG1	mouse anti-human	**Dako-Agilent Technologies; Santa Clara, California, USA**	paraffin	1/200	30 min
CD4	GC50A1	IgM	mouse anti-sheep	**VMRD, Inc; Pullman, Washington, USA**	cryostat	1/50	4 hours
CD8	CACT80C	IgG1	mouse anti-sheep	**VMRD, Inc; Pullman, Washington, USA**	cryostat	1/200	60 min
CD79a	HM57	IgG1	mouse anti-human	**Dako-Agilent Technologies; Santa Clara, California, USA**	paraffin	1/50	3 hours

Interpretation of the results

Positive cell counting for markers of T-lymphocytes subpopulations (CD3+, CD4+, CD8+), B-lymphocytes and plasmocytes (CD79a+) in the epidermis and dermis was performed under a light microscope. The tissue slides were evaluated by two independent veterinary pathologists (DD and DT). The examiners assessed the expression pattern and evaluated the localization of the immunollabelling cells. The positive cells were determined by a semiquantitative morphometric protocol according to Walton et al. 2008 and modified as follows: - *(none);* + *(sparse/very few);* ++ *(few);* +++ *(some);* ++++ *(many);* +++++ *(abundant).*

For each skin biopsy the CD4:CD8 ratio was estimated as a ratio of integers (in a range of 1:1 to 6:1) by comparing the densities of dermal lymphocytic subpopulations into IHC sections.

Statistical analysis

Student's t-test was used to calculate and compare the mean of CD4:CD8 ratios separately on facial and foot skin between scabietic and control sheep. A p-value equal to or less than 0.05 was considered significant. Statistical analysis was performed using SPSS 16.0 software for Windows (SPSS Inc.)

A digital representative image of each tissue slide was captured (Figures 3-9), using the NIKON ECLIPSE E-200 microscope equipped with Fi1-L2 Digital System (NIKON, Japan).

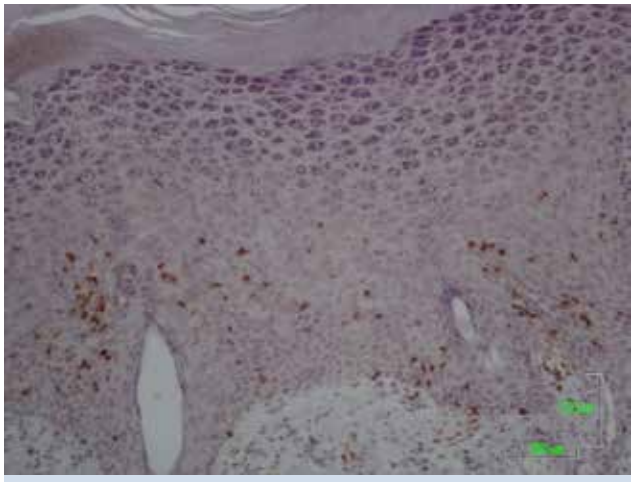

Figure 3. Paraffin section of facial skin from a ewe with sarcoptic mange: exocytosis of CD3+ T-lymphocytes in hyperplastic epidermis. IHC, EnVision™ detection system

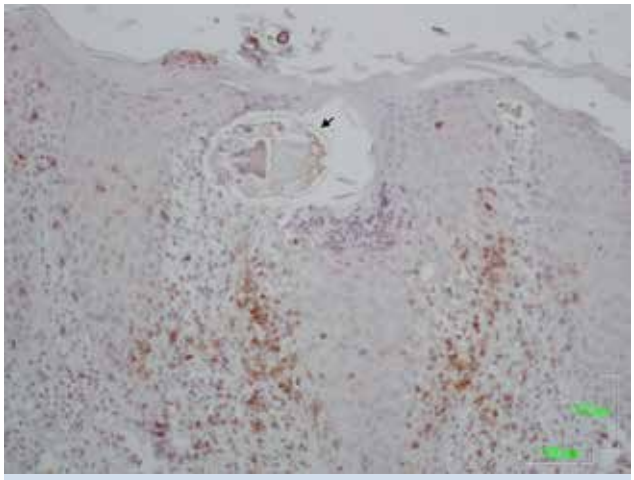

Figure 4. Paraffin section of foot skin from a ewe with sarcoptic mange: infiltration of CD3+ T-lymphocytes in upper dermis (dermoepidermal area) and exocytosis toward a *Sarcoptes scabiei* mite (arrow), burrowing in epidermis. IHC, EnVision™ detection system

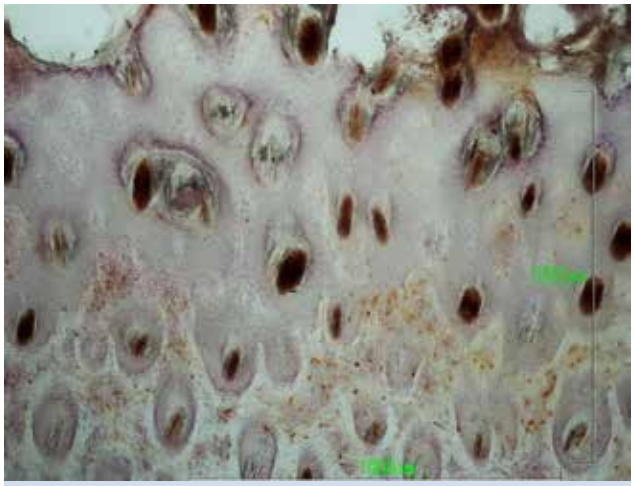

Figure 5. Cryostat section of foot skin from a ewe with sarcoptic mange: infiltration of CD4+ T-lymphocytes in upper dermis. IHC, EnVision™ detection system

Figure 6. Cryostat section of foot skin from a ewe with sarcoptic mange: mild infiltration of CD8+ T-lymphocytes in upper dermis, as well as focal lymphocytic exocytosis in epidermis. IHC, EnVision™ detection system

Figure 7. Paraffin section of foot skin from a ewe with sarcoptic mange: there are few CD79a+ cells in the dermis. IHC, EnVision™ detection system.

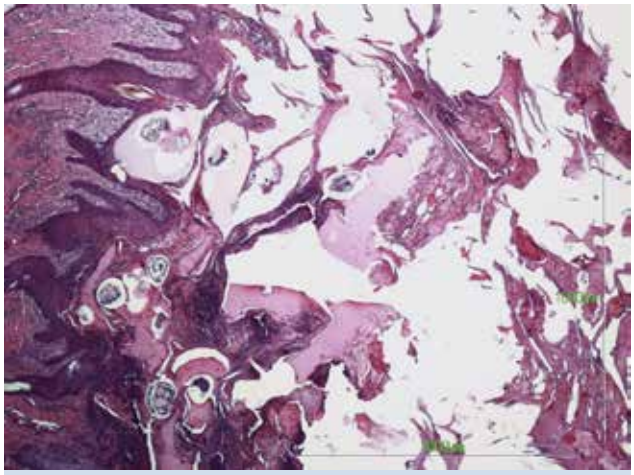

Figure 8. Paraffin section of scrotum skin from a ram with sarcoptic mange: occurrence of many *Sarcoptes scabiei* mites in thick serocellular crusts. Haematoxylin-Eosin stain

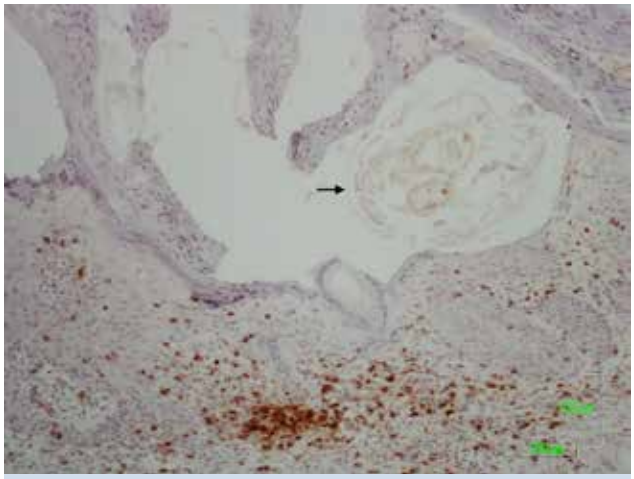

Figure 9. Paraffin section of scrotum skin from a ram with sarcoptic mange: notice the *Sarcoptes scabiei* mite (arrow) burrowing into parakeratotic stratum corneum associated with exocytosis of CD3+ T-lymphocytes in epidermis and severe focal dermoepidermal inflammation. IHC, EnVision™ detection system

RESULTS

a) Dermatitis due to *Sarcoptes scabiei*

In all affected ewes and rams, skin biopsy sections from the face and feet showed similar histopathological features. No significant differences between the prevalence of mites on the two different body sides were observed. In the vast majority of sections mites could not be observed, while in a small percentage of biopsies 1-2 mites were found per section. The main histopathological lesions of all facial and feet biopsies were epidermal hyperplasia, orthokeratotic hyperkeratosis, patchy hypergranulosis, acanthosis and pronounced rete ridge formation In a few sections in which mite (s) were present, focal parakeratotic hyperkeratosis was observed around the parasitic burrow, as well as spongiosis, especially beneath or at the sides of the burrow. Moreover, multifocal crusting was observed in some sections. The epidermis was characterized by exocytosis of eosinophils and neutrophils, forming micropustules in some fields, as well as T-lymphocytes (CD3+), found either scattered (Figure-3) or centered beneath the sites containing a mite (Figure-4). In particular, T-cells were cytotoxic CD8+cells, since CD4+ and CD79a+ cells were completely absent throughout the epidermis.

In the dermis, histopathology revealed a mixed inflammatory infiltrate, with high numbers of eosinophils and lymphocytes and lower one of histiocytes. The distribution pattern of inflammatory infiltrate was mainly dermoepidermal to deep perivascular and/or diffuse; and rarely focal periadnexal, especially when the parasitic burrows were located at the infundibulum of hair follicles. The immunohistochemical staining revealed that the predominant lymphocytes in dermal infiltrates were T-lymphocytes (CD3+), with the main subpopulation being CD4+ (Figure-5) as opposed to CD8+ cells (Figure-6). In each anatomical region, the CD4+:CD8+ ratio ranged from 4:1 to 5:1. B-lymphocytes and plasmocytes (CD79a+) cells were sporadically seen (Figure-7). The semi-quantitative evaluation of lymphocytic subpopulations and CD4+:CD8+ ratio are presented in Table-2.

The 2 biopsies from scabietic scrotum showed a relatively larger number of mites in hyperplastic epidermis in comparison to facial and foot biopsies of the same individual. Each skin section of scrotum contained 4-7 mites at multiple levels within stratum corneum. Severe orthokeratotic hyperkeratosis along with extensive parakeratosis and severe crusting and spongiosis were observed (Figure-8). Exocytosis of lymphocytes, eosinophils and neutrophils was seen in epidermis. Immunohistochemistry revealed a mild epidermal exocytosis and severe focal dermoepidermal infiltration of CD3+ cells (Figure-9). Moreover, the dermal CD4+:CD8+ ratio was estimated to 4:1 approximately.

B) Healthy skin (controls)

The skin of healthy sheep (controls) showed a normal histological structure, as it was expected. No evidence of epidermal hyperplasia was seen. Eosinophils and neutrophils were not observed. Very few lymphocytes were seen around blood vessels at the upper dermis. Semi-quantitative evaluation of lymphocyte subpopulations in the dermis and CD4+:CD8+ ratios are presented in Table-2.

Table 2. Semi-quantitative evaluation of the dermal lymphocyte subpopulations of facial and foot skin (scabietic vs. healthy skin)

Lymphocytes	Facial skin with scabies (n=34)	Healthy facial skin (n=10)	Front foot skin with scabies (n=34)	Healthy front foot skin (n=10)
CD3+	+++++	+	+++++	+
CD4+	++++	+	++++	+
CD8+	++	+	++	+
CD79a+	- to +	-	- to +	-
CD4:CD8 (mean)	**4,7 : 1**	**1,2 : 1**	**4,4 : 1**	**1,5 : 1**

- (none); + (sparse/very few); ++ (few); +++ (some); ++++ (many); +++++ (abundant)

DISCUSSION

The current study presents histopathological and immunohistochemical findings, concerning cases of sarcoptic mange in adult sheep with chronic extensive lesions.

As far as the histopathology of sarcoptic mange in domestic and wild mammals, the main features on the basis of which the distinction between the two forms of the disease is made are the number of mites throughout the hyperplastic epidermis, as well as the dominant cell type infiltrating dermis (Pence and Ueckermann, 2002; Mauldin and Peters-Kennedy, 2016). Thus, the histopathological lesions of all facial and feet biopsies of our ovine chronic cases are compatible with the "classical (hypersensitivity) form" of the disease, because very few mites were observed in stratum corneum, while the dermal inflammatory infiltrate was mixed, consisting mainly of eosinophils and lymphocytes (Pence and Ueckermann, 2002; Mauldin and Peters-Kennedy, 2016).

On the contrary, in the sections of the scrotum of the two rams many mites were seen at multiple levels in the stratum corneum, while intense orthokeratotic hyperkeratosis was observed, as well as parakeratosis. The increased number of mites in parakeratotic stratum corneum is the main feature of the "hyperkeratotic (Norwegian) form" of sarcoptic mange (Pence and Ueckermann, 2002). However, the mixed inflammatory infiltrate in scrotum sections composed mainly by eosinophils and lymphocytes is a diagnostic feature not related to the "Norwegian form" of sarcoptic mange (Pence and Ueckermann, 2002; Mauldin and Peters-Kennedy, 2016). It is worth noting that the Norwegian form of sarcoptic mange has not been recorded in sheep to date, even in cases where more than 1-2 mites have been observed into histopathological sections. For example, during an experimental study of ovine sarcoptic mange the presence of many mites has been recorded in chronic lesions (7-9 weeks post infection) without being considered as Norwegian scabies. In these experimental cases, the inflammatory infiltrate was mixed consisting of lymphocytes, eosinophils, macrophages and a few neutrophils (Ibrahim and Abu-Samra, 1987). On the other hand, the presence of many *Sarcoptes scabiei* mites with the complete absence of eosinophils in inflammatory infiltrate has been recorded in a case of fatal sarcoptic mange in Blue Sheep (*Pseudois nayaur*), suggesting the lack of an appropriate immune response to the parasite or other coping strategies because there has been no abatement of the clinical signs in affected animals over several years (Dagleish et al., 2007).

In addition, according to modern literature, the diagnosis of "Norwegian form" of sarcoptic mange is complex, based both on the histopathological features and on CD4:CD8 lymphocytic ratio in dermal lesions (Bhat et al., 2017). The role of CD4+ and CD8+ lymphocytes as well as all immunohistochemical findings of the present study are discussed below.

Immunohistochemistry has already been applied in some ovine ectoparasitoses. The immunophenotype of lymphocyte subpopulations has been studied in healthy ovine skin (Gorrell et al., 1995; McElroy et al., 1998), as well as in skin lesions induced by the ectoparasites *Lucilia cuprina, Hyalonema anatolicum* and *Psoroptes ovis* (Bowles et al., 1992; Boppana et al., 2005; Van den Broek et al., 2005), or infectious agents, such as orf and sheep pox (Jenkinson et al., 1992; Gulbahar et al., 2006).

Our study demonstrated a significant infiltration of T-cells (CD3+) into ovine scabietic skin, suggesting a local T-cell mediated immune response (Arlian et al., 1997; Salvadori et al, 2016; Bhat et al., 2017). The exocytosis of cytotoxic CD8+ T-lymphocytes throughout epidermis or centered beneath the sites in stratum corneum containing a mite is a characteristic feature, which has also been reported in experimental sarcoptic mange in dogs (Arlian et al., 1997). Also, CD8+ lymphocytes may induce dysregulated keratinocyte apoptosis contributing to the elicitation and progress of epidermal hyperproliferation (Salvadori et al, 2016). The predominance of CD4+ T-lymphocytes in the dermis is a feature similar to that has been observed in humans, pigs and dogs with the hypersensitivity form of sarcoptic mange (Gallardo et al., 2002; Bhat et al., 2017). CD4+:CD8+ ratio in the dermal infiltrate has been evaluated to at least 4:1, as it has also been reported in humans with ordinary scabies (Cabrera et al., 2005) and sheep with *Lucilia cuprina* myiasis (Bowles et al., 1992). It is likely that the ectoparasite-derived antigens in ovine skin cause similar immune responses regardless of the parasite involved.

In general, *Sarcoptes scabiei* mites, as they penetrate and burrow through the epidermis, produce a variety of antigens (both secretory and excretory) evoking a complex immune response (Arlian and Morgan, 2017). The type of immune response is largely dependent on the general immune status of the host (Pence

and Ueckermann, 2002). In humans and animals the classical (hypersensitivity) form of sarcoptic mange elicits a combined type-I and type-IV reactions (Pence and Ueckermann, 2002; Skerratt, 2003; Espinosa et al., 2017; Niedringhaus et al., 2019).

The high number of CD4+ cells (helper T-lymphocytes) in dermal infiltrate in ovine sarcoptic mange suggests the recognition of specific parasitic antigens by these cells, as it has also been reported in bovine skin infestation by lice (Milnes et al., 2007). The interaction of mite antigens with specific CD4+ cells may lead to massive influx of eosinophils observed in sheep psoroptic mange lesions (van den Broek et al., 2005).

Cutaneous eosinophilia due to *Sarcoptes scabiei* infestation corresponds to type-I (immediate) hypersensitivity reaction, as it has been commonly observed in dermatitis associated with other ectoparasites (Skerratt, 2003; Nimmervoll et al., 2013). Studies on type-IV (delayed-type) hypersensitivity reactions in humans, pigs, and sheep have shown that the participating lymphocytes are mostly CD4+ T-cells with a fewer CD8+ T-cells (Pyrah and Watt, 1995; Jorundsson et al., 1999). Moreover, T-lymphocytes entered the dermis in sarcoptic mange corresponds to a type-IV (delayed) hypersensitivity response (Skerratt, 2003). The inflammatory dermal infiltrate observed in this study likely suggests a combined type-I and type-IV hypersensitivity reaction. Thus, our cases are considered chronic cases of the hypersensitivity form of sarcoptic mange ("classical or ordinary form").

In human dermatopathology, the predominance of CD8+ T-cells with few CD4+ T-cell in the dermal infiltrate and absence of B-cells are significant immunohistochemical features of "Norwegian scabies" (Gallardo et al., 2002; Walton et al. 2008). As far as the two cases of rams, their scrotal lesions could not be considered as a "hyperkeratotic (Norwegian) form" of sarcoptic mange. The dermal inflammatory infiltrate was composed equally by eosinophils and CD3+ T-lymphocytes whith the predominance of CD4+ T-helper cells. These features are compatible to the hypersensitivity form of sarcoptic mange ("classical or ordinary" scabies). This does not rule out the possibility of "Norwegian scabies" existence in few infected immunocompromised sheep individuals, especially in flocks with endemic sarcoptic mange for very long time even years. A study in pigs has also provided evidence that in herds with long-standing exposure to *Sarcoptes scabiei*, the infection becomes highly over dispersed with large mite populations present only in a few pigs and in specific body areas (Goyena et al., 2013).

The differences in histopathological features of cutaneous inflammation, between different body regions in an individual, are correlated with the different degree of disease severity and chronicity in each region (Van den Broek et al., 2004; Nimmervoll et al., 2013). In ovine psoroptic mange, the histopathological lesions at the advancing margin of an extensive lesion were more severe than those at the initial site of infestation, and this was reflected by the numbers of mites present (Van den Broek et al., 2004). According to the history, the scabietic lesions on the scrotum followed those on the face and legs. Moreover, the regional differences in the cutaneous microarchitecture and lipids (Lyne and Hollis, 1968; Arlian and Morgan, 2017) and the interaction of mite with local microenvironment and microbiome (De Candia et al., 2019) may have an impact on the involved immunopathologic mechanisms and parasitic load.

T-lymphocytes (CD3+ cells) include CD4+, CD8+ and γδ+ subpopulations. The lymphoid system of ruminants contains a large number of γδ+ T cells, in contrast to human, dog and cat. In sheep, they are more prevalent in lambs than in ewes (Hein and Mackay, 1991; Watson et al., 1994). In healthy ovine skin, γδ+ T-cells are the predominant lymphocyte subpopulation in wooled body regions regardless of age (McElroy et al., 1998). Some immunohistochemical studies have tried to investigate their involvement both in innate and adaptive immune mechanisms in ovine skin diseases (Bowles et al. 1992; Jorundsson et al. 1999; Boppana et al., 2005; van den Broek et al., 2005; Gulbahar et al. 2006).

As far ovine sarcoptic mange, there have been no reports on the role of γδ+ T-cells play in its immunopathogenesis. Even if the chronic cases of recent study involve adult sheep, with lesions located only in the non-wooled body regions, the participation of γδ+ T-cells in the inflammatory infiltrate should not be considered negligible, because these cells have not been immunohistochemically investigated. This limitation of our study on the specific role of γδ+ T-cells in immunopathology of ovine sarcoptic mange could be the subject of a future research study. In fact, the evaluation of the nature of γδ+ T-cell cutaneous response in ruminants would require both cell immunophenotyping and investigation of the local cytokines profile (Milnes et al., 2007; Shu et al., 2009).

CONCLUSSION

In adult sheep the dermal inflammatory infiltrate in chronic scabietic lesions is mixed, dominated by eosinophils and lymphocytes equally. The immunophenotypical characterization of lymphocytes subpopulations showed almost exclusively T-cell population (CD3+) with predominant T-helper cells (CD4+) versus T-cytotoxic cells (CD8+) in 4:1 to 5:1 ratios. The mixed inflammatory infiltrate combined with the immunohistochemical features suggest a type-I and type-IV hypersensitivity reaction during the course of the disease. In conclusion, all our chronic cases in adult sheep are recorded into the hypersensitivity form of sarcoptic mange ("classical or ordinary" scabies) and no cases of the hyperkeratotic form of the disease ("Norwegian or crusted" scabies) were found.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest to declare.

REFERENCES

Abu-Samra MT, Hago BE, Aziz MA, Awad FW (1981) Sarcoptic mange in sheep in the Sudan. Ann Trop Med Parasitol 75: 639-645.

Arlian LG, Rapp CM, Stemmer BL, Morgan MS, Moore PF (1997) Characterization of lymphocyte subtypes in scabietic skin lesions of naive and sensitized dogs. Vet Parasitol 68: 347-358.

Arlian LG, Morgan MS (2017) A review of Sarcoptes scabiei: past, present and future. Parasit Vectors 10: 297.

Bhat SA, Mounsey KE, Liu X, Walton SF (2017) Host immune responses to the itch mite, Sarcoptes scabiei, in humans. Parasites & Vectors 10: 385.

Boppana DK, Wikel SK, Raj DG, Manohar MB, Lalitha J (2005) Cellular infiltration at skin lesions and draining lymph nodes of sheep infested with adult *Hyalomma anatolicum anatolicum* ticks. Parasitology 131: 657-667.

Bowles VM, Grey ST, Brandon MR (1992) Cellular immune responses in the skin of sheep infected with larvae of *Lucilia cuprina*, the sheep blowfly. Vet Parasitol 44: 151-162.

Cabrera R, Guarda R, Palisson F, Gonzalez S (2005) Parasitic infections. In: Bos JN (Ed), Skin Immune System, 3rd ed, CRC Press, Boca Raton: pp 680-681.

Dagleish MP, Ali Q, Powell RK, Butz D, Woodford MH (2007) Fatal *Sarcoptes scabiei* infection of blue sheep (*Pseudois nayaur*) in Pakistan. J Wildl Dis 43: 512-517.

De Candia AL, Leverett KN, von Holdt BM (2019) Of microbes and mange: consistent changes in the skin microbiome of three canid species infected with *Sarcoptes scabiei* mites. Parasites & Vectors 12:488

Espinosa J, Raez-Bravo A, Lopez-Olvera JR, Perez JM, Lavin S, Tvarijonaviciute A, Cano-Manuel FJ, Fandos P, Soriguer RC, Granados JE, Romero D, Velarde R (2017) Histopathology, microbiology and the inflammatory process associated with *Sarcoptes scabiei* infection in the Iberian ibex, *Capra pyrenaica.* Parasites & Vectors 10: 596.

Fthenakis GC, Papadopoulos E, Himonas C, Leontides L, Kritas S, Papatsas J (2000) Efficasy of moxidectin against sarcoptic mange and effects on milk yield ewes and growth of lambs. Vet Parasitol 87: 207-216.

Fthenakis GC, Karagiannidis A, Alexopoulos C, Brozos C, Papadopoulos E (2001) Effects of sarcoptic mange on the reproductive performance of ewes and transmission of *Sarcoptes scabiei* to newborn lambs. Vet Parasitol 95: 63-71.

Gallardo F, Barranco C, Toll A, Pujol RM (2002) CD30 antigen expression in cutaneous inflammatory infiltrates of scabies: a dynamic immunophenotypic pattern that should be distinguished from lymphomatoid papulosis. J Cutan Pathol 29: 368-373.

Gorrell MD, Townsend WL, Ladds PW (1995) The distribution of lymphocyte subpopulations in normal and acanthotic ovine skin. Vet Immunol Immunopathol 44: 151-167.

Goyena E, Ruiz de Ybanez R, Martinez-Carrasco C, Balseiro A, Alonso de Vega F, Casais R, Prieto M, Garcia-Marin JF, Berriatua E (2013) On the aggregated nature of chronic *Sarcoptes scabiei* infection in adult pigs. Vet Parasitol 192: 301-306.

Gulbahar MY, Davis WC, Yuksel H, Cabalar M (2006) Immunohistochemical evaluation of inflammatory infiltrate in the skin and lung of lambs naturally infected with sheeppox virus. Vet Pathol 43: 67-75.

Hein WR, Mackay CR (1991) Prominence of gamma delta T cells in the ruminant immune system. Immunol Today 12: 30-34.

Hidalgo Arguello MR, Diez-Banos N, Martinez-Gonzalez B, Rojo-Vazquez FA (2001) Efficacy of moxidectin 1% injectable against natural infection of *Sarcoptes scabiei* in sheep. Vet Parasitol 102: 143-150.

Ibrahim KE, Abu-Samra MT (1987) Experimental transmission of a goat strain *Sarcoptes scabiei* to desert sheep and its treatment with ivermectin. Vet Parasitol 26: 157-164.

Jenkinson DM, Hutchison G, Reid HW (1992) The B and T cell responses to orf virus infection of ovine skin. Vet Dermatol 3: 57-64.

Jorundsson E, Press CM, Ulvund M, Landsverk T (1999) Prominence of gammadelta T cells in the elicitation phase of dinitrochlorobenzene-induced contact hypersensitivity in lambs. Vet Pathol 36: 42-50.

Liu X, Walton SF, Murray HC, King M, Kelly A, Holt DC, Currie BJ, McCarthy JS, Mounsey KE (2014) Crusted scabies is associated with increased IL-17 secretion by skin T cells. Parasite Immunol 36: 594-604.

Lyne AG, Hollis DE (1968) The skin of sheep: a comparison of body regions. Aust J Biol Sci 21: 499-527.

McElroy MC, Bassett HF, Monaghan ML, Davis WC (1998) The distribution of lymphocyte subsets in normal ovine skin. Vet Immunol Immunopathol 62: 65-71.

Mauldin EA, Peters-Kennedy J (2016) Integumentary System. In: *Jubb, Kennedy and Palmer's Pathology of Domestic Animals*, 6th Edit., MG Maxie, Ed., Elsevier, St. Louis, pp 509-736.

Milnes AS, Bailey M, Knowles TG, Coles GC, L. E. Green LE M. J. Day MJ (2007) An immunohistochemical assessment of the cutaneous immune response to louse infestation in cattle. J Comp Path 136: 240-249.

Niedringhaus KD, Brown JD, Sweeley KM, Yabsley MJ (2019) A review of sarcoptic mange in North American wildlife. IJP: Parasites and Wildlife 9: 285–297

Nimmervoll H, Hoby S, Robert N, Lommano E, Welle M, Ryser-Degiorgis MP (2013) Pathology of sarcoptic mange in red foxes (Vulpes vulpes): macroscopic and histologic characterization of three disease stages. J Wildl Dis 49: 91-102.

Pence DB, Ueckermann E (2002) Sarcoptic mange in wildlife. Rev Sci Tech 21: 385-398.

Perez J, Ortega J, Bravo A, Diez-Banos P, Morrondo P, Moreno T, Martinez-Moreno A (2005) Phenotype of hepatic infiltrates and hepatic lymph nodes of lambs primarily and challenge infected with Fasciola hepatica, with and without triclabendazole treatment. Vet Res 36: 1-12.

Pyrah ITG, Watt NJ (1995) Immunohistological study of the cutaneous delayed type hypersensitivity reaction in sheep. Vet Immunol Immunopathol 48: 299-312.

Rahbari S, Nabian S, Bahonar AR (2009) Some observations on sheep sarcoptic mange in Tehran province, Iran. Trop Anim Health Prod 41: 397-401.

Rodriguez-Cadenas F, Carbajal-Gonzalez MT, Fregeneda-Grandes JM,

Aller-Gancedo JM, Huntley JF, Rojo-Vazquez FA (2010) Development and evaluation of an antibody ELISA for sarcoptic mange in sheep and a comparison with the skin-scraping method. Prev Vet Med 96: 82-92.

Salvadori C, Rocchigiani G, Lazzarotti C, Formenti N, Trogu T, Lanfranchi P, Zanardello C, Citterio C, Poli A (2016) Histological lesions and cellular response in the skin of alpine chamois (Rupicapra r. rupicapra) spontaneously affected by sarcoptic mange. Biomed Res Int 2016: 3575468.

Shu D, Pfeffer A, Pernthaner A. Cutaneous cytokine gene expression and cellular responses in lambs infested with the louse, *Bovicola ovis*, and following intradermal injection of crude louse antigen. Vet Immunol Immunopathol 2009; 129: 82-92.

Skerratt LF (2003) Cellular response in the dermis of common wombats (*Vombatus ursinus*) infected with *Sarcoptes scabiei var. wombati*. J Wildl Dis 39: 193-202.

Van den Broek AH, Else RW, Huntley JF, Machell J, Taylor MA, Miller HR (2004) Early innate and longer-term adaptive cutaneous immunoinflammatory responses during primary infestation with the sheep scab mite, *Psoroptes ovis*. J Comp Pathol 131: 318-29.

Van den Broek AH, Huntley JF, Mackellar A, Machell J, Taylor MA, Miller HR (2005) Characterisation of lesional infiltrates of dendritic cells and T cell subtypes during primary infestation of sheep with *Psoroptes ovis*, the sheep scab mite. Vet Immunol Immunopathol 105: 141-150.

Vismarra A, Mangia C, Passeri B, Brundu D, Masala G, Ledda S, Mariconti M, Brindani F, Kramer L, Bacci C (2015) Immunohistochemical study of ovine cystic echinococcosis (*Echinococcus granulosus*) shows predominant T cell infiltration in established cysts. Vet Parasitol 209: 285-288.

Walton SF, Beroukas D, Roberts-Thomson P, Currie BJ (2008) New insights into disease pathogenesis in crusted (Norwegian) scabies: the skin immune response in crusted scabies. Br J Dermatol 158: 1247-1255.

Watson DL, Colditz IG, Andrew M, Gill HS, Altmann KG (1994) Age-dependent immune response in Merino sheep. Res Vet Sci 57:152-8.

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Research article
Ερευνητικό άρθρο

Assessment of the Microbiological Profile, Species Diversity and Antimicrobial Susceptibility of Recovered Bacteria from Retail Honeys in Turkey

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ABSTRACT: The aim of this study was to assess the microbiological profile, coliform and staphylococcal species diversity, and the antimicrobial susceptibility of coliform and other Gram-negative bacteria recovered from retail honeys in Turkey. A total of 150 honey samples, including extracted honey and comb honey samples, were purchased from honey sellers. The honey samples were analyzed for total mesophilic aerobic bacteria (TMAB), total mesophilic anaerobic bacteria (TMAnB), coliforms, *Escherichia coli* (*E. coli*), *Staphylococcus* spp., lactic acid bacteria (LAB), yeasts, and molds. All presumptive coliform and Staphylococcus isolates were identified at species level and then Gram-negative isolates were screened for antimicrobial susceptibility. TMAB, TMAnB, LAB, yeasts and molds mean counts (log cfu/g) in the samples were 3.26±1.08, 3.0±0.89, 2.93±0.52, 2.90±0.83, 1.80±0.53, respectively. Eighteen point seven percent and 15.3% of extracted and comb honey contained coliform and *Staphylococcus* spp., respectively, with a mean count (MPN/g) of 8.06±1.23 and 0.71±0.66. TMAB, *Staphylococcus* spp. and yeast contamination rates were significantly higher in the extracted honeys ($P<0.05$). Presumptive coliform and *Staphyloccus* spp. isolates were mostly identified as *Klebsiella pneumoniae, Proteus mirabilis, Serratia marcescens,* and *Staphylococcus hominis* and *Staphylococcus epidermidis*, respectively. Among coliform and non-coliform Gram-negative recovered isolates, antimicrobial resistance was highest against ceftriaxone (92.4%) and cefepime (91.5%) followed by tigecyclin (46.2%). The results obtained in this study provide insight on the microbiological profile of honey and the diversity of coliform and Staphylococcus species in honey samples. Moreover, these results show that honey, which is considered beneficial for human health, may contain antibiotic-resistant bacteria.

***Keywords*:** Honey, microbiological profile, coliform, Staphylococcus, antimicrobial susceptibility

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INTRODUCTION

Owing to its several beneficial biological effects, including antioxidant (Aljadi and Kamaruddin, 2004), antimicrobial (Gomes et al., 2010), and anti-inflammatory (Tonks et al., 2003) activity, the consumption of honey, produced by honeybees (*Apis mellifera*), contributes to the health and well-being of humans. In the past few years, the production and consumption of honey have displayed a steady increase at the global level. This is attributed to the increase in the global population, the interest of consumers of all age groups, including young people, in natural food products, and the variety of food products containing honey (Garcia, 2018). To date, literature reports on honey have mostly focused on the physicochemical properties (El Sohaimy et al., 2015; Chakir et al., 2016; Boussaid et al., 2018; Kavanagh et al., 2019) and antimicrobial activity (Mercan et al., 2007; Sherlock et al., 2010; Stagos et al., 2018) of honey. However, although honey stops the growth of many microorganisms because of its composition (high concentration of sugar and a low water activity), throughout the different stages of the production chain from the hive to the table [primary (pollen, flower, honeybee digestive tract) and secondary (human, equipment, containers, wind, dust, soil etc.)], honey can be contaminated by microorganisms, which may alter the shelf life of the product and/or cause foodborne diseases (Olaitan et al., 2007; Grabowski et al., 2017). Honey being consumed without undergoing any prior heat treatment or preservation techniques requires strict attention to be paid to good manufacturing practices during its production. Previously reported studies from different countries have shown that retail honey can contain vegetative and spore-forming bacteria, yeast and mold (Ceauşi et al., 2009; Kačániová et al., 2012; Dümen et al., 2013; Erkan et al., 2015; Kunová et al., 2015; Moujanni et al., 2017; Combarros-Fuertes et al., 2019). Besides, most of these studies focused on determining microbiological profile rather than microbial diversity at species level from the samples. Mostly, the reported studies for microbiological profile varies globally but these are influenced by the detection methods which have different sensitivity and specificity, the region and the study design. In recent years, the rapid development of antibiotic resistance in several bacteria, and reports showing the role of bacteria originating from food, animals, and the environment in certain infectious diseases affecting humans (Manges, 2016; Bhatta et al., 2016; WHO, 2016), have increased the importance of genus/species identification and antimicrobial resistance detection in bacteria isolated not only from human clinical specimens but also from food, animal and environmental samples. Antibiotic resistant Gram negative bacteria are a serious problem in clinical settings and increase the morbidity and mortality in humans (Cosgrove, 2006; Kollef et al., 2008). There is very limited data in literature on antibiotic resistant Gram negative bacteria, including coliforms, isolated from honey (Hleba et al., 2014). However, the few reported studies on isolates from the digestive tracts of honey bees have found the Gram negative bacteria to be resistant to different antibiotic classes (Tian et al., 2012; Bezirtzoglou et al., 2016; Gasper et al., 2017; Kačániová et al., 2017). To the best our knowledge, there is no previous study that systemically focused on the determination of microbiological profile including coliform and Staphylococcus species diversity and antibiotic susceptibility in coliform and Gram negative bacteria recovered from retail honeys in Turkey. Therefore, the aim of this study was i) to assess the microbiological profile of honeys ii) to determine both coliform and Staphylococcus isolates at genus and species level, and iii) to screen antimicrobial susceptibility in the recovered coliform and non-coliform Gram negative bacteria isolates.

MATERIAL AND METHODS

Study design and sample collection

A cross-sectional study was conducted from July 2017 to June 2018 in the Diyarbakir province located in the Southeast Anatolia Region of Turkey. In total, 150 honey samples were collected from different sale points including honey sellers and markets in four districts (Baglar, Kayapinar, Yenisehir and Sur) of Diyarbakir province. The number of samples per area was determined according to the relative population size of the districts (TUİK, 2016). The sample numbers of the analyzed honey types (extracted or comb honey) were determined in view of the consumption levels of extracted honey and comb honey in Turkey (Soylu et al., 2018; Baki et al., 2017). Eventually, 106 extracted honey (71%) and 44 comb honey (29%) samples were collected. The samples were collected into sterile 100-ml containers (labelled with numbers, place and date of collection) and transferred in cold boxes at 4°C to the laboratory of the Department of Food Hygiene and Technology of Dicle University for microbiological analysis.

Microbiological analysis

For microbiological analysis, honey samples were

taken aseptically, using a sterile spatula and/or scalpel (for comb honey), from the sample containers. Ten grams of each honey sample was mixed with a ninefold volume of 0.1% peptone water in a sterile plastic bag, and homogenized for 60 s with a stomacher (Easy Mix-G560E, France). Subsequently, 10-fold serial dilutions were prepared of each sample with 0.1% peptone water. The pour plate technique was used for enumerating total aerobic mesophilic bacteria (TMAB), total anaerobic mesophilic bacteria (TMAnB), lactic acid bacteria (LAB), yeasts and molds in the honey samples. The TMAB count was enumerated on plate count agar (PCA) after incubation at 30°C for 72 hours as described in the ISO 4833-1:2013 standard (ISO 2013). The TMAnB count was enumerated on PCA after incubation at 30°C for 72 hours under anaerobic conditions. Lactic acid bacteria were enumerated on de Man Rogosa and Sharpe Agar (MRSA) incubated at 37°C for 48 hours. Molds and yeasts were enumerated on potato dextrose agar supplemented with 10% tartaric acid, which was incubated at 22±1°C for 5-7 days.

Coliform and *E. coli* counts were performed using the most probable number (MPN) method as described in the U.S. Food and Drug Administration's Bacteriological Analytical Manual (FDA/BAM 2002). From each sampling bag containing 10 g of honey + 90 ml of 0.1% peptone water, aliquots of 10 ml, 1 ml and 0.1 ml were taken and transferred to tubes containing 10 ml (double-strength), 10 ml (single-strength), and 10 ml (single-strength) of lauryl sulphate tryptose (LST) broth, respectively. All tubes were incubated at 35°C±0.5°C for 24-48 hours. Briefly, after presumptive positives were determined, confirmation tests were performed by transferring a loopful of suspension into brilliant green lactose broth (BGLB). The MPN was calculated and species distribution was determined using the Vitek 2 system, according to manufacturer's instructions (Biomerieux, France). For *E. coli* counts, a loopful of suspension, from each presumptive positive tube mentioned above, was transferred to a tube containing *E. coli* broth (EC). The EC tubes were incubated at 44.5°C for 24-48±2h and then examined for gas production. After gently agitating each gassing EC tube, a loopful of broth was streaked on Levine's eosin-methylene blue (L-EMB) agar for isolation. The plates were incubated at 35°C±0.5°C for 18-24 h. Then presumptive colonies were confirmed with the Vitek 2 system (Biomerieux, France).

Staphylococcus spp. counts were determined using 9 test tubes containing 10, 9.9, and 9 ml of tryptic soy broth, 10% NaCl, and 1% sodium pyruvate (TSBNS) (three tubes each) as described in the FDA's BAM (FDA/BAM 2001). From each sampling bag containing 10 g of honey + 90 ml of 0.1% peptone water (corresponding to a dilution of 1:10), aliquots of 10 ml, 1 ml and 0.1 ml were taken and transferred to the tubes containing 10 ml of TSBNS broths, respectively. These tubes were incubated at 37°C for 48±2 hours. Briefly, positive tubes were confirmed by streaking a loopful of suspension onto Baird-Parker agar (BPA) and one or more suspected black colonies from each positive BPA plate were confirmed and identified at the species level using the Vitek 2 system (Biomerieux, France). Following confirmation, the MPN of *Staphylococcus* spp. was calculated based on the proportion of confirmed turbid TSBNS tubes for three consecutive dilutions.

Antibiotic susceptibility testing of the isolates

Antibiotic susceptibility tests were performed on all of the coliform and non-coliform isolates obtained from the honey samples. The susceptibility tests were conducted using the BD Phoenix™ 100 Automatic Microbiology Identification System in accordance with the manufacturer's instructions (BD Diagnostic Instrument Systems, Sparks, MD, USA). A Phoenix NMIC-400/ID Panel, of which the following antibiotics were part of, was used: amikacin, amoxicillin-clavulanate, ampicillin, ciprofloxacin, colistin, gentamicin, netilmicin, tigecycline, trimethoprim-sulfamethoxazole, aztreonam, cefepime, ceftazidime, ceftriaxone, imipenem and meropenem. The minimal inhibitory concentration (MIC) values were interpreted as susceptible, intermediate, or resistant according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2017). All isolates with intermediate susceptibility were re-classified as susceptible.

Statistical analysis

Data analysis was performed with the SPSS statistical software version 24 (IBM SPSS, IBM Corporation, USA). The chi-square test was used to compare differences between microorganism presence and the honey types. Statistical differences between the honey types and mean values of microorganisms were determined using Student's t-test. $P < 0.05$ was considered significant.

RESULTS

Microbiological profile in honeys

The mean numbers determined in the 150 honey samples are given Table 1. The mean TMAB, TMAnB, mold and LAB counts (log cfu/g) of the extracted

honey samples, which were 3.37±1.07, 3.07±0.91, 2.94±0.43, and 2.88±0.49, respectively, were higher than the counts of the comb honey samples (Table 1). However, there were no significant differences in the mean counts of microorganisms between the extracted and comb honey samples (P>0.05). Out of the 150 analyzed honey samples, 94 (62.7%), 34 (22.7%), 28 (18.7%), 25 (16.7%), 23 (15.3%), 21 (14%) and 11 (7.3%) were contaminated at detectable levels (>10 cfu/g or >3 MPN/g) of TMAB, TMAnB, coliforms, yeasts, *Staphylococcus* spp., LAB and molds, respectively. None of the samples were contaminated with *E. coli* within a detectable number (<3 MPN/g). Comparison of honey types showed that 49.3%, 14.7% and 14% of the extracted honey samples, and 13.3%, 2% and 1.3% of the comb honey samples were contaminated with TMAB, yeasts and *Staphylococcus* spp., and these contamination rates were found to be statistically significant (P<0.05) (Table 2).

Distribution of coliform and Staphylococcus isolates

Out of the 150 honey samples analyzed in the present study, 28 (18.7%) contained coliforms, resulting in 106 isolates. Forty-three (40.5%) isolates were identified as *K. pnuemonia*, *E. cloacae* and *K. oxytoca*, all of which are coliform bacteria, whilst the remaining 63 isolates (59.4%) were identified as *P. vulgaris*, *S. marcescens* and *P. mirabilis* (Table 3). The analysis of the 150 honey samples for *E. coli* with the most probable number method revealed turbidity in the EC broth tubes of 11 (7.3%) samples. However, none of the suspected isolates obtained from the EC broth tubes were confirmed as *E. coli*. Of the 150 honey samples tested, 23 (15.3%) were found to be contaminated with Staphylococcus, and a total of 30 strains were isolated. Out of the 25 *Staphylococcus* spp. isolates obtained from extracted honey samples, 10 were *S. hominis*, 8 were *S. epidermidis*, 6 were *S. hemolyticus*, and 6 were *S. capitis*. Furthermore, out of the 5 *Staphylococcus* spp. isolates obtained from comb honey samples, 2 were *S. hominis*, 1 was *S. epidermidis*, 1 was *S. hemolyticus*, and 1 was *S. capitis* (Table 3). None of the analyzed honey samples was contaminated with *S. aureus* in detectable numbers.

Table 1. Microbial counts in honeys

Variable	Overall counts (N:150)			Honey types					
				Extracted honey (n:106)			Comb honey (n:44)		
	Range (Min.-Max.)	Mean±SD	Median	Range (Min.-Max.)	Mean±SD*	Median	Range (Min.-Max.)	Mean±SD*	Median
TMAB[x]	1.13-5.08	3.26±1.08	3.41	1.13-5.08	3.37±1.07	3.45	1.17-4.33	2.84±1.04	3.15
TMAnB[x]	1.30-4.37	3.00±0.89	3.04	1.30-4.37	3.07±0.91	3.16	1.77-3.14	2.48±0.55	2.66
Molds[x]	1.17-2.79	1.80±0.53	2.75	1.77-4.39	2.94±0.43	1.78	1.77-3.50	2.64±0.75	2.31
Yeasts[x]	1.77-4.39	2.90±0.83	1.97	1.17-2.10	1.66±0.84	2.75	1.47-2.79	2.19±0.66	2.65
Lactic acid bacteria[x]	1.84-3.99	2.93±0.52	2.90	1.84-3.58	2.88±0.49	2.85	2.70-3.99	3.34±0.91	3.05
Coliform[y]	0.30-46	8.06±1.23	0.74	0.30-29.0	6.76±9.65	0.74	0.30-46.0	12.81±1.98	0.64
Staphylococcus spp.[y]	0.36-2.30	0.71±0.66	0.36	0.36-2.30	0.65±0.59	0.53	0.36-2.30	1.33±1.37	1.33

[x] log cfu/g
[y] MPN/g
SD: Standard deviation
*There was no significant difference in the mean counts of microorganisms between honey types.
None of samples was found to be contaminated with *E. coli* in detectable numbers (<3 log MPN/g).

Table 2. Microbial contamination rates in honey samples (N:150, %)

Microorganism	Honey types		Overall
	Extracted honey (n:106)	Comb honey (n:44)	
TMAB	49.3[a]	13.3[b]	62.7
TMAnB	18.7[a]	4[a]	22.7
Coliform	14.7[a]	4[a]	18.7
Yeasts	14.7[a]	2[b]	16.7
Staphylococcus spp.	14[a]	1.3[b]	15.3
LAB	11.3[a]	2.7[a]	14
Molds	5.3[a]	2[a]	7.3

[a.b.c]: Values in the same row that are not followed by the same uppercase letter are significantly different (P<0.05).

Table 3. Distribution of coliform, non-coliform Gram negative and *Staphylococcus* spp. isolates identified in honeys*

Microorganism		Honey type	
		Extracted honey (no. of isolates)	Comb honey (no. of isolates)
Coliform bacteria	x		
Klebsiella pneumonia	26 (60.4)	23	3
Enterobacter cloacae	11 (25.6)	8	3
Klebsiella oxytoca	6 (14)	6	Not detected
Non-coliform Gram (-) bacteria	y		
Proteus vulgaris	38 (60.3)	24	14
Serratia marcescens	17 (27)	9	8
Proteus mirabilis	8 (12.7)	7	1
***Staphylococcus* spp.**	z		
S. hominis	10 (33.3)	8	2
S. epidermidis	8 (26.6)	7	1
S. hemolyticus	6 (20)	5	1
S. capitis	6 (20)	5	1

* Of the 150 honey samples analyzed, 28 (18.7%) and 23 (15.3%) were contaminated with coliform and Staphylococcus, respectively.
x: No. of isolates with (% of the 43 isolates)
y: No. of isolates with (% of the 63 isolates)
z: No. of isolates with (% of the 30 isolates)

Resistance pattern of the coliform and non-coliform Gram-negative isolates

The antibiotic resistance of the isolates was highest to ceftriaxone (92.4%) and cefepime (91.5%) followed by tigecyclin (46.2%), trimethoprim-sulfamethoxazole (43.4%), netilmicin (43.4%), amoxicillin-clavulanate (43.4%), ceftazidime (36.8 %), aztreonam (36.8%), and colistin (30.1%) (Table 4). The isolates with the highest percentages of resistance to the different antibiotics tested were *P. vulgaris* and *P. mirabilis*. Of the isolates, 83% were found to be multi-drug resistant (resistant to at least three different classes of antibiotics).

Table 4. Antimicrobial susceptibility pattern of coliform and non-coliform Gram negative bacteria recovered from honeys

Bacterial isolate	No. of resistant isolates with (%)										
	AMC	CIP	COL	GEN	NET	TIG	TMP- SUL	AZT	CEF	CEFT	CFTX
***Proteus vulgaris*(n:38)**	38(100)	0(0)	NA	0(0)	38(100)	38(100)	38(100)	0(0)	38(100)	0(0)	38(100)
***Klebsiella pneumonia*(n:26)**	0(0)	0(0)	26(100)	10(38)	0(0)	0(0)	0(0)	26(100)	26(100)	26(100)	26(100)
***Serratia marcescens*(n:17)**	NA	0(0)	NA	0(0)	0(0)	0(0)	0(0)	4(24)	17(100)	4(24)	17(100)
***Enterobacter cloacae*(n: 11)**	NA	3(27)	0(0)	0(0)	0(0)	3(27)	0(0)	3(27)	3(27)	3(27)	4(36)
***Proteus mirabilis*(n:8)**	8(100)	8(100)	NA	0(0)	8(100)	8(100)	8(100)	0(0)	7(87.5)	0(0)	7(87.5)
***Klebsiella oxytoca*(n:6)**	0(0)	0(0)	6(100)	4(67)	0(0)	0(0)	0(0)	6(100)	6(100)	6(100)	6(100)
Overall (n: 106)	46 (43.4)	11(10.3)	32 (30.1)	14 (13.2)	46(43.4)	49(46.2)	46(43.4)	39(36.8)	97(91.5)	39(36.8)	98(92.4)

AMC: Amoxicillin-Clavulanate; CIP: Ciprofloxacin; COL: Colistin; GEN: Gentamicin; NET: Netilmicin; TIG: Tigecycline; TMP-SUL:Trimethoprim-Sulfamethoxazole; AZT: Aztreonam; CEF: Cefepime; CEFT: Ceftazidime; CFTX: Ceftriaxone.
NA: The isolate has intrinsic resistance to certain antibiotic.
All isolates were pan susceptible/sensitive to amikacin, imipenem and meropenem

DISCUSSION

Total mesophilic aerobic bacteria, total anaerobic bacteria, yeast and mold counts are important parameters used to determine the microbial quality of honey. Kunová et al. (2015) reported that the total viable count ranged from 1.87 log cfu/g to 3.87 log cfu/g with a median level of 2.52 log cfu/g, based on the analysis of honey samples originating from the Czech Republic, Slovakia and Germany. Pucciarelli et al. (2014) reported that the mean TMAB count of the analyzed honey in Argentina was as 3.13 log cfu/g, Rozanska and Osek (2012) reported a TMAB count ranging from $1.9x10^2$ cfu/g to $4.6x10^3$ cfu/g for five honey types of different botanical origin in Poland. The results determined in the present study for mean TMAB count is similar with the studies mentioned above. However, in a study on honey samples obtained from different stores in Turkey the determined mean TMAB count was higher at 6.98 log cfu/g (Erkan et al., 2015). The physico-chemical composition of honey is favourable for the survival of the spores and vegetative forms of some anaerobic and facultative bacteria, even if at a low level. In the present study, 22.7% of the 150 honey samples analyzed were contaminated with TMAnB and the mean TMAnB count was 3.0±0.89 log cfu/g. Kačániová et al. (2012) reported vegetative anaerobic bacteria levels of 0% in 20 honey samples from Slovakia and 1% in 20 honey samples from Poland. In the same study, the counts of vegetative anaerobic bacteria in the two positive samples were found to be as 1 log cfu/g and 1.54 log cfu/g. Different from other microorganisms, yeasts and molds are capable of long-term survival and even growth in honey. In the analyzed honey samples, 25 (16.7%) and 11 (7.3%) were contaminated with yeast and mold, respectively, in the present study. Similarly, Moujanni et al. (2017) reported to have detected higher yeasts (40%) than molds (32%) in 109 Moroccan honey samples. These results suggest that honey contamination with yeasts occurs at a higher rate, which is attributed to yeasts having a higher capability of surviving and growing in media with high sugar concentrations (Tysett et al., 1981).

The source of lactic acid bacteria in honey is mainly plants, the digestive tract of honeybees and soil. However, these bacteria, which are widespread in the environment, can pass into honey under improper production, processing and storage conditions. In the present study, the mean number of LAB was determined to be 2.93±0.52 log cfu/g. Similar to the present study, Vazquez-Quiñones (2018) determined the presence of lactic acid bacteria at a level above 2 log cfu/g (>10^2 cfu/g) in honeys from Mexico, and Duman et al. (2008) determined a number of lactic acid bacteria ranging between 10^2 - 10^3 cfu/g in honeys from Turkey.

Coliform bacteria, the counts of which are used as an indicator of the sanitary quality of foodstuffs, belong to four genera, namely, *Escherichia*, *Klebsiella*, *Citrobacter* and *Enterobacter*. Some coliform species has also been known to cause clinically important infections in humans (Armbruster et al., 2017). In two of the very few studies, in which the number of coliform bacteria in honey was determined by the MPN method, Pucciarelli et al. (2014) reported the average number of coliforms as 1.45 MPN/g in honey, whilst Vazquez-Quiñones (2018) reported a coliform number of <3 MPN/g. While these results are quite lower than those obtained in the present study, some other literature reports point out the detection of higher coliform numbers (Combarros-Fuertes et al., 2019; Dümen et al., 2013). The contamination of honey with coliform bacteria may occur via the digestive tract of honeybees, pollens, the environment, equipment and personnel hygiene (Silva et al., 2017). In the present study, none of the honey samples contained a detectable level of *E. coli* by MPN method. Similar to the present study, Leme et al. (2018) and Combarros-Fuertes et al. (2019) reported not to have detected *E. coli* in any of the honey samples they analyzed. On the other hand, Dümen et al. (2013) reported to have detected *E. coli* in 18 (3.6%) out of 500 honey samples, and determined that the number of *E. coli* ranged from <10 cfu/g to $3.4x10^1$ cfu/g. To our knowledge, only very few literature reports are available on the systematic investigation of the species distribution of coliform bacteria contaminating honey. Although honey shows antimicrobial activity against several clinically important pathogens, including *P. mirabilis, P. vulgaris*, *E. cloacae, E. aerogenes* and *K. pneumoniae*, the detection of these bacteria in honey, even at low levels, in the present study, demonstrates that these bacteria can be in honey (Snowdon et al., 1996; McLoone et al., 2016).

Bacteria of the genus Staphylococcus, which are part of the natural microflora of both humans and animals, are ubiquitous and include coagulase-positive and coagulase-negative species, known to bear significance in terms of food safety and public health (Hennekinne et al., 2012; Becker et al., 2014). In a study on honey obtained from beehive combs with sterile syringes it was reported that while 2 (7.14%) of the 28 Yateí honey samples contained coagulase-negative *Staphylococcus* spp., none were contaminated with coagulase-positive *Staphylococcus* spp. (Puciarelli et al., 2014). In another study from different tree spe-

cies, the presence of coagulase-positive *Staphylococcus* spp. was detected in 65 (11.7%) of the 552 honey samples (Ceauşi et al., 2009). When comparing those reports with the present study, the higher contamination rates of *Staphylococcus* spp. in the present study was attributed not only to primary and secondary or cross contaminations, but also to the use of the most probable number method, which enables the detection of *Staphylococcus* spp. numbers less than 10 cfu/g. In the present study, *S. aureus* having not been detected in any of the samples was attributed to the antimicrobial activity of honey against many pathogens, including *S. aureus*, owing to its characteristic composition, structure and microbial flora (Sherlock et al., 2010).

In the present study, it was determined that the overall contamination rate of extracted honey with TMAB, TMAnB, coliforms, *Staphylococcus* spp., yeasts, molds and lactic acid bacteria was higher than that of comb honey. This demonstrated that the microbial quality of extracted honey is relatively lower than that of comb honey. In a study that analyzed honey samples from different points of a honey processing unit was reported that postharvest extracted honey contained increased numbers of TMAB, yeasts and molds, and that coliform bacteria, was not detected in comb honey (Fernandez et al., 2017). In their study on the comparison of the cold and hot extraction methods used to obtain extracted honey from comb honey, Gallez and Fernández (2009) determined that the microbial contamination of extracted honey occurred with the use of both methods and the cold extraction method posed a greater risk of contamination than the hot extraction method. The increased risk of microbial contamination associated with each step of the production of extracted honey from comb honey at honey processing units is in agreement with the lower microbial quality determined for extracted honey, compared to comb honey.

The Turkish Food Codex by-law on microbiological criteria and the 2073/2005 numbered microbiological criteria for foodstuffs of the European Commission (EC) do not enforce any limit for TMAB, TMAnB, coliform, *Staphylococcus* spp., lactic acid bacteria, yeast and mold count of honey (Turkish Food Codex, 2011; European Commission, 2005). Therefore, the microorganism counts determined in the present study were not assessed for conformity to any legal requirements. However, the determined values for the studied microorganisms in the present study are not considered hazardous to humans.

Antibiotic resistance rapidly increasing among bacteria is a major public health concern at the global level (WHO, 2016). The non-legal use of different antibiotic groups, such as tetracyclines, streptomycin, macrolides, and sulphonamides for the treatment of honeybee diseases may cause antibiotic residues in honey. The high levels of multi-drug resistant coliform and non-coliform Gram-negative bacteria detected in the present study not only suggest that drugs are used improperly, but also point out the impact of multiple factors, including the close contact of honeybees with the environment, the vector role of honeybees in transferring antibiotic-resistant bacteria to the hive, the ability of antibiotic-resistant bacteria to colonize in the honeybee gut, and the contamination of honey with antibiotic-resistant bacteria at different stages, from production to the consumer (Tian et al., 2012; Kačániová et al., 2017).

CONCLUSIONS

E. coli and *S. aureus* having not been detected in any of the honey samples analyzed in the present study shows that the sanitary quality of the honey was acceptable. The presence of clinically important bacteria for humans from the analyzed samples indicates that these bacteria can be found in honey. Findings in this study indicated that honey, can act as a potential vehicle for the transmission of antibiotic-resistant bacteria and thus, pose a health risk to consumers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Aljadi AM, Kamaruddin MY (2004) Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. Food Chem 85:513-518.

Armbruster CE, Smith SN, Johnson AO, De Ornellas V, Eaton KA, Yep A, Mobley HL (2017) The Pathogenic Potential of Proteus Mirabilis is Enhanced by Other Uropathogens During Polymicrobial Urinary Tract İnfection. Infect Immun 85:E00808-16.

Baki F, Saner G, Adanacıoğlu H, Güler D (2017) Türkiye'de süzme çam

balına yönelik tüketici tercihlerinin konjoint analizi: İzmir ili örneği. Balkan and Near Eastern J Soc Sci 3: 50-57.
Becker K, Heilmann C, Peters G (2014) Coagulase-negative staphylococci. Clin. Microbiol Rev 27:870-926.
Bezirtzoglou E, Voidarou C, Stavropoulou E (2016) Emerging antibiotic resistance in honey as a hazard for human health. J Bacteriol Mycol 2:6-12.
Bhatta DR, Cavaco LM, Nath G, Kumar K, Gaur A, Gokhale S, Bhatta DR (2016) Association of Panton Valentine Leukocidin (PVL) genes with methicillin resistant Staphylococcus aureus (MRSA) in Western Nepal: a matter of concern for community infections (a hospital based prospective study). BMC Infect Dis 16:199.
Boussaid A, Chouaibi M, Rezig L, Hellal R, Donsì F, Ferrari G, Hamdi S (2018) Physicochemical and bioactive properties of six honey samples from various floral origins from Tunisia. Arab J Chem 11:265-274.
Ceauşi C, Ţogoe I, Tudor L, Furnaris F (2009) The evaluation of microbiological quality of some bee honey sorts. Bull Univ Agric Sci Vet 66:273-277.
Chakir A, Romane A, Marcazzan GL, Ferrazzi P (2016) Physicochemical properties of some honeys produced from different plants in Morocco. Arab J Chem 9:946-954.
Combarros-Fuertes P, Valencia-Barrera RM, Estevinho LM, Dias LG, Castro JM, Tornadijo ME, Fresno JM (2019) Spanish honeys with quality brand: a multivariate approach to physicochemical parameters, microbiological quality and floral origin. J Apic Res 58:92-103.
Cosgrove SE (2006) The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. Clin Infect Dis 42:82-89.
Duman AB, Sezer Ç, Oral NB (2008) Kars'ta Satışa Sunulan Süzme Balların Kalite Niteliklerinin Araştırılması. Kafkas Univ Vet Fak Derg 14:89-94.
Dümen E, Akkaya H, Öz GM, Sezgin FH (2013) Microbiological and parasitological quality of honey produced in İstanbul. Turk. J Vet Anim Sci 37:602-607.
El Sohaimy SA, Masry SHD, Shehata MG (2015). Physicochemical characteristics of honey from different origins. Ann Agric Sci 60:279-287.
Erkan ME, Vural A, Guran HS, Durmusoglu H (2015) Microbiological investigation of honey collected from Şırnak province of Turkey. J Hell Vet Med Soc 66:22-26.
European Commission (2005) Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union, 338: 1-26
Fernandez LA, Ghilardi C, Hoffmann B, Busso C, Gallez LM (2017) Microbiological quality of honey from the Pampas region throughout the extraction process. Rev Argent Microbiol 49:55-61.
Gallez L, Fernández LA (2009) Mieles Del Sistema Serrano De Ventania: Evaluación De La Calidad Microbiológica En El Circuito De La Planta De Extracción. Rev Argent Microbiol 41:163-167.
Garcia NL (2018) The current situation on the International honey market. Bee World 95:89-94.
Gasper J, Terentjeva M, Kántor A., Ivanišová E, Kluz M, Kačániová M (2017) Identification of Apis mellifera Gut Microbiota with MALDI-TOF MS Biotyper. Scientific Papers Animal Science & Biotechnologies 50:192-196.
Gomes S, Dias LG, Moreira LL, Rodrigues P, Estevinho L (2010) Physicochemical, microbiological and antimicrobial properties of commercial honeys from Portugal. Food Chem Toxicol 48:544-548.
Grabowski NT, Klein G (2017) Microbiology and foodborne pathogens in honey. Crit Rev Food Sci Nutr 57:1852-1862.
Hennekinne JA, De Buyser ML, Dragacci S (2012) Staphylococcus aureus and its food poisoning toxins: characterization and outbreak investigation. FEMS Microbiol Rev 36:815-836.
ISO (2003) EN ISO 4833 Microbiology of Food and Animal Feeding Stuffs- Horizontal Methods for Enumeration of Microorganisms-Colony-Count Technique at 30 Degrees, International Standardization Organization, Geneva.
Kačániová M, Gasper J, Brindza, Schubertová Z, Ivanišová E (2017) Bacteria of Apis mellifera gastrointestinal tract: counts, identification and their antibiotic resistance. Agrobiodivers. Improv Nutr Health Life Qual 1:210-215
Kačániová M, Hleba L, Džugan M, Pasternakiewicz A, Kňazovická V, Pavelková A, Felsöciová S, Petrová J, Rovná K, Kluz M, Grabek-Lejko D (2012) Microbiological properties and antimicrobial effect of Slovakian and Polish honey having regard to the water activity and water content. J Microbiol Biotechnol Food Sci 2:272-281.
Kavanagh S, Gunnoo J, Passos TM, Stout JC, White B (2019) Physicochemical properties and phenolic content of honey from different floral origins and from rural versus urban landscapes. Food Chem 272:66-75.
Kollef KE, Schramm GE, Wills AR, Reichley RM, Micek ST, Kollef MH (2008) Predictors of 30-day mortality and hospital costs in patients with ventilator-associated pneumonia attributed to potentially antibiotic-resistant gram-negative bacteria. Chest 134:281-287.
Kunová S, Kačániová M, Haščík P, Čuboň J (2015) Microbiological and chemical quality of Slovak and European honey. J Microbiol Biotechnol Food Sci 4:41-44.
Leme LM, Montenegro HR, Dos Santos LDR, Sereia MJ, Valderrama P, Março PH (2018) Relation between near-infrared spectroscopy and physicochemical parameters for discrimination of honey samples from Jatai weyrauchi and Jatai angustula bees. Food Anal Methods 11:1944-1950.
Manges AR (2016) Escherichia coli and urinary tract infections: the role of poultry-meat. Clin Microbiol Infect 22:122-129.
Mercan N, Guvensen A, Celik A, Katircioglu H (2007) Antimicrobial activity and pollen composition of honey samples collected from different provinces in Turkey. Nat Prod Res 21:187-195.
Moujanni A, Terrab Benjelloun A, Eddoha R, Nasser B, Benbachir M (2017) Microbiological quality of moroccan labeled euphorbia resinifera honey. J Microbiol Biotechnol Food Sci 6:1188-1194.
Olaitan PB, Adeleke OE, Iyabo OO (2007) Honey: a reservoir for microorganisms and an inhibitory agent for microbes. Afr Health Sci 7:159-165
Pucciarelli AB, Schapovaloff ME, Kummritz S, Señuk IA, Brumovsky LA, Dallagnol AM (2014) Microbiological and physicochemical analysis of Yateí (Tetragonisca Angustula) honey for assessing quality standards and commercialization. Rev Argent Microbiol 46:325-332.
Rozanska H, Osek J (2012) Effect of storage on microbiological quality of honey. B Vet I Pulawy 56:161-163.
Sherlock O, Dolan A, Athman R, Power A, Gethin G, Cowman S, Humphreys H (2010) Comparison of the antimicrobial activity of Ulmo honey from Chile and Manuka honey against methicillin-resistant Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa. BMC Complement Altern Med 10:1-5.
Silva MS, Rabadzhiev Y, Eller MR, Iliev I, Ivanova I, Santana WC (2017) Microorganisms in honey. In V. De Alencar Arnaut De Toledo (Ed.), Honey analysis, IntechOpen, pp. 233-245.
Snowdon JA, Cliver DO (1996) Microorganisms in Honey. Int J Food Microbiol 31:1-26.
Soylu M, Silici S (2018) Üniversite öğrencilerinin bal tüketim tercihleri. J Hum Sci 15:386-398.
Stagos D, Soulitsiotis N, Tsadila C, Papaeconomou S, Arvanitis C, Ntontos A, Karkanta F, Adamou-Androulaki S, Petrotos K, Spandidos D, Kouretas D, Mossialos M (2018) Antibacterial and antioxidant activity of different types of honey derived from Mount Olympus in Greece. Int J Mol Med 42:726-734.
TEPGE (2020) Aricilik. No: HU-01. https://arastirma.tarimorman.gov.tr/tepge/Menu/27/Tarim-Urunleri-Piyasalari [accessed 03 February 2020].
Tian B, Fadhil NH, Powell JE, Kwong WK, Moran NA (2012) Long-term exposure to antibiotics has caused accumulation of resistance determinants in the gut microbiota of honeybees. MBio 3:e00377-12.
Tonks AJ, Cooper RA, Jones KP, Blair S, Parton J, Tonks A (2003) Honey stimulates inflammatory cytokine production from monocytes. Cytokine 21:242-247.
TUİK (2016) Turkish Statistical Institute. http://www.tuik.gov.tr [accessed 11 May 2018]
Turkish Food Codex (2011) Communiqué on Microbiological Criteria. https://www.resmigazete.gov.tr/eskiler/2011/12/20111229M3-6.htm [accessed 12 October 2019].
Tysett C, Rosseau M (1981) Problem of microbes, hygiene of commercial honey. Rev Med Vet 132:591-600.
US FDA's Bacteriological Analytical Manual (FDA/BAM) (2001) *Staphylococcus aureus.* https://www.fda.gov/food/laboratory-methods-food/bam-staphylococcus-aureus [accessed 21 October 2019].
US FDA's Bacteriological Analytical Manual (FDA/BAM) (2002) Enumeration of Escherichia coli and the Coliform Bacteria. https://www.fda.gov/food/laboratory-methods-food/bam-4-enumeration-escherichia-coli-and-coliform-bacteria [accessed 21 October 2019].
Vazquez-Quiñones CR, Moreno-Terrazas R, Natividad-Bonifacio I, Quiñones-Ramírez EI, Vázquez-Salinas C (2018) Microbiological assessment of honey in México. Rev Argent Microbiol 50:75-80.
World Health Organization (WHO) (2016) Critically important antimicrobials for human medicine: ranking of antimicrobial agents for risk management of antimicrobial resistance due to non-human use. https://apps.who.int/iris/bitstream/handle/10665/255027/9789241512220-eng.pdf. [accessed 12 June 2019].

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Research article
Ερευνητικό άρθρο

Dynamic changes of psychrotrophic bacterial populations in Algerian refrigerated raw cow milk

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ABSTRACT: The aim of the present study is to evaluate the microbial dynamic changes in raw cow milk during refrigeration. Milk samples were collected from two dairy farms situated in basin and mountain areas in the region of Mila, Algeria. Cold storage of milk samples at 4 °C was used to promote psychrotrophic microbial growth during 10 days. The microbial flora of milk was determined by culture methods and the identification of bacterial isolates was carried using cultural, morphological and biochemical criteria. The variations in total viable count and kinetic parameters of microbial growth were analyzed during refrigeration using Baranyi and Roberts Model. The initial microbial counts ranged from 2.94 ±0.04 to 3.87 ±0.11 log CFU * mL^{-1} and the maximum growth did not exceed 7.00 ±0.12 log CFU * mL^{-1}. The hygienic limit was surpassed after 2 days in the basin farm and later after 6 days in mountain farm. The generation time varied from 1.20 ±0.55 to 4.18 ±0.22 days in the basin farm and mountain farm respectively. The psychrotrophic bacterial populations were identified as *Acinetobacter*, *Pseudomonas*, *Aeromonas*, *Chryseobacterium*, *Enterococcus* and *Lactobacillus*. The notable delay in microbial growth in milk from the mountain farm compared to the one from basin could be related to indoor/outdoor feeding mode adopted in mountain opposed to indoor feeding in the basin farm. This prolonged shelf-life suggests the possible presence of antimicrobial molecules coming from plants grazed around the farm and the selection in the microbiota of some microbial species with antagonist potential.

Keywords: raw cow milk, refrigerated storage, microbial growth parameters, psychrotrophic bacterial populations, shelf-life

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INTRODUCTION

In many parts of the world milk and dairy products are indispensable constituents of human nutrition and play an important role in human health and the development of life (FAO, 2013; Quigley et al., 2013). The prolonged storage of milk by refrigeration favors the growth of psychrotrophic bacteria, this category of microorganisms have become a fundamental limitation for dairy products shelf-life (De Jonghe et al., 2011). Initially, they account for less than 10% in refrigerated raw milk, than they evolve as the major microorganisms constituting more than 70% to 90% of the microbiota (Decimo et al., 2014). Particularly, psychrotrophs by their proteases and lipases extracellular enzymes play a leading role in the deterioration of dairy products (Cempírková and Mikulová, 2009).

Numerous bacterial strains were isolated from refrigerated milk, they belong to the genera *Pseudomonas*, *Stenotrophomonas*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Bacillus*, *Listeria* and *Enterococcus* (Lafarge et al., 2004; Hantsis-Zacharov and Halpern, 2007a; Boubendir et al., 2011). The majority of psychrotrophic bacteria are known for the spoilage of milk and dairy products. However, some species can be considered as human pathogenic bacteria by producing toxins and / or showing resistance to antibiotics (Samaržija et al., 2012). Dynamic changes of psychrotrophic flora in milk during 24 h of refrigeration (Lafarge et al., 2004) and in creamed milk from different geographical origins (Franciosi et al., 2011) have been investigated. Vyletělova et al. (2000) evaluated microbial growth dynamics of mesophilic and psychrotrophs in cow's raw milk in different times of storage and temperatures. Gargouri et al. (2013) confirmed that the dynamics of psychrotrophic bacteria counts (PBC) in cow's raw milk conserved at 4 °C was linked to storage time and the initial contamination value. Interesting relationships were elucidated between the geographical origin of milk and both genetic diversity and antimicrobial activity, suggesting the profound correlation between the region and some genotypic and metabolic conducts of milk microbiota (Silvetti et al., 2014).

The data on milk microbial dynamics during refrigeration particularly in Algeria is missing, thus the aim of the present study is to evaluate the dynamics of microbial growth during all the time of milk refrigeration in two dairy farms, one in the basin and the other in the mountain of Mila, Algeria.

MATERIALS AND METHODS

Milk sampling

Raw milk samples were collected from two dairy farms located in the region of Mila (Algeria) at the locality of El-Kherba (36° 27'N / 6° 15'E) in the basin and Hamala (36° 34'18 "N / 6° 20'24) in the mountain. In the basin (farm A), the cows feed only inside principally on silage, barley, oats and hay, while in the mountain (farm B), the cows feed both inside and outside, the prevalent plants grazed around this farm were *Thymus*, *Origanum*, *Malva*, *Hibiscus*, *Rosmarinus* and *Olea* leaves (Ouelbani et al., 2016). At each farm, five raw milk samples were collected from healthy cows "Française Frisonne Pie noir". The teat ends were cleaned by wiping using dry paper towels, the preliminary jets were discarded and 25 mL of raw milk sample were directly collected from each of the four teats (i.e., a total of 100 mL per cow) and transferred to the laboratory into individual sterile flasks at 4 °C.

Enumeration of microorganisms

Raw milk samples were processed as soon as they arrived to the laboratory. They were analyzed immediately at the first (time 0) day and at different times (2, 4, 6, 8 and 10 days) over 10 days of refrigerated storage at 4 °C. Serial dilutions (10^{-1}-10^{-5}) were prepared for each sample; 1 ml of each dilution was placed on Sterile Standard Plate Count (SPC) agar, a standard medium corresponding to the American Public Health Association formulation for milk, water, food and dairy products (Oxoid CM0463). Plates were incubated at 37 °C for 48 h, those with the number of colonies 30 to 300 were considered for counting. All the experiments were performed in three replications per milk sample at each time point and results were expressed as mean values. To control the occurrence of hemolytic bacteria as indicators of probable pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus* and *Staphylococcus aureus*, 0.1 ml from the dilution 10^{-1} were also plated on 5% (v/v) horse blood Columbia agar added with Cefazolin at 20 mg * L^{-1} (Sandoz Gmbh, Kundl, Austra), plates were incubated for 48 h at 37 °C (Boubendir et al., 2016). The enumeration of *Lactobacillus* was carried by adding 1 mL of the dilution 10^{-1} to sterile dishes, and liquefied MRS Agar (45°C) is poured into the dish and mixed carefully. Once the medium is solidified, a new layer of uninoculated MRS Agar is poured on the surface to make a layer-plate; the plates were incubated for 48 h at 37 °C.

Identification

At each sampling time (0, 6 and 10 days), colonies with different morphologies (color, shape, and size) were selected and purified by streaking on the same medium. The identification of the presumed bacteria was realized using cultural, morphological and biochemical criteria. The main identification tests used were: Gram staining, mobility (at 25 and 37 °C), catalase presence, oxidase presence, methyl red test (MR test), indole production, Voges-Proskauer reaction (VP test), nitrate reduction, H_2S production (TSI test), urease presence, aesculin hydrolysis and hemolytic activity.

Data analysis

Mean data relative to the repetitions of total bacterial counts on SPC agar medium for milk samples during storage at 4 °C were analyzed using Baranyi and Roberts Model (Baranyi and Roberts, 1995) in order to obtain the microbial growth parameters, i.e., maximum growth rate (μ_{max}), lag phase length (λ) and maximum cell load attained (X_{max}).

RESULTS AND DISCUSSION

Microbial dynamic changes during refrigeration of milk

The dynamics of microbial growth on SPC agar during 10 days of storage at 4 °C are shown in Figure 1, while Table 1 reports the parameters of microbial growth obtained. The initial number of psychrotrophic bacteria in raw milk samples collected from the two farms (A and B) were 3.87 ±0.11 and 2.94 ±0.04 log CFU * mL^{-1} respectively. These values did not exceed the hygienic limit (4.69 log CFU * mL^{-1}) reported by Cempírková and Mikulová (2009) and Gargouri et al. (2013). This denotes the respect of good farming practices, good health state of animals and appreciable hygienic farm environment. The environment where dairy cows are housed and milked influences the microbial contamination of milk by udder preparation techniques, milking machines protocols, the procedures of cleaning and disinfecting milking machines, milk tanks and the hygiene of personnel. Other principal factors are the refrigeration rate of the milk at the required temperature and the time of storage (Rasolofo et al., 2010; Samaržija et al., 2012). The present level of initial microbial contamination of raw milk was almost similar to those reported in other farms globally. The initial contamination of cow's raw milk from mountain farms and foothills areas of Southern and Western Bohemia, registered the mean value of 3.46 log CFU * mL^{-1} at different temperatures (4, 6.5 and 10 °C) (Cempírková and Mikulová, 2009). Another study in Brazil observed an initial PBC of 3.5 log CFU * mL^{-1} in the raw milk sample stored at 7 °C (Machado et al., 2013). In the region of Sfax (Tunisia) the level of PBC in raw milk was 2.89 CFU * mL^{-1} after one day of cold storage at 4 °C (Gargouri et al., 2013). In Ireland the mean value of initial contamination of milk at different temperatures (2, 4 and 6 °C) was 2.87 log CFU * mL^{-1} (O'connell et al., 2016). In the semi-arid area of Algeria (region of Biskra), the initial PBC in raw milk refrigerated at 4 °C ranged from 2.5 to 3.5 log CFU * mL^{-1} (Boubendir et al., 2016).

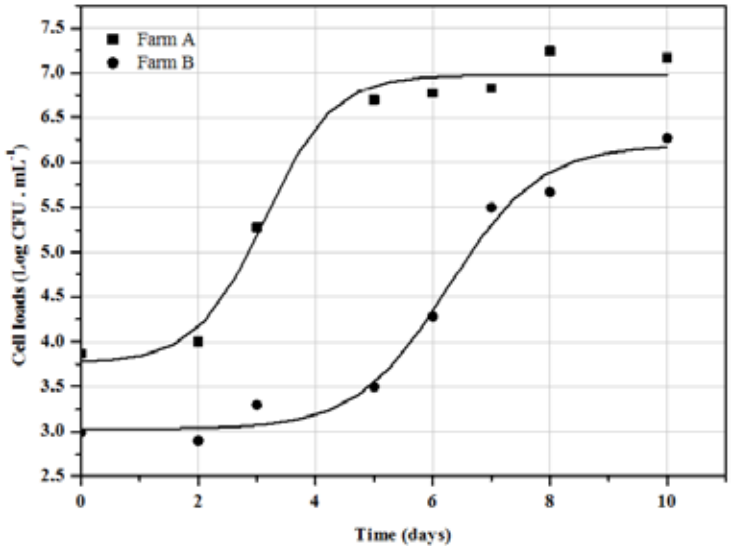

Figure 1. Growth curves of total viable bacteria count obtained by Baranyi and Roberts Model in milk refrigerated during 10 days at 4 °C, from two dairy farms in the basin (A) and mountain (B) in the region of Mila, Algeria

Table 1. Microbial growth parameters obtained by Baranyi and Roberts Model of mean count data of total viable bacteria over refrigerated storage of milk samples, from two dairy farms in the basin (A) and mountain (B) in the region of Mila, Algeria

Farm / Location	Geographical coordinates	Altitude (m)	Initial value[a]	λ^b	$\mu_{max}{}^c$	X_{max}
Farm A (Basin)	36°27'N/6°15'E	598	3.87 ± 0.11	1.20 ± 0.55	0.78 ± 0.16	7.00 ± 0.12
Farm B (Mountain)	36°34'N/6°20'E	829	2.94 ± 0.04	4.18 ± 0.22	0.73 ± 0.05	6.29 ± 0.07

[a] (log CFU * mL^{-1}), [b] Lag phase length (days), [c] Maximum growth rate (Δlog CFU * mL^{-1} *day), X_{max}: Maximum cell load (log CFU * mL^{-1})

The maximum growth levels (X_{max} value) reached during the stationary phase, after 10 days of milk refrigeration, were 7.00 ±0.12 and 6.29 ±0.07 log CFU * mL^{-1} in farm A and B respectively. The hygienic limit of milk was surpassed after 2 days in farm A and later after 6 days in farm B. In Slovenia, the PBC augmented on the second day of collection up 4.61 log CFU * mL^{-1} (Perko, 2011), and in Spain registered 7.04 log CFU * mL^{-1} (De Garnica et al., 2011). In Tunisia, the hygienic limit was surpassed after 2 days of cold storage (Gargouri et al., 2013). In Ireland the PBC level reached was 7.00 log CFU * mL^{-1} (Paludetti et al., 2018). In Brazil, according to Ribeiro Junior et al. (2018) the amount of psychrotrophs augmented to 5.32 log CFU * mL^{-1}. In Algeria at the region of Biskra, the X_{max} level attained in the stationary phase did not exceed 5.25 log CFU * mL^{-1} (Boubendir et al., 2016), while in the region of Djelfa, the level of psychrotrophs attained the threshold of 5.25 log CFU * mL^{-1} at the end of the 5th day of conservation (Yabrir et al., 2018). The difference in maximum growth levels observed in different parts of the world could be due to the variations in the temperature of cooling and storage time of milk.

The generation times (λ values) obtained in the present study ranged from 1.20 ±0.55 to 4.18 ±0.22 days in farm (A) and (B) respectively. Few studies evaluated the microbial parameters of growth during milk refrigeration. Cempírková and Mikulová (2009) in Southern and Western Bohemia registered a lag phase length of 2 days; Vithanage et al. (2017) in Australia have observed that the lag phase of psychrotrophs continues to 3 days. In Algeria, Boubendir et al. (2016) remarked an extended lag phase ranging from 6.74 to 10.50 days, while Yabrir et al. (2018) observed a lag phase of 3 days. On the other hand, the value of lag phase length obtained in milk from mountain was higher than those calculated according to ComBase predictive models (http://www.combase.cc) at the temperature of 4°C. The predicted lag time for *Listeria monocytogenes / innocua* was 4.15 days, *Aeromonas hydrophila* (3.36 days), *Pseudomonas* (1.68 days) and *Yersinia enterocolitica* (1.09 days).

The notable delay in microbial growth registered in milk collected from the mountain farm compared to the one from basin suggests the influence of specific environmental factors in dairy farms. The shelf-life of milk samples collected from the mountain adopting inside/outside feeding mode was elongated compared to the one of basin adopting only inside feeding mode. Indeed, the variations of milk microflora composition have been linked to changes in cow feeding environment. The microbial composition of milk and its activity is influenced by grazing from inside to outside and the nature of forage (Sanz Sampelayo et al., 2007). The variation in durations of lag phase in refrigerated raw milk collected from different geographies could be explained by the presence of antimicrobial natural molecules in milk. Numerous bioactive molecules of plants grazed by cows have a fundamental role for rumen health and display an antimicrobial activity. Furthermore, the concentration in milk of a variety of volatile compounds, such as terpenes is enhanced when cows are grazing in herb-rich pasture (Michel et al., 2001; Larsen et al., 2012).

The delay in microbial growth observed in the mountain farm could be related to the possible presence of antimicrobial molecules coming from the plants grazed around the farm. A variety of bioactive molecules including thymol, carvacrol, eugenol derived from plants, demonstrated antibacterial against both gram-positive and gram-negative pathogens (Ananda Baskaran et al., 2009). The antagonist effect of carvacrol, eugenol, and thymol against the main bacterial mastitis pathogens in milk was demonstrated, particularly against *S. aureus, Escherichia coli, Streptococcus agalactiae* and *S. dysgalactiae* (Gaysinsky et al., 2007; Ananda Baskaran et al., 2009).

According to Darsanaki et al. (2012), the cow

can graze olives leaves with a confirmed inhibitory effect against pathogenic bacteria. Olive leaves aqueous extract exhibited antimicrobial activity against *P. aeruginosa, S. aureus, E. coli, K. pneumoniae* and *B. subtilis*. Also, *Malva* plant with *M. sylvestris* and *M. parviflora* extracts, have demonstrated antimicrobial activity against a large variety of pathogenic bacteria such as *P. aeruginosa*, *E. coli* and *S. aureus* (Sharifi-Rad et al., 2019). In addition, *Hibiscus* extracts have shown a good antimicrobial activity in microbiological medium and in milk, especially against *S. aureus* and *E. coli* (Higginbotham et al., 2013). *Rosmarinus officinalis* essential oil has demonstrated antibacterial activity on milk microorganisms against *Staphylococcus spp., Bacillus cereus* and *Aeromonas hydrophila* (Paşca et al., 2015).

The evolution of psychrotrophic bacterial populations in mountain milk

The evolution of psychrotrophic bacterial populations in mountain milk stored at 4 °C during 10 days is reported in Figure 2. In the first day (time 0), before refrigeration, the bacterial populations were initially detected at low levels in fresh raw milk, they were identified as *Acinetobacter*, *Pseudomonas*, *Aeromonas*, *Chryseobacterium*, *Enterococcus* and *Lactobacillus*, the initial population microbial load varied from 1.00 to 2.51 log CFU * mL^{-1}. After 6 days of refrigeration, a rise in the number of the totality psychrotrophic bacterial populations is remarked except for *Chryseobacterium*, the maximum microbial growth reached 3.31 log CFU * mL^{-1} with *Enterococcus* being the dominant population in the microbiota. At the end of refrigeration after 10 days, *Enterococcus* and *Lactobacillus* populations surmount largely in number the others psychrotrophs with 4.41 and 4.00 log CFU * mL^{-1} respectively. The *Chryseobacterium* regains growth and attained 2.14 log CFU * mL^{-1}, while the growth of *Acinetobacter*, *Pseudomonas* and *Aeromonas* seems to be reduced. However, the absence of *Listeria*, *Staphylococcus*, *Bacillus* and other hemolytic pathogenic bacteria is remarked during all the time of refrigeration.

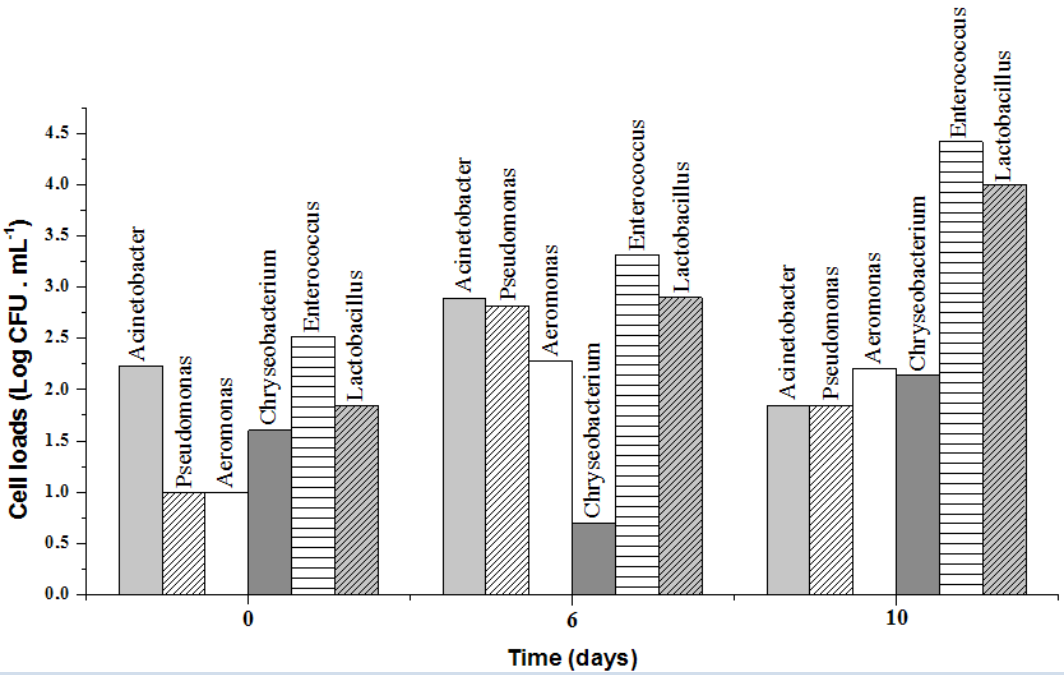

Figure 2. The evolution of psychrotrophic bacterial populations in mountain milk stored at 4°C during 10 days in the region of Mila, Algeria

Psychrotrophic bacteria were frequently isolated in cooled milk and dairy products environments, their origin can be from forage, feed, bedding material, faeces and soil, dust; when in contact with cow's teat skin the milk may be contaminated (Gleeson et al., 2013). Usually in untreated milks, *Pseudomonas* and *Acinetobacter* genera are isolated during the first days of cold storage (Rasolofo et al., 2010; Vithanage et al., 2014), and both exhibit essentially lipolytic activity (Hantsis-Zacharov and Halpern, 2007a). According to Gislene et al. (2015), the genus *Pseudomonas* is detected in raw milk during the first day at a low level,

however the numbers of *Pseudomonas* increase after 6 days of refrigeration. The genera *Acinetobacter* and *Pseudomonas* are recognized as the major psychrotrophic groups in refrigerated raw milk with high spoilage potential (Machado et al., 2015; Yuan et al., 2018).

In this study *Aeromonas* grow well at low temperature, this finding is in accordance with the results of Lafarge et al. (2004). Also, Mankai et al. (2012) isolated *Aeromonas hydrophila* from refrigerated raw milk conserved at 4 °C. This genus can contaminate milk by infecting the udder tissues and multiplying in mammary tissues (Nahla, 2006). Similarly, *Chryseobacterium* strains are part of the psychrotolerant and proteolytic microflora that causes numerous defects in food products (Hantsis-Zacharov and Halpern, 2007b; Machado et al., 2017). This genus is commonly regarded as a usual resident of soils and plants and is known for its aptitude to degrade toxic plant composites, comprising polyphenols (Lopez et al., 2004; Bekker, 2011). The members of the genus *Chryseobacterium* demonstrated antagonistic activities against human pathogenic bacteria such as *P. aeruginosa*, *L. monocytogenes*, *Salmonella typhi* and *S. aureus* (Lim et al., 2011).

Enterococcus was the dominant bacterial population during all the period of refrigeration; this finding is in accordance with the study of Mcauley et al. (2015). In Turkey, Citak et al. (2005) showed that *E. faecalis* was the major *Enterococcus* in raw milk. Giannino et al. (2009) registered the presence of *E. faecalis*, *E. faecium* in raw milk, establishing their essential role as origin of the typical fermenting microflora. Furthermore, enterococci are used to prolong the shelf-life and enhance the hygienic safety of food since they produce antimicrobial substances for example lactic acid, bacteriocins (enterocins) and hydrogen peroxide. Nevertheless, the presence of virulence factors and the emergence of multiple resistance to antibiotics among enterococci in food isolates require a careful safety evaluation before biotechnical use (Franz et al., 2007). Bacteriocins incite a great attention being commonly active against numerous gram-positive food borne pathogens such as *Clostridium botulinum*, *L. monocytogenes* and *S. aureus* (Morandi et al., 2012).

Lactobacillus population manifests here an unusual physiological adaptation to cold environment manifesting a psychrotrophic behavior at low temperature and was present all the period of cold storage. Usually, *Lactobacillus* grows as mesophilic and shows a maximum growth at 37 °C (Toqeer et al., 2006). Mami et al. (2012) demonstrated that *Lb. plantarum* can inhibit the growth of *L. monocytogenes*, *S. aureus* and other populations of undesirable bacteria in milk. This inhibition could result from the production of organic acids, especially lactic acid, which reduce pH environment, hydrogenous peroxide (H_2O_2), diacetyl (2, 3-butanediona), reuterin, bacteriocins and carbon dioxide (CO_2) (Gutiérrez-Cortés et al., 2017).

CONCLUSION

There is a notable delay in microbial growth in milk collected from the mountain farm adopting inside/outside feeding mode compared to the one from basin adopting only inside feeding. This prolonged shelf-life suggests the possible presence of antimicrobial bioactive molecules from plants grazed by cows outside the farm and the selection in the microbiota of some microorganisms with antagonist potential. The dynamic changes of bacterial populations habiting refrigerated milk could be in favor of long shelf-life and safety against pathogenic bacteria and spoilage agents. Taking into consideration the reinforcement of the data for the implementation a predictive model, the period of time will be prolonged and other measurements will be achieved in future works. Also, the exploration of other bacteria inhabiting refrigerated milk, the typing of the isolates using molecular tools and their kinetic study during all the time of refrigeration could be further developed.

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CONFLICT OF INTEREST

None of the authors have any conflicts of interest to declare regarding this work.

REFERENCES

Ananda Baskaran S, Kazmer GW, Hinckley L, Andrew SM, and Venkitanarayanan K (2009) Antibacterial effect of plant-derived antimicrobials on major bacterial mastitis pathogens in vitro. J Dairy Sci 92: 1423-1429.

Baranyi J, Roberts TA (1995) Mathematics of predictive food microbiology. Int J Food Microbiol 26: 199-218.

Bekker A (2011) Growth and spoilage characteristics of *Chryseobacterium* species in milk. Magister Scientiae thesis -Food Microbiology University of the Free State, South Africa.

Boubendir A, Hamidechi MA, Mostakim M, El Abed S, Ibnsouda S, Koraich I (2011) Incidence of *Listeria spp.* and other psychrotrophic bacteria in raw bovine milk in the North East of Algeria. Rev Med Vet-Toulouse 162: 265-269.

Boubendir A, Serrazanetti DI, Hamidechi MA, Vannini L, Guerzoni ME (2016) Changes in bacterial populations in refrigerated raw milk collected from a semi-arid area of Algeria. Ann Microbiol 66: 777-783.

Cempírková R, Mikulová M (2009) Incidence of psychrotrophic lipolytic bacteria in cow's raw milk. Czech J Anim Sci 54: 65-73.

Citak S, Yucel N, Mendi A (2005) Antibiotic resistance of enterococcal isolates in raw milk. J Food Process Pres 29: 183-195.

Darsanaki RK, Aliabadi MA, Rokhi ML, Nourbakhsh M, Raeisi G (2012) Antimicrobial activity of olive leaf aqueous extract. Ann Biol Res 3: 4189-4191.

Decimo M, Morandi S, Silvetti T, Brasca M (2014) Characterization of gram-negative psychrotrophic bacteria isolated from Italian bulk tank milk. J Food Sci 79: 2081-2090.

De Garnica ML, Santos JA, Gonzalo C (2011) Influence of storage and preservation on microbiological quality of silo ovine milk. J Dairy Sci 94: 1922-1927.

De Jonghe V, Coorevits A, Van Hoorde K, Messens W, Van Landschoot A, De Vos P, Heyndrickx M (2011) Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. Appl Environ Microbiol 77: 460-470.

FAO (2013) Milk and Dairy Products in Human Nutrition. Food and Agricultural Organization of the United Nations, Rome, Italy.

Franciosi E, De Sabbata G, Gardini F, Cavazza A, Poznanski E (2011) Changes in psychrotrophic microbial populations during milk creaming to produce Grana Trentino cheese. Food Microbiol 28: 43-51.

Franz CM, van Belkum MJ, Holzapfel WH, Abriouel H, Gálvez A (2007) Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. FEMS Microbiol Rev 31: 293-310.

Gargouri A, Hamed H, Elfeki A (2013) Analysis of raw milk quality at reception and during cold storage: combined effects of somatic cell counts and psychrotrophic bacteria on lipolysis. J Food Sci 78: M1405-M1411.

Gaysinsky S, Taylor TM, Davidson PM, Bruce BD, Weiss J (2007) Antimicrobial efficacy of eugenol microemulsions in milk against *Listeria monocytogenes* and *Escherichia coli* O157:H7. J Food Protect 70: 231-237.

Giannino ML, Marzotto M, Dellaglio F, Feligini M (2009) Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods. Int J Food Microbiol 130:188-195.

Gislene B, Favarin L, Luchese RH, Mcintosh D (2015) Psychrotrophic bacteria in milk: How much do we really know?. Braz J Microbiol 46: 313-321.

Gleeson D, O'connell A, Jordan K (2013) Review of potential sources and control of thermoduric bacteria in bulk tank milk. Irish J Agr Food Res 52: 217-227.

Gutiérrez-Cortés C, Suárez H, Buitrago G, Moreno CD (2017) Isolation and evaluation of the antagonist activity of lactic acid bacteria in raw cow milk. Agronomía Colombiana 35: 357-364.

Hantsis-Zacharov E, Halpern M (2007a) Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. Appl Environ Microbiol 73: 7162-7168.

Hantsis-Zacharov E, Halpern M (2007b) *Chryseobacterium haifense sp.* nov., a psychrotolerant bacterium isolated from raw milk. Int J Syst Evol Microbiol 57: 2344-2348.

Higginbotham KL, Burris K, Zivanovic S, Davidson PM, Stewart CN (2013) Antimicrobial activity of *Hibiscus sabdariffa* aqueous extracts against *Escherichia coli* O157:H7 and *Staphylococcus aureus* in a microbiological medium and milk of various fat concentrations. J Food Protect 77: 262-268.

Lafarge V, Ogier JC, Girard V, Maladen V, Leveau JY, Gruss A, Delacroix-Buchet A (2004) Raw cow milk bacterial population shifts attributable to refrigeration. Appl Environ Microbiol 70: 5644-5650.

Larsen MK, Hymøller L, Brask-Pedersen DB, Weisbjerg MR (2012) Milk fatty acid composition and production performance of Danish Holstein and Danish Jersey cows fed different amounts of linseed and rapeseed. J Dairy Sci 95: 3569-3578.

Lim CS, Oh YS, Lee JK, Park AR, Yoo JS, Rhee SK, Roh DH (2011) *Flavobacterium chungbukense sp.* nov., isolated from soil. Int J Syst Evol Microbiol 61: 2734-2739.

Lopez MJ, NICHOLS NN, DIEN BS, MORENO J, BOTHAST RJ (2004) Isolation of microorganisms for biological detoxification of lignocellulosic hydrolysates. Appl Microbiol Biotechnol 64: 125-131.

Machado SG, Bazzolli DMS, Vanetti MCD (2013) Development of a PCR method for detecting proteolytic psychrotrophic bacteria in raw milk. Int Dairy J 29: 8-14.

Machado SG, DA Silvf LA, Bazzolli DMS, Heyndrickx M, Costa PM, Vanetti MCD (2015) *Pseudomonas spp.* and *Serratia liquefaciens* as Predominant Spoilers in Cold Raw Milk. J Food Sci 80: M1842-M1849.

Machado SG, Baglinière F, Marchand S, Van coillie E, Vanetti MCD, De block J, Heyndrickx M (2017) The Biodiversity of the Microbiota Producing Heat-Resistant Enzymes Responsible for Spoilage in Processed Bovine Milk and Dairy Products. Front Microbiol 8: 1-22.

Mami A, Hamedi AR, Henni J, Kerfouf A, Kihal M (2012) Screening of autochthonous Lactobacillus species from Algerian raw goats' milk for the production of bacteriocin-like compounds against *Staphylococcus aureus*. Afr J Biotechnol 11: 4595-4607.

Mankai M, Boulares M, Ben Moussa O, Karoui R, Hassouna M (2012) The effect of refrigerated storage of raw milk on the physicochemical and microbiological quality of Tunisian semihard Gouda-type cheese during ripening. Int J Dairy Technol 65: 250-259.

Mcauley CM, Britz ML, Gobius KS, Craven HM (2015) Prevalence seasonality and growth of Enterococci in raw and pasteurized milk in Victoria, Australia. J Dairy Sci 98: 8348-8358.

Michel V, Hauwuy A, CHAMBA JF (2001) Raw cow milk microflora: diversity and influence of conditions of production. Dairy Sci Technol 81: 575-592.

Morandi S, Cremonesi P, Povolo M, Brasca M (2012) *Enterococcus lactis sp*. nov from Italian raw milk cheeses. Int J Syst Evol Microbiol 62: 1992-1996.

Nahla TK (2006) Study on mesophilic *Aeromonas* in milk and some milk products in Port Said city. J Appl Sci Res 11: 1037-1041.

O'connell A, Ruegg PL, Jordan K, O'brien B, Gleeson D (2016) The effect of storage temperature and duration on the microbial quality of bulk tank milk. J Dairy Sci 99: 3367-3374.

Ouelbani R, Bensari S, Mouas TN, Khelifi D (2016) Ethnobotanical investigations on plants used in folk medicine in the regions of Constantine and Mila (North-East of Algeria). J Ethno Pharm 194: 196-218.

Paludetti LF, Jordan K, Kelly AL, Gleeson D (2018) Evaluating the effect of storage conditions on milk microbiological quality and composition. Irish J Agr Food Res 57: 52-62.

Pașca C, Mărghitaș LA, Dezmirean D, Bobiș O, Bonta V, Mărgăoan R, Chirilă F, Fit N (2015) The assessment of the antibacterial activity of some plant extracts on normal and pathogenic microflora from milk. Anim Sci Biotechnol 48: 166-172.

Perko B (2011) Microbiological quality of raw milk. Mljekarstvo 61: 114-124.

Quigley L, O'sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, Cotter PD (2013) The complex microbiota of raw milk. J Dairy

Sci 96: 4928-4937.
Rasolofo EA, St-gelais D, Lapointe G, Roy D (2010) Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. Int J Food Microbiol. 138: 108-118.
Ribeiro Júnior JC, De oliveira AM, Silva FG, Tamanini R, De oliveira ALM, Beloti V (2018) The main spoilage-related psychrotrophic bacteria in refrigerated raw milk. J Dairy Sci 101: 75-83.
Samaržija D, Zamberlin Š, Pogacic T (2012) Psychrotrophic bacteria and milk quality. Mljekarstvo 62: 77-95.
Sanz Sampelayo MR, Chilliard Y , Schmidely P, Boza J (2007) Influence of type of diet on the fat constituents of goat and sheep milk. Small Ruminant Res 68: 42-63.
Sharifi Rad J, Lalanne GM, Alvarez AJH, Taheri Y, SHaheen SH, Kregiel D, Antolak H, Pawlikowska E, Jokanovic MB, Rajkovic J, Hosseinabadi T, Masic BL, Baghalpour N, Mohajeri M, Martins N (2020) Malva species: Insights on its chemical composition towards pharmacological applications. Phytother Res 34: 546-567.
Silvetti T, Morandi S, Brasca M (2014) Biopreservation potential of *Enterococcus faecalis* isolated from Italian traditional raw milk cheeses. J Food 12: 210-217.
Toqeer A, Kanwal R, Avub N (2006) Influence of temperature on growth pattern of *Lactococcus lactis, Streptococcus cermoris* and *Lactobacillus acidophilus* isolated from camel milk. J Biotechnol 5: 481-488.
Vithanage NR, Yeager TR, Jadhav SR, Palombo EA, Datta N (2014) Comparison of identification systems for psychrotrophic bacteria isolated from raw bovine milk. Int J Food Microbiol 189: 26-38.
Vithanage NR, Dissanayake M, Bolge G, Palombo EA, Yeager TR, Datta N (2017) Microbiological quality of raw milk attributable to prolonged refrigeration conditions. J Dairy Res 84: 92-101.
Vyletělova M, Hanuš O, Urbanova E, Kopunecz P (2000) The occurrence and identification of psychrotrophic bacteria with proteolytic and lipolytic activity in bulk milk samples at storage in primary production conditions. Czech J Anim Sci 45: 373-383.
Yabrir B, Zobiri A, Laoun A, Titouche Y, Chenouf NS, Ranebi D, Isselnane S, Mati A (2018) Bacteriological behavior of ewe's raw milk collected in Algerian area steppe and refrigerated at 4°C or 7°C. Livestock Res Rural Dev 30(2). http://www.lrrd.org/lrrd30/2/byab30023.html
Yuan L, Sadiq FA, Liu TJ, LI Y, GU J, Yang HY (2018) Spoilage potential of psychrotrophic bacteria isolated from raw milk and the thermo-stability of their enzymes. J Zhejiang Univ-SC B 19: 630-642.

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Research article
Ερευνητικό άρθρο

Hematological and Biochemical Parameters of Pregnant and Lactating Goats in Rangeland of Cholistan Desert, Bahawalpur, Pakistan

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ABSTRACT: Jattal goats (n=90) of approximately 2-6 years of age being reared in Cholistan desert of Pakistan were studied during January to December, 2015 to examine the alterations in hematochemical parameters of Jattal goats at different reproductive phases while feeding on natural vegetations of desert areas of Cholistan. These were divided into three equal groups (non-pregnant, pregnant and lactating). Blood samples were collected by jugular vein puncture from goats of these three groups. Hematological parameters, white blood cells (WBC), lymphocytes (LYM), monocytes (MON), granulocytes (GRA), red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and red cell distribution width RDW were recorded.

The results obtained revealed that all these parameters were generally lower in non-pregnant goats. The study revealed that significant higher values of hemoglobin (Hb), mean cell hemoglobin (MCH) and mean corpuscular volume (MCV) were observed in the lactating goats ($P<0.05$). Pregnant goats showed a significantly ($P<0.05$) higher values of leucocytes (WBC) than lactating and non-pregnant goats. Plasma sodium (Na) and potassium (K) concentration were markedly lower in lactating goats. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly lower in pregnant goats. The Plasma concentration of cholesterol and triglycerides were higher in pregnant goats.

In conclusion in present study changes in some hematochemical parameters have been determined in pregnant, non-pregnant and lactating goats of Jattal breed.

Keywords: Jattal goat, haematochemical, Cholistan desert.

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INTRODUCTION

Goats play a vital role in the livestock subsector of the Pakistani agricultural economy. Pakistan hosts 68.4 million goats and constitutes significant source of milk and meat for millions of Pakistani people. Livestock division contributes about 58.9% in total agricultural value increased and about 11.3% of country GDP (Anonymous, 2018). Keeping of small ruminants is necessary practice in rural parts of Pakistan as it plays a vital role in the socio-economic profile through provision of an income and employment. Goats consume a large number of grasses, bushes, shrubs, tree leaves and crop residues that would otherwise go waste and cause environmental pollution. The goats can be milked any time of the day and are therefore named as the 'moving refrigerators'. Goat meat is preferred over other meat in Pakistan because it is leaner (Jindal, 1984). The Jattal is one of the important breeds of goat maintained in Cholistan areas of Pakistan. Farmers prefer it over other breeds because it has high prolificacy with three kidding in two years (Lashari and Tasawar, 2010).

Diet, age, sex, pregnancy and estrus are known to affect the biochemical and hematological parameters (Balikci *et al.,* 2007). Blood is a reliable and main medium for measuring the health condition of animals. Research has confirmed the incident of modifications in certain constraints during lactation and gestation period after birth in several animal species (Ozyurtlu *et al.,* 2007), however, no indicated values are existing for blood constraints before and during pregnancy in the Jattal goat. Therefore, the present work focused on the chosen and biochemical parameters in pregnant, and lactating Jattal goats being reared in Cholistan desert of Pakistan.

MATERIALS AND METHODS

Location and climate of the study area

This research was performed on the Jattal breed of goat reared in Cholistan desert, Punjab, Pakistan. This is the seventh largest desert of the world. Cholistan desert comprises about 26,000 km^2 situated between latitude 27 to 29 N and longitude 69ºto 75º E at height of about 112 m above sea level (Ali *et al*., 2009).

The Cholistani environment is arid subtropical with meager rainfall, low relative humidity, high temperature and strong summer winds. It is the driest and hottest area of Pakistan, with summer spanning May through October. Randomly selected artificial/natural reservoirs and ponds, called Tobas. (Farooq *et al.,* 2015). It is the homeland of many precious animal genetic resources. Most of the Cholistan is covered with wide range of nutritious and drought tolerance species of plants.

Experimental Period and Animals

Ninety adult female goats ranging between 2-5 years of age were used in a study approved by the Ethical Review Committee for the Use of Animals, under the administrative control of the Office of Research, Innovation, and Commercialization of The Islamia University, Bahawalpur. Written consent was obtained from the Cholistani pastoralists involved in our study. The selected flocks were under natural grazing with seasonal/perennial grasses along with tree looping. Important species of natural cholistan vegetation consumed by goats were: *Cynodon dactylon, Ochthochola compressa, Solanum surattense, Tribulus longipetalus Tribuluslongipetalus, Tribulus terristris, Pulicaria crispa, Avera javanica, Haloxylon salicornicum, Calligonum polygoxoidis.*

These goats were divided into three equal groups (n=30/group) *viz* .non-pregnant, pregnant (more than 90 days) and lactating. All animal were clinically normal and healthy and were free of endo/ectoparasites.

Blood collection

For hematology and biochemical parameters 10 mL blood samples were taken from every goat by jugular vein puncture into clean test tubes and stored as two aliquots: un-clotted for hematological examination and clotted for harvesting serum. While the animal was manually restrained, for the sake of standardization of collection technique all samples was collected by same person from animals restrained by same technique.

Transportation of samples was made in an ice box to the Physiology Laboratory, Department of Life Sciences, the Islamia University of Bahawalpur, Pakistan, refrigerated and analyzed within 12h for the assessment of hematological parameters by using an automated hematology analyzer (Mythic 18; Orphee, USA). Entire samples were examined on the same day.

Blood samples for biochemical analyses were centrifuged at 3500 rpm for 10-15 min., and the serum was harvested in serum collection containers. The biochemical parameters included triglycerides (TGs) cholesterol, aspartate aminotransferase (AST), ala-

nine aminotransferase (ALT), Sodium (Na^+) and Potassium (K^+) which were assessed using the biochemistry analyzer (CHEM 100, Japan).

Statistical Analysis

Statistical analysis was conducted through MINITAB. The mean values (±SEM) for the hematochemical parameters were calculated. Variation between the mean values for three groups was attained through ANOVA. $P<0.05$ was considered as statistically significant.

RESULTS

Hematochemical Parameters

Table 1 shows the mean+ SE values for red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin(MCH), mean cell hemoglobin concentration (MCHC) and red cell distribution width RDW in non-pregnant, pregnant and lactating goats.

A gradual increase 3.41±0.17x106/µL, 3.73±0.13 x106/µL and 3.93±0.25 x106/µL in the number of erythrocyte was examined in the peripheral blood of non-pregnant, pregnant and lactating goats respectively. The haemoglobin (Hb) level was significantly lower ($P<0.05$) in pregnant and lactating goats as compared to non-pregnant goats. The haematocrit (HCT) value was higher in pregnant and lactating goats as compared to non-pregnant goats. The mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and red cell distribution width (RDW) were significantly ($P<0.05$) lower in pregnant and lactating goats as compared to non-pregnant goats.

Table 2 shows the mean + SE values for white blood cells (WBC), lymphocytes (LYM), monocytes (MON), granulocytes (GRA).

The mean leukocyte (WBC) value was 17.32±1.70x10^3/µL in non-pregnant goats and prominent higher 24.44±2.08x10^3/µl was detected in pregnant goats and significantly lower 20.78±1.65 x10^3/µL was recorded in lactating goats. The difference was statistically significant ($P<0.05$). Lymphocyte (LYM) mean ± SEM value was 9.04±1.14x103/µL in non-pregnant goats. The highest value recorded 13.77±1.56 x103/µL in pregnant goats and the lactating goats also showed higher values of Lymphocyte as compared to non-pregnant goats. The mean±SEM value of monocytes (MON) was 0.84±0.05x103/µL in non-pregnant goats which was significantly ($P<0.05$) lower than lactating and pregnant goats.

Apparently higher 9.67±0.79x103/µL value of granulocytes (GRA) was recorded in pregnant goats as compared to non-pregnant and lactating goats but statistically the difference was non-significant ($P>0.05$).

The result of biochemical parameters of the non-pregnant, pregnant and lactating goats are shown in Table 3. There were statistically significant ($P<0.05$) differences in the values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol and triglycerides between the non pregnant and pregnant goats. Aspartate aminotransferase (AST), cholesterol and triglycerides levels were higher in pregnant and lactating goats than non-pregnant, while a lower value of alanine aminotransferase (ALT) was observed in pregnant and lactating goat. There was significant decreasing trend in the sodium and potassium parameters, but the decrease was not statistically significant ($P>0.05$).

Table 1. The mean ± SEM values of red blood cell parameters of non pregnant, pregnant and lactating goats maintained in Cholistan desert of Bahawalpur

Parameters	Non-Pregnant (n=30)	Pregnant (n=30)	Lactating (n=30)
RBC (10^6/ µL)	3.41±0.17	3.73±0.13	3.93±0.25
Hb (g/dl)	7.93±0.20	7.32±0.16*[a]	7.05±0.25*[b]
HCT (%)	10.08±0.52	10.69±0.38	11.34±0.72
MCV ((μm^3)	29.60±0.16	28.67±0.31*[a]	28.83±0.19*[b]
MCH (Pg)	24.14±0.99	20.47±0.86	18.70±0.85*[b]
MCHC (g/dl)	81.39±3.21	69.25±2.29*[a]	64.83±2.92*[b]
RDW(%)	16.86±0.84	14.94±0.37*[a]	13.68±0.48*[b]
RDW-SD (μm^3)	24.17±0.31	23.09±0.39	21.37±0.67*[b]

Table 2. The mean ± SEM values of white blood cell parameters of Non pregnant, pregnant and lactating goats maintained in Cholistan desert of Bahawalpur

Parameters	Non-Pregnant (n=30)	Pregnant (n=30)	Lactating (n=30)
WBC (10^3/ µL)	17.32±1.70	24.44±2.08*a	20.78±1.65
LYM (10^3/ µL)	9.04±1.14	13.77±1.56	10.96±1.08
MON (10^3/ µL)	0.84±0.05	1.00±0.09	1.04±0.08
GRA (10^3/ µL)	7.43±0.67	9.67±0.79	8.77±0..69
LYM (%)	50.04±2.32	54.00±2.54	52.33±2.13
MON (%)	5.30±0.27	4.48±0.38	5.13±0.27
GRA (%)	44.66±2.14	41.54±2.32	42.56±2.07

Table 3. The mean ± SEM values of biochemical parameters of Non-pregnant, pregnant and lactating goats maintained in Cholistan desert of Bahawalpur

Parameters	Non-Pregnant (n=30)	Pregnant (n=30)	Lactating (n=30)
AST(IU/L)	36.57±7.50*[a]	36.43±4.83*[a]	39.29±5.13*[b]
ALT(IU/L)	26.43±4.33*[a]	20.71±3.15*[b]	23.14±2.83*[b]
Cholesterol (mg/dL)	56.86±6.92*[a]	76.14±6.69*[b]	70.71±5.44*[b]
Triglyceride (mg/dL)	54.86±6.01	74.43±3.91*[a]	63.43±3.15
Sodium(MEQ/L)	152.9±12.00*[a]	131.57±4.27*[b]	128.57±4.44*[b]
Potassium(MEQ/L)	3.96±0.21	3.54±0.23	3.90±0.31

DISCUSSION

This is the first study of Jattal goats of Colistan which was designed to determine salient hemato-chemical parameters among non-pregnant, pregnant and lactating goats maintained in Cholistan desert of Bahawalpur.

Hematochemical blood factors are most important for the livestock management as they provide essential indicators that may help to judge the animal's health status (Coles, 1986). It is accepted that numerous parameters like nutrition, age, stress, environment, muscle activity, gestation and disease have much effects on blood picture (Meyer and Harvey, 2004; Klinkon *et al.,* 2012).

In present study red blood cell (RBC) counts showed non-significant (P>0.05) difference among the non pregnant, pregnant and lactating goats. But pregnant and lactating goats had apparently higher RBCs count. This confirms the result of Pospisil (1987) who reported that there were no differences in red blood cell picture of 16 pregnant 30 non-pregnant and 20 lactating female goats older than 3 years.

Similar results have been documented in pregnant ewe, equine, pigs and canines. During the final stages of pregnancy the hemodilution effect boost in plasma levels of red blood cells (Jain, 1993). Azab and Abdel-Maksoud (1999) also found the same results in pregnant goats. The hemodilution in ruminants decreases the flow of blood in the capillary vessels but it may improve the flow of blood through the capillary vessels of placenta to enhance the diffusion of Oxygen and other nutrients to the embryo (Yılmaz, 2000).

The present RBCs results were however different to that reported by Garkal *et al.* (2016) who described erythrocyte count decrease during pregnancy in buffaloes. This decrease may be due to the under stress condition due to the destruction of erythrocytes (Muna *et al.,* 2003).

The present study demonstrated that leucocyte counts were significantly (P<0.05) higher in pregnant and lactating goats as compared to non-pregnant goats. Present results are comparable to that described by Jain (1993), in that WBCs volume slowly boost up during the period of gestation upto the day of parturition. Lymphopenia and eosinopenia may be the cause of leucocytosis at the time of parturition. The neutrophils, lymhocytes and eosinopiles studies were comparable to those reported by (Azab and Abdel-Maksoud, 1999). The significant increase in the leukocytes count observed in pregnant goats is consistent with the earlier reports of Fortagne and Schafer (1989) who described an increase in the total leukocyte count in pregnant goats. Sandabe and Yahi, (2000) noted a significant increase in the leukocyte count of pregnant Sahel goats. This might be due to increase in

the bone marrow activity as well as, pregnancy stress. According to Dellmann and Brown (1987) the stress probably excite the discharge of main features called leucocytosis inducing factor (LIF) and colony stimulating factors (CSF) which are known to boost haemopoietic activities and blood cells mobilization into circulation.

The results of biochemical parameters in the present study showed that the cholesterol concentration was significantly ($P<0.05$) higher in pregnant and lactating goats as compared to non pregnant goats. These results were comparable as reported by Waziri *et al.* (2010) in Sahel does. Similarly Biagi *et al.,* 1988 observed increased cholesterol level during pregnancy in sheep and Saanen goats respectively.

The differences in the level of biochemical parameters between the sources are enormous and results from nutrition breed, environment, season, age, stage of pregnancy, milk yield differences in the goats, time of sampling and analytical methods (Hassan *et al*., 1986).

The triglyceride concentration was significantly higher in pregnant goats as compared to lactating and non-pregnant goats. Krokavec *et al.* 1992 reported the same result according to the present study. A variation in the results of triglycerides levels was reported by Nazifi *et al.* (2002) in Iranian goats.

A significant decrease in plasma sodium level was found in lactating goats in the present study. A non significant variation of K was observed among non-pregnant, pregnant and lactating goats. But apparently low potassium level was observed in pregnant group. The decrease in K level during pregnancy might be due to the mineral corticoid activity of progesterone (Azabe and AbedlMaksoud, 1999) where the amount of discharge of potassium are increased which may be cause to reduce this electrolyte.

Alanin aminotransferase (ALT) was higher in the non-pregnant goats and lower in pregnant and lac tating goats. The decrease in Alanin aminotransferase (ALT) activity in the pregnant goats was due to uterine and reproductive hormonal changes during pregnancy stage. The present results are comparable with Tainturier *et al.* (1984) who informed that alanin aminotransferase action reduced in the last months of pregnancy and that it remained stable in the start of lactation. Present study proved that lactating goats shoe increased levels of Alanin aminotransferase (ALT).

The highest activity of aspartate aminotransferase (AST) concentration was recorded in lactating goats and decreased in dry and pregnant groups. But apparently increased in the lactating goats was recorded. Tainturier *et al.* (1984) reported that AST activity in dairy cattle changes occasionally during pregnancy and lactation. This increase might be due to the release of this enzyme from liver metabolism during lactating period.

CONCLUSION

This study revealed that significant higher values of hemoglobin (Hb), mean cell hemoglobin (MCH) and mean corpuscular volume (MCV) were observed in the lactating goats ($P<0.05$). Pregnant goats showed a significantly ($P<0.05$) higher values of leucocytes (WBC) than lactating and non-pregnant goats. Plasma sodium (Na) and potassium (K) concentration were markedly lower in lactating goats. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly lower in pregnant goats. The plasma concentration of cholesterol and triglycerides were higher in pregnant goats.

CONFLICT OF INTEREST

None declared.

REFERENCES

Ali I., M.S. Chaudhry and U. Farooq. 2009. Camel rearing in Cholistan desert of Pakistan. Pakistan Veterinary Journal. 29: 85-92.

Anonymous. 2018. Economic Survey of Pakistan Livestock wing, Islamabad 2017-18.

Azab M.E.H.A. and Abdel-Maksoud 1999. Changes in some hematological and biochemical parameters during prepartum and postpartum periods in female Baladi goats. Small Ruminant Research. 34: 77–85.

Balikci E. A., A. Yaldiz and F. Gurdogan. 2007. Blood metabolic concentrations during pregnancy and postpartum in Akkaraman ewe. Small Ruminant Research. 67: 247-251.

Biagi G., M. Bagliacca, A. Leto and A. Romagnoli. 1988. The use of metabolic profile test in a Saanen goat herd. Ann. Fac. Med. Vet. Pisa. 51: 395-410.

Coles E. H. 1986. Veterinary Clinical Pathology. 3rd Edition. W. B Sanders Company. Philadalphia. 10-20.

Dellmann H. D. and E. M. Brown. 1987. Textbook of Veterinary Histology. 3rd Edition. Lea and Febiger, Philadelphia. 71-95.

Farooq U., S.A. Mahmood, A. Ijaz, N. Ahmad, M. Idris and M.T. Abbas. 2015. Evaluation of post thaw sperm parameters and fertility of Cholistani service bulls. Turkish Journal of Veterinary and Animal Sciences. 39: 472–479.

Fortagne M. and M. Schafer. 1989. Haematological parameters of goats in the period from pregnancy to lactation. Archiv für Experimentelle Veterinaermedizin. 43: 223-230.

Garkal R. A., P.M. Kekan, S. D. Kharde, V. K. Munde, B. W. Narladkar and M.S. Vaidya. 2016. Effect of different housing systems on haematological parameters of buffaloes. Buffalo Bulletin 35 (2): 191-197.

Hassan G. A., F. D. El-Nouty, M. A. Samak and M. H. Saleem. 1986. Relations between milk productions and some blood constituents in Egyaptianbaladi goats. Beiter. Trop. Landwritsch Veterinamed. 24: 213-219.

Jain N. C. 1993. Essentials of Veterinary Hematology, Comparative Hematology of Common Domestic Animals. Lea and Febiger, Philadelphia, PA. 44-46.

Jindal S. K., 1984. Goat Production. Cosmo Publications, New Delhi, India.

Klinkon M., G. Gorjanc, Z. Klinkon, J. Ježek and M. Simčič. 2012. Blood parameters of Cika calves and bulls. Veterinary Clinical Pathology. 41: 40.

Krokavec M., K. Simo, A. Martinko and M. Krokavcova. 1992. Metabolic indicator in large scale breeding in goats during various seasons. Journal of Veterinary Medicine. 37: 879-889.

Lashari M. H. and Z. Tasawar. 2010. Effect of hCG given on day 12 post-mating on ovarian function and embryo survival in Beetal goats in southern Punjab, Pakistan. Turkish Journal of Veterinary and Animal Sciences. 34(6): 513-517.

Meyer D. J. and J. W. Harvey. 2004. Veterinary Laboratory Medicine: Interpretation and Diagnosis (3rd ed.), Saunders, St. Louis, p. 5.

Muna Ahmed M. M., M. E. Awad and M. E. S. Barri. 2003. Some micromineral profile in the serum of female camels as affected by the physiological state. Pakistan Journal of Biological Sciences. 6: 1853- 1855.

Nazifi S., H. R. Gheisari and F. Shaker. 2002. Serum lipid and lipoprotein and their correlation with thyroid hormone in the clinically healthy goats. Veterinarski Arhiv. 72: 249-257.

Ozyurtlu N., S. Y. Gürgöze, S. Bademkıran, A. Şimşek and R. Çelik. 2007 İvesikoyunlarındadoğumöncesivesonrasıdönemdekibazıbiyokimyasalparametrelerve mineral maddedüzeylerininaraştırılması. F Ü Sag BilDerg. 21: 33–36 (in Turkish).

Pospisil J., F. Kase and J. Váhala. 1987. Basic haematological values in the Cameroon goat (Capra hircus). Comparative Biochemistry and Physiology. A Comparative Physiology. 88(3): 451-454.

Sandabe, U. K. and D. Yahi. 2000. Effect of pregnancy on some haematological parameters in Sahel goats. Annals of Borno. 27: 326-330.

Tainturier D. J., P. Braun, A. G. Rico and J. P. Thouvenot. 1984. Variation in blood composition in dairy cows during pregnancy and after calving. Research in Veterinary Science. 37: 129-131.

Waziri M. A., A. Y. Ribadu and N. Sivachelvan. 2010. Changes in the serum proteins, hematological and some serum biochemical profiles in the gestation period in the Sahel goats. Veterinarski Arhiv. 80 (2): 215-224.

Yılmaz B. 2000. Blood and Blood Circulatory. second edition. Feryal Matbaacilik, Ankara, Turkey, pp. 307-308.

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Case report
Κλινικό περιστατικό

Canine trypanosomosis: a case report

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ABSTRACT: Trypanosomosis is a haemoprotozoan infection affecting a broad range of wild and domestic animals including dogs. Infected dogs may die within 2-4 weeks in the acute and fatal form of the disease. A dog was presented with the complaint of anorexia, persistent recumbency and loss of body weight which lasted for few days. On clinical examination, the affected dog revealed rise in temperature (105°F), lachrimation, anaemia, unilateral corneal opacity, swelling of throat, enlargement of lymph nodes and severe loss of bodily condition. Wet smear, thin blood smear and hematological parameters were observed. Motile trypanosomes were found in wet smear, while trypanosomes with a characteristic flagellum, kinetoplast and undulating membrane were present outside the red blood cells in the thin smear. The dog was successfully recovered with a single dose of diminazene aceturate. This report may provide a way forward to establish effective and safe therapeutic protocols for the control of canine trypanosomosis.

Keywords: Dog; trypanosomes; corneal opacity; thin smear; diminazene aceturate

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INTRODUCTION

Trypanosomosis is a haemoprotozoan infection affecting a broad range of wild and domestic animals including dogs. Dogs are affected by two groups of trypanosomes: (i) mechanically transmitted; *Trypanosoma* (*T.*) *evansi* and (ii) biologically transmitted salivarian trypanosomes i.e. *T. brucei* and *T. congolense*. The latter trypanosomes are transmitted biologically by tsetse flies while former species are mechanically transmitted either by eating infected carcasses or by biting insects like *Tabanus* and *Stomoxys* (Green, 2006; Urquhart et al., 1996). *Trypanosoma evansi* causes surra disease mostly in camel and horses but adult dogs have also been widely affected (Rashid et al., 2008; Defontis et al., 2012; Rashid et al., 2014). In tropical and subtropical regions, trypanosomosis is prevalent and although canine trypanosomosis is not frequent but outbreaks of canine disease have been reported from South America, Brazil, Iran, Tunisian, India, Germany and Afghanistan (Ian et al., 2004; Morteza et al., 2007; Eloy and Lucheis, 2009;Rjeibi et al., 2015). In Algeria, *T. evansi* and *T. congolense* have been identified in dogs through molecular investigation (Medkour et al., 2020). Canine trypanosomosisis characterized by neurologic, ocular and vascular signs in infected dogs (Bhardwaj et al., 2015). Infected dogs die within 2-4 weeks in the acute and fatal form of the disease (Soulsby, 1982). Clinical manifestations of the disease may include intermittent fever (39°C-41°C), anaemia, anorexia leading to emaciation, progressive weakness, oedema of the abdominal wall and legs, enlarged superficial lymph nodes, edema of head and throat (to be differentiated from rabies), myocarditis, paresis of the hindquarters (sometimes) and ocular signs include conjunctivitis, lachrymation, and corneal opacity (Savani et al., 2005; Sonika et al., 2007; Da Silvaet al., 2009). A single dose of diminazene aceturate has been found to effective against canine infection among the available trypanosomacidal drugs including diminazene, quinapyramine and suramine (Kumar, 2017). This report presents a case of canine trypanosomosis and therapeutic response with diminazene aceturate.

CASE HISTORY

A male greyhound dog of 3-year age was brought to outdoor clinic of KBCMA College of Veterinary and Animal Sciences Narowal, sub campus of University of Veterinary and Animal Sciences, Lahore with the complaint of anorexia, persistent recumbency and loss of body weight from last few days. The owner of the dog reportedthat he also has another greyhound dog (2. 5-year-old) at home which looked normal.

On clinical examination of the dog these parameters were observed: temperature (105°F), heart rate (152 bpm), respiration (56 breaths per min), lachrimation, anaemia (markedly pale mucous membrane), unilateral corneal opacity (Figure 1), swelling of throat, enlargement of lymph nodes and severe loss of bodily condition. Next day, blood examination of the second dog, which had normal body temperature and submandibular oedema, was also performed.

Figure1. Corneal opacity in the dog

Blood sample was collected aseptically on the owner's consent. The blood was observed for parasites. For this purpose, marginal ear vein was pricked after the application of methylated spirit and drop of blood was taken on the slide. Then, thin smear was made, air dried, fixed in methanol and stained with Field's stain (A & B). The thin smear was observed under the oil immersion lens (X100). Blood from cephalic vein was taken for the observation of a direct smear (wet smear) and for examining other heamatological parameters. All the blood sampling on the animals was conducted during the process of diagnosing and treatment in the veterinary practice.

RESULTS

Wet smear revealed the presence of large number of motile trypanosomes. On the observation of thin smear of dog 1, trypanosomes with a characteristic flagellum, kinetoplast and undulating membrane were found outside the red blood cells (Figure 2). Results of haematological parameters have been summarized in the Table 1. The thin smear of second dog was also positive for trypanosomes (Figure 3). Babesio-

sis, theileriosis and anaplasmosis were excluded after examining a blood smear. Both the dogs were treated with diminazene aceturate (3. 5mg/kg b. w., intramuscularly) and other supportive therapy. With a single dose of diminazene aceturate, each dog recovered after follow up of 15 days.

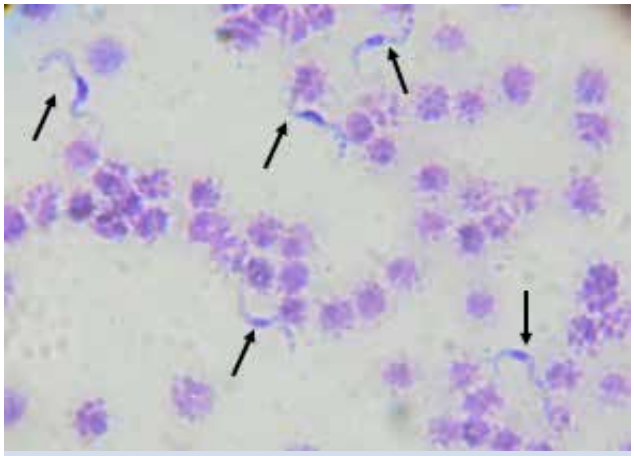

Figure2. *Trypanosoma* spp., dog 1, Field's-stained thin blood smear (X100)

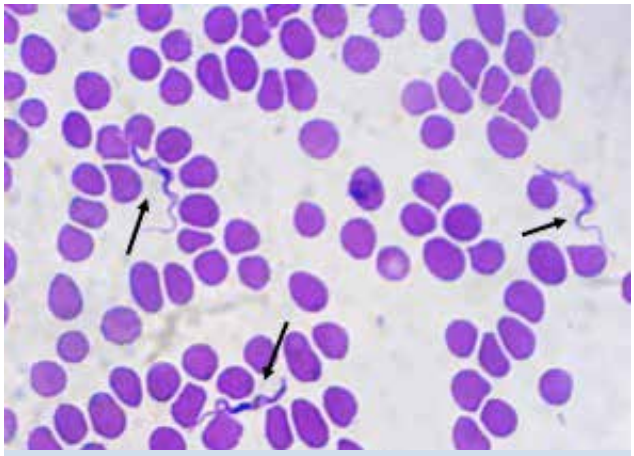

Figure3. *Trypanosoma* spp., dog 2, Field's-stained thin blood smear (X100)

Table 1: Haematological evaluation of dogs infected with trypanosomosis

Haematological parameter	Observed value (Dog 1)	Observed value (Dog 2)	Reference value
Platelets (10^9 /L)	150	250	200–500
Erythrocytes (10^{12}/L)	4.5	5.5	5.5-8.5
Leucocytes (10^9 /L)	12	10	6–17
Basophils (10^9 /L)	0.02	0.01	rare
Monocytes (10^9 /L)	0.88	0.86	0.15–1.35
Eosinophils (10^9 /L)	0	0	0 0.1–1.25
Haemoglobin (g/dL)	8	11.5	12–18
Haematocrit (%)	30	38	37–55

DISCUSSION

Trypanosomosis is of great economical and medical significance infecting a broad range of mammalian host including canines (Desquesnes et al., 2013). Despite that, reports on clinical disease in canines are limited (Aref et al., 2013; Rjeibi et al., 2015). The trypanosomes like *T. evansi, T. congolense, T. brucei*, and *T. cruzi* can infect dogs (Stephen, 1970; Abenga et al., 2005). The clinical signs and morphological features of the trypanosomes found in the present study are in agreement with several reports (Savani et al., 2005; Sonika et al., 2007; Urquhart et al., 1996; Da Silva et al., 2009).

Since 1955, diminazene aceturate has been used as drug of choice for the treatment of trypanosomes infection in livestock. Diminazene use is limited in canine and equine species, besides being an effective trypanocidal drug (Desquesnes et al., 2013). Relapsing parasitaemia has been observed after the use of single dose of diminazene (intramuscular administration) at 3. 5 mg/kg in equines (Tuntasuvan et al., 2003) and at 5 mg/kg in dogs (Rjeibi et al., 2015). The relapsing parasitaemia may represent that drug, in case of central nervous system involvement, is not capable to cross the blood-brain barrier, its concentration in the plasma is not sufficient or drug resistance has developed (Da Silva et al., 2009). The factors like low therapeutic index, variable pharmacokinetics, excessive drug concentration in plasma, high doses, and adequate doses (given at short intervals) may contribute towards diminazene aceturate toxicosis (Flores et al., 2014; Echeverria et al., 2019). The mortality in dogs after diminazene diaceturate (Rjeibiet al., 2015) and diminazene aceturate (Echeverria et al., 2019) therapy was also observed due to injury to the central nervous system. The recovery of the dog with diminazene aceturate was observed in this study which has also been observed by Gunaseelan et al. (2009) and Kumar (2017). Treatment of canine trypanosomosis with Cymelarsan® (Rhone Meriux, France) and mag-

nesium chloride has also been reported (Rashid et al., 2014).

Hematological analyses of blood of infected animals revealed anemia, hypoproteinemia and thrombocytopenia in this reported case of trypanosomosis which is in agreement with Rjeibi et al. (2015). In a similar study, dog infected with trypanosomes represented increased erythrocyte sedimentation rate (ESR) and decreased packed cell volume (PCV) and haemoglobulin (Rashid et al., 2008). Refractory hypoglycaemia occurs in dogs infected with *T. congolense* (Deschamps et al., 2016).

It is concluded, based on morphological features, that this is a case of trypanosomosis but for species confirmation, molecular assays are required and single dose of diminazene aceturate may recover infected dogs. To our knowledge, it is first case report of trypanosomosis in dogs in district Narowal, Pakistan which was successfully recovered with diminazene aceturate. The previous studies in Pakistan reported trypanosomes infection mostly in camels, equines, cattle, sheep, goat, bear and puma, while only two studies reported in dog so far; one in Faisalabad (2014) and other in Lahore (2008). Furthermore, one case of human African trypanosomiasis has also been reported in one of the Pakistan Army Troops in 2011, deployed to various countries of Africa as UN peace keepers. Such kind of reports may provide a way forward to establish effective and safe therapeutic protocols for canine trypanosomes.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

REFERENCES

Abenga JN, Ezebuiro CO, Kehinder D, Fajinmi AO, Samdi S (2005) Studies on anaemia in Nigerian local puppies infected with Trypanosoma congolense. Nigerian Institute for Trypanosomiasis Research, Kaduna, Nigeria. Vet Archiv 75:65-174.

Bhardwaj RK, Singh R, Himalini, Virampal Singh (2015) Trypanosomiasis in a Bakerwali dog- a case report. Indian J Anim Sci 7:113-114.

Da Silva AS, Zanette RA, Wolkmer P, Costa MM, Garcia HA, Lopes STA, Santurio JM, Teixeira MMG, Monteiroa SG (2009) Diminazene aceturate in the control of *Trypanosoma evansi* infection in cats. Vet Parasitol 165:47-50.

Defontis M, Richartz J, Englmann N, Bauer C, Schwierk VM, Büscher P, Moritz A (2012) Canine *Trypanosoma evansi* infection introduced into Germany. Vet Clin Pathol 41:396-374.

Deschamps JY, Desquesnes M, Dorso L, Ravel S, Bossard G, Charbonneau M, Garand A, Roux FA (2016) Refractory hypoglycaemia in a dog infected with *Trypanosomacongolense*. Parasite 23:1.

Desquesnes M, Dargantes A, Lai D, Lun Z, Holzmuller P, Jittapalapong S (2013) Trypanosoma evansi and surra: a review and perspectives on transmission, epidemiology and control, impact, and zoonotic aspects. Biomed Res Int 2013:321237.

Echeverria JT, Soares RL, Crepaldi BA, Oliveira GG, Silva PMPD, Pupin RC, Martins TB, Cleveland HPK, Ramos CADN, Borges FA (2019) Clinical and therapeutic aspects of an outbreak of canine trypanosomiasis. Rev Bras Parasitol Vet 28:320-324.

Eloy LJ, Luchesis SB (2009) Canine trypanosomiasis: etiology of infection and implication for public health. J Venom Anim Toxins incl Trop Dis 15:589-611.

Flores MM, Pereira PR, Mazzanti A, Kommers GD, Fighera RA (2014) Aspectosepidemiológicos, clínicos e anatomopatológicos da intoxicação por aceturato de diminazenoemcães. Pesq Vet Bras 34:667-674.

Green CE (2006) Infectious diseases of dogs and cats. 3rd ed, Elsevier Inc: pp 676- 680.

Gunaseelan L, Senthil KK, Selvaraj P, Kathiresan D (2009) Haematobiochemical changes in a case of canine trypanosomiasis. TamilnaduJ Vet &Ani Sci 4:122-123.

Ian M, Peter HH, Michael AM (2004) The Trypanosomiases. 1st ed, CABI Publication, Wallingford.

Kumar S (2017) Trypanosomosis in dog - a case report. ExplorAnim Med Res7:220-222.

Medkour H, Laidoudi Y, Lafri I, Davoust B, Mekroud A, Bitam I, Mediannikov O (2020) Canine vector-borne protozoa: Molecular and serological investigation for *Leishmania spp., Trypanosoma spp., Babesia spp.,* and *Hepatozoon spp.* in dogs from Northern Algeria. Vet Parasitol Regional Studies and Reports 19:100353.

Morteza H, Dariush S, Sedighe, Seyed N, Ramin M (2007) *Trypanosoma evansi* in three dogs in Iran. Comp Clin Pathl 16:69-71.

Rashid A, Rasheed K, Hussain A (2008) Trypanosomiasis in dog; a case report. Iran J Arthropod Borne Dis 2:48-51.

Rashid I, Firyal S, Tariq A, Muhammad G, Saqib M, Zafar MS Use of cymelarsan® and manganese chloride for treatment of the canine trypanosomiasis. Adv Anim Vet Sci 2:104-105.

Rjeibi MR, Hamida TB, Dalgatova Z, Mahjoub T, Rejeb A, Dridi W, Gharbi M (2015) First report of surra (*Trypanosoma evansi* infection) in a Tunisian dog. Parasite 22:3.

Savani, ES, Nunes VL, Galati EA, Castilho TM, Araujo FS, Ilha IM, Camargo MC, D'auria SR, Floeter-Winter LM (2005) Occurrence of co-infection by *Leishmania* (*Leishmania*) *chagasi* and *Trypanosoma* (*Trypanozoon*) *evansi* in a dog in the state of Mato Grosso do Sul, Brazil. Mem Inst Oswaldo Cruz100:739-741.

Sonika S, Sharma G, Juyal PD (2007) Trypanosomosis in a German shepherd dog_a case report. J Vet Parasitol 21:81-82.

Soulsby EJL (1982) Helminths, Arthropods and protozoa of domesticated animals. 7th ed, Bailliere Tindall, London.

Stephen LE (1970) Clinical Manifestation of Trypanosomosis in Livestock and other animals. HW Hulligan ed.

Urquhart GM, Armour J, Duncan JL, Dunn AM, Jennings FW (1996) Veterinary Parasitology. 2nd ed, Blackwell Publishing (MA), UK: pp 212-218.

Tuntasuvan D, Jarabrum W, Viseshakul N, Mohkaew K, Borisutsuwan S, Theeraphan A, Kongkanjana N (2003) Chemotherapy of surra in horses and mules with diminazene aceturate. Vet Parasitol 110:227-233.

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