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ΠΕΚΕ (Ε. Μπατζαλέξη)
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Πατισίων 158, 11257 Αθήνα

**EUROPEAN COLLEGES OF VETERINARY SPECIALISTS
ΕΥΡΩΠΑΪΚΑ ΚΟΛΕΓΙΑ ΕΙΔΙΚΕΥΜΕΝΩΝ ΚΤΗΝΙΑΤΡΩΝ**

				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 Veterinary Microbiological Institute of Votanicos, from where all members 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, Institute 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 capabilities, 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 members. 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 execution of the veterinary profession.

Furthermore, the role of the HVMS has been determinative on the decision making of the Ministry of Agriculture on veterinary 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 Companion Animals (founding member)**
- **Veterinary Society of the Balkan and the Black Sea (founding member)**

The HVMS has a total of 1220 members many of which have been distinguished in the scientific field (University Professors, 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. All 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
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Spec. Secretary:	Konstantinos Chandras
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Member:	Apostolos Rantsios

Hereditary myelopathies in dogs

R.D. Baka , Z.S. Polizopoulou 

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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	Physiotherapy	Poor
Dystrophic myelopathies					
<i>Afgan hound myelopathy</i>	Autosomal recessive mode of inheritance	Afgan hound, Kooikerhondje	C6-T2 or T3-L3 spinal cord	None	Poor
<i>Rottweiler leukoencephalopathy</i>	Suspected inherited disorder	Rottweiler, Rottweiler crossbreed	Cervical spinal cord, brainstem, cerebellum	None	Poor
<i>Rottweiler neuroaxonal dystrophy</i>	Inherited disorder	Rottweiler	Cervical spinal cord	None	Guarded
<i>Spongiform leukoencephalopathy</i>	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 Brainstem/Cerebellar	Dorsolateral funiculi of the white matter: demyelinating lesions 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 (Kathmann et al. 2006). Initially the disease is thought to be breed-specific to German Shepherds (GSD), thus named German shepherd myelopathy (Braund and Vandeveld, 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 signs (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 homozygosity 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 de-

velop 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 post-mortem 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 (Kathmann et 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). Tho-

racic 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; Christman, 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 leuko-dystrophy, 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 mu-

tation 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 Chiari-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 or 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 syringes are not necessarily in more pain than those with smaller syringes (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 syringes are in pain and dogs with smaller syringes 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 cavitory 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, Rottweiler 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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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; Yalçın 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 faecalis*. 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 *Liste-*

ria 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	<i>Escherichia coli</i> and <i>Staphylococcus aureus</i> <i>Listeria monocytogenes</i> <i>Agrobacterium tumefaciens</i> <i>Escherichia coli</i> Bovine mastitis in organic dairy farming: <i>Staphylococcus aureus</i> , <i>Staphylococcus epidermidis</i> , <i>Staphylococcus hyicus</i> , <i>Staphylococcus xylosus</i> and <i>Escherichia coli</i> <i>Pseudomonas aeruginosa</i>	Zhang et al., 2016 Abdollahzadeh et al., 2018 Lee et al., 2020 Kosariet al., 2020 Zhu et al., 2016 Elcocks et al., 2020
Thyme	<i>Listeria monocytogenes</i> <i>Escherichia coli (E. coli) O157:H7</i> <i>Streptococcus mutans</i> <i>Staphylococcus aureus</i> <i>Streptococcus pyogenes</i> <i>Aspergillus flavus</i>	Sarengaowaet al., 2019 Guo et al., 2020 Abdel Hameed et al., 2020 Mohammed et al., 2020 Maqbulet al., 2020 Khaliliet al., 2015
Thyme & Cinnamon	<i>Salmonella</i> Species <i>Salmonella</i> species	Al-Nabulsiet al., 2020 Olaimat et al., 2019
Thyme & Oregano	<i>Listeria monocytogenes</i>	Cho et al., 2020

Pine oil	<i>Escherichia coli</i> O157:H7, <i>Listeria</i> , and <i>Campylobacter</i> species	Wells et al., 2015
Eucalyptus	Multi-drug resistant <i>Acinetobacter baumannii</i> <i>Escherichia coli</i>	Knezevic et al., 2016 Kareem et al., 2020
Eugenol	Verotoxin producing <i>Escherichia coli</i>	Ezzeldeen et al., 2015
Tea tree oil	Multi-drug resistant <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Acinetobacter baumannii</i> , and <i>Pseudomonas aeruginosa</i>	Oliva et al., 2018
MEO	Environmental mastitis: <i>Staphylococcus aureus</i> , <i>Staphylococcus chromogenes</i> , <i>Staphylococcus sciuri</i> , <i>Staphylococcus warneri</i> , <i>Staphylococcus xylosus</i> and <i>Escherichia coli</i> Bovine respiratory pathogen: <i>Mannheimia haemolytica</i> Bovine endometritis: <i>Escherichia coli</i> , <i>Fusobacterium necrophorum</i> , <i>Trueperella pyogenes</i> , <i>Staphylococcus aureus</i> Urinary tract infection: Multidrug-resistant strains of <i>Escherichia coli</i> , <i>Enterococcus</i> spp., <i>Candida albicans</i> and <i>Candida famata</i> <i>Leishmania</i> , <i>Plasmodium</i> and <i>Trypanosoma</i> species Arthropod disease vector: female <i>Ixodes ricinus</i>	Fratini et al., 2014 Amat et al., 2017 Paiano et al., 2020 Ebaniet al., 2018 Le et al., 2018 Kulmaet al., 2017
Oregano	<i>Salmonella</i> species	Mohan and Purohit, 2020
Oregano & Rosemary	<i>Escherichia coli</i> O157:H7	Diniz-Silva et al., 2020
Oregano & Carvacrol	<i>Escherichia coli</i> 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
<i>Plectranthus barbatus</i> oil	Larvicides against malaria, dengue and Japanese encephalitis mosquito vectors	Govindarajan et al., 2016
<i>Marrubium vulgare</i> oil	Bovine reproduction system pathogen: <i>Trichomonas vaginalis</i>	Akbari et al., 2018
<i>Arisaemafargesii</i>	Larvicidal activity against <i>Aedes</i> mosquitoes	Huang et al., 2020
<i>Myristica fragrans</i>	<i>Escherichia coli</i> , <i>Staphylococcus aureus</i>	Kiarsiet et 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; Barbarstani 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, Bakhshwain, 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; Yalçın 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 Increase in BWG, FI and improvement in FCR	Attia et al., 2017 Pournazariet al., 2017
	0.5-1 g/kg		
	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 Improvement of BWG, FI, FCR. Beneficial effects on cholesterol, immunity and antioxidant status	Wade et al., 2018 Ismail et al., 2019
	5 g/kg		
	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. Showed anticoccidial effects	Ahmadian et al., 2020 Lee et al., 2020
Thyme and Carvacrol	60 and 120 mg/kg	Positive effects on intestinal health	Aydin and Yildiz, 2020
Thyme oil and Black cumin oil	250 and 100 mg/kg	Improved ADG, FCR, carcass yield and decreased cholesterol level Increased BW, WG and immune response	Hassan, 2019 Witkowskaet al., 2019
Thyme and Peppermint	100 mg/kg each		
Oregano	NS	Increased ADG, ADFI and antioxidant status Improved performance and meat quality as increased breast meat redness and reduced yellowness Beneficial effect on the growth performance Improved performance, carcass yield and immunity	Riet al., 2017 Cázares-Gallegos et al., 2019 Hn et al., 2019 Eleret al., 2019
	150 mg/kg		
	200-600 mg/kg		
Oregano and Thyme	NS	Improvement of WG, immune parameters and intestinal morphology Improvement of total tract retention of DM, increase of LAB and reduction of E.coli Improvement of BW, FCR and LAB Improved immunity and showed antiviral effects against ND and IBD	Parvizi et al., 2020 Park and Kim, 2018 Ruben et al., 2018 El-Shall et al., 2020
	300-900 mg/kg		
	4% each		
MEO	0.03%	Improved immunity and showed antiviral effects against ND and IBD	Pathak et al., 2017 Yang et al., 2019
	0.01%		
Cinnamon	500 mg/kg	Increased villi height and immunity, reduction of <i>Salmonella</i> and <i>Clostridium</i> counts improved the immune status, antioxidant ability and cecal microbiota	Mohammadi et al., 2019 El-Gogary, 2020
	NS		
Rosemary	300 mg/kg	Improvement in FCR, immunity and concentration of Se in liver and breast muscles of broiler	Abbasi et al., 2019
Rosemary, Thymus & Satureja	0.5-1.0 g/kg	Beneficial effect on lipid profile, immunity, antioxidant status Improved immune responses, antioxidants and intestinal microflora	Tayeb et al., 2019
	300 ppm		
Rosemary & Thyme	5-10 g/kg each	Significant effect on live BW, FI and dressing percentage	Barbarestaniet al., 2020
Lavender oil	600 mg/kg	Increased growth performance, intestinal morphometrics, villi height, antioxidant status and gut bacteria balance, reduced E. coli	
Laying hen			
Oregano	50-250 mg/kg	No effect on FI, FCR, egg production and egg shell characteristics	Cufadar, 2018

Thyme	50-200 mg/kg 300 mg/kg 2%	Enhanced immune response Improvement in antioxidant status during HS period Showed hypolipidemic and antioxidative effects along with improved immunity without effecting performance and egg quality	Migliorini et al., 2019 Beyziet al., 2020 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 Ghanima et al., 2020
Quil			
Thyme	200-400 ppm	Improved FCR	Dehghaniet al., 2018
Eucalyptus	150-450 mg/kg 0.1%	Increased BW, ADG, FI and antioxidant status Enhanced productive performance, eggshell quality, immunocompetence and reduces number of broken eggs	Gumuset al., 2017 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-Vidales et 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 diseases, 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 depend-

ing 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 conceivable 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
MEO	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
	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
Thyme	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	Kurniawati et al., 2020
Clove & Rosemary	3.5 g/animal/day	Decrease in the lipid oxidation.	Rivaroli et al., 2016
	100 ul/L	Decreased AN, VFA concentration and MP	Baraz et al., 2018
Coriander oil	100 g/day	Increased MY and reduced MP	Hamdani et al., 2019
	2 g and 4 g / animal/day	No effect on carcass quality. Affect oxidation	de Oliveira Monteschioet al., 2017
Cashew and caster	14 mL/cow/day	Increase of FI, ND and MY. Decrease in ruminal AN concentration	Matloupet al., 2017
	2 g/day	No effect on FI and N digestibility. Alter ruminal pH	Coneglianet al., 2019
Buffaloes			
Ajwain oil	1-2 ml/day 0.05%	Increased DM intake, ADG and protein metabolism Reduced MP, A:P ratio and improved ND	Pawaret al., 2019 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-Essawy et 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			
<i>Callistemon viminalis</i> 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 Dileret al., 2017 Janacua-Vidaleset al., 2019 Mercatiet al., 2020 Cázares-Gallegos et al., 2019
	0.125-3.0 ml/kg	Rainbow trout	NE			
	2000 ppm	Pig meat	+	+		
	NS	Pig meat	+	+		
	200-600 mg/kg	Broiler meat	+	+		
Oregano & linseed	3% and 0.6 %	Goat meat	+	+		Rotondiet al., 2018
Rosemary	200-400 mg/kg	Lamb meat	+		+	Ortuñoet al., 2014 Mohammadi et al., 2019
	300 mg/kg	Broiler meat	+	+		
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 Plachaet al., 2019 Beyziet al., 2020 Yalçinet al., 2020 Gumuset al., 2017 Onel and Aksu, 2019
	0.1%	Broiler meat	+	+		
	300 mg/kg	Layer meat and egg	+			
	2%	Layer meat and egg	+	+		
	150-450 mg/kg	Quil meat	+	+		
	600 mg/kg	Broiler meat	+	+		
Thyme & Clove	4 MIC and 2 MIC respectively	Minced beef	+		+	(Zengin and Baysal, 2015)
Clove	0.25%	Fresh chicken sausage	+		+	Sharma et al., 2017 El-Essawy et al., 2019
	2ml/d	Sheep meat	+	+		
Basil EO	0.062, 0.125 and 0.25%	Beef burger	+	+	+	Sharafati Chaleshtori et al., 2015 Falowoet al., 2019 Kumar et al., 2018
	2 and 4%	Cattle meat	+	+	+	
	0,25, 0.50, 0.75%	Mutton nuggets	+	+	+	
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) Tekceet al., 2020 Giller et al., 2020 Pukrop et al., 2019 Lei et al., 2019
	250-750 ml/1000 L	Broiler meat	+	+		
	25 g/day	Cattle meat	+	+		
	1 g/d	Cattle meat	+	+		
	2 mg/kg	Goat meat	+	+		

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.

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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-butanate) 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.

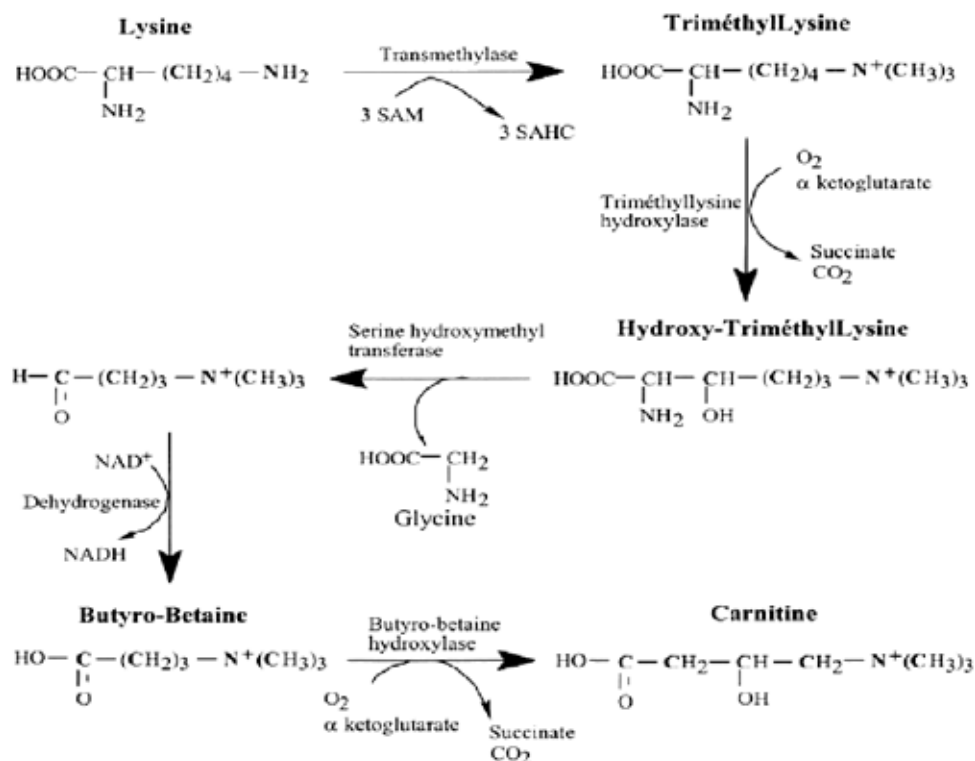


Figure 1. Biosynthesis of L-carnitine (Arslan, 2006).

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 co-factor 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. Hrnear 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
Hrnecar 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 broil-

ers. 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 the breast 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 oligosaccharide 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 titers in the 18th and 28th day of life using Terbutaline and L-carnitine in the diet 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
MirzapourSarab 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 titer against 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 titer against Newcastle virus
Fallah et al., 2016	100 mg/kg l-carnitine + 100 mg/kg terbutaline	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 paraoxonizations 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/kg L- carnitine + 1, 2 mg/kg gemfibrozil	Reduced total serum cholesterol and triglyceride
Arslan and Tufan, 2018	100 mg/kg L- 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 facilitated the 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 the microbial 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, <i>Escherichia coli</i> 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 myoglobin (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.

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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 (Schröder-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 (Schröder-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 (Lahrman 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; Lahrman 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%***				Beckskei 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 (Schröder-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 scarring. 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. Healed tail damage also increases the risk significantly.	Valros et al. (2004)
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 (Ursi-

nus 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.	Lahrman 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	Lahrman 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.	Lahrman 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-Abattoir)
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; Schröder-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 (Schröder-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 extent 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 liters. 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 (Schröder-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, investivable 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 (Schröder-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.

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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 perspec-

tive. 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.

$S/P = (OD \text{ of unknown sample} - OD \text{ of negative-control sample}) / (OD \text{ of positive-control sample} - OD \text{ of negative-control sample})$. Samples having an S/P ratio < 0.2 were considered negative, whereas samples with an S/P ratio ≥ 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 significantly 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 multivariate 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	0.001*
2-3 years	264	39 (14.8%)	10.8-19.76	
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	0.7*
Mid stage (3-6 months)	62	10 (16.1%)	8.41-28.13	
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	0.4*
20-30	233	39 (16.7%)	12.31-22.3	
>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	1 st trimester	33	3 (9%)
	2 nd trimester	185	19 (10.3%)
	3 rd trimester	290	48 (16.6%)
	Total	711	114 (16%)

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			
1 st trimester		ref	
2 nd trimester	1.1	0.31-4.10	0.8
3 rd trimester	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.

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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; Karapehliyan 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 H₂O₂ 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 bilirubin, 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 \pm 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, fT₃, and fT₄ 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 ($\mu\text{mol/L}$)	70.09 \pm 3.40 ^a	50.96 \pm 3.54 ^b
TAC (mmolTrolox Equiv./L)	2.13 (1.78-2.61) ^a	2.86 (2.11-3.12) ^b
TOC ($\mu\text{mol H}_2\text{O}_2$ Equiv./L)	4.41 \pm 0.07 ^a	3.54 \pm 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 \pm 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 \pm 0.02	0.45 \pm 0.01
Zn (ppm)	0.77 \pm 0.06	0.71 \pm 0.04
Se (ppb)	13.77 \pm 1.31	15.70 \pm 0.88
Fe (ppm)	0.50 \pm 0.03	0.48 \pm 0.02
Ca (mg/dL)	9.5 (6.46-15.15)	9.15 (3.41-9.68)
Mg (mg/dL)	4.39 \pm 0.17	4.19 \pm 0.20
Mn (ppm)	1.88 \pm 0.13 ^a	3.05 \pm 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 \pm 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 \pm 2.88 ^a	19.00 \pm 1.99 ^b
Total cholesterol (mg/dL)	82.28 \pm 5.74	74.84 \pm 4.33
LDL-C (mg/dL)	38.07 \pm 3.21	35.46 \pm 2.53
Urea (mg/dL)	14.28 \pm 1.08	15.53 \pm 1.35
Creatinine (mg/dL)	0.7 (0.6-0.9)	0.8 (0.6-0.9)
Total bilirubin (mg/dL)	0.18 \pm 0.005	0.19 \pm 0.008
Glucose (mg/dL)	70.64 \pm 2.76 ^a	62.84 \pm 3.46 ^b
Total protein (g/dL)	6.65 (6.30-8.60)	6.70 (4.50-8.10)
Albumin (g/dL)	2.80 \pm 0.07	2.81 \pm 0.10
AST (U/L)	87 \pm 4.59	94.46 \pm 5.46
GGT (U/L)	18.85 \pm 0.98	17.07 \pm 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 \pm 1.18	12.85 \pm 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 thyroxine (fT ₄) ng/dL	0.79 \pm 0.04	0.83 \pm 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 thyroxine values were given as mean \pm 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-1 β , 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 activi-

ties. 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 α} (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.

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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 *Pseudomonas* 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 fluorescent 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'GGTTCACCTTGTTACGACTT3') 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 hemorrhag-

ic 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 ₂ S 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

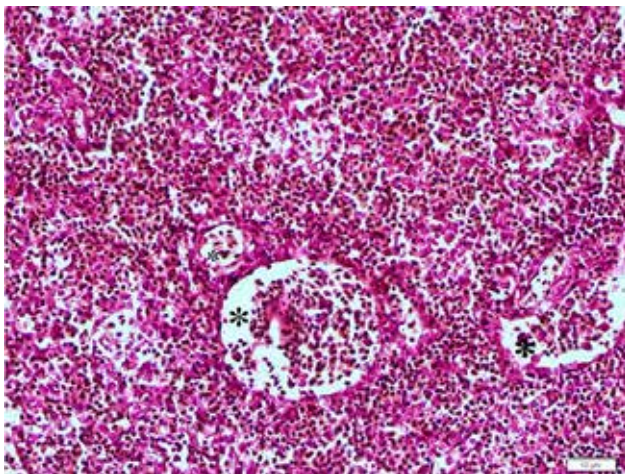


Figure 3. Discharge of the white pulp (arrowed), congestion (*) of spleen vessels (H&E) x1000

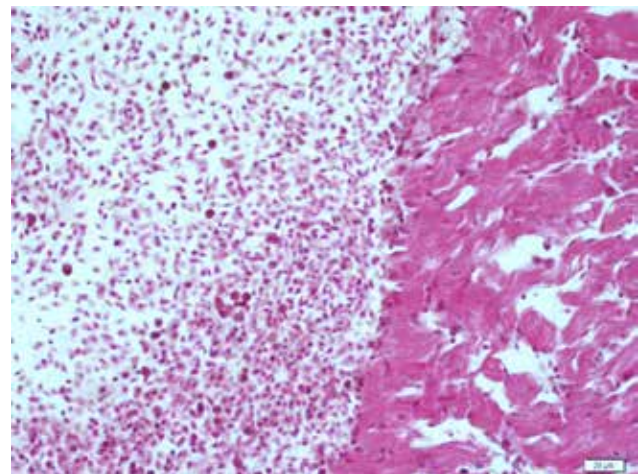


Figure 4. The polymorph-nuclear leukocyte infiltration (arrowed) and hypertrophy of the heart muscle cells (H&E) x400

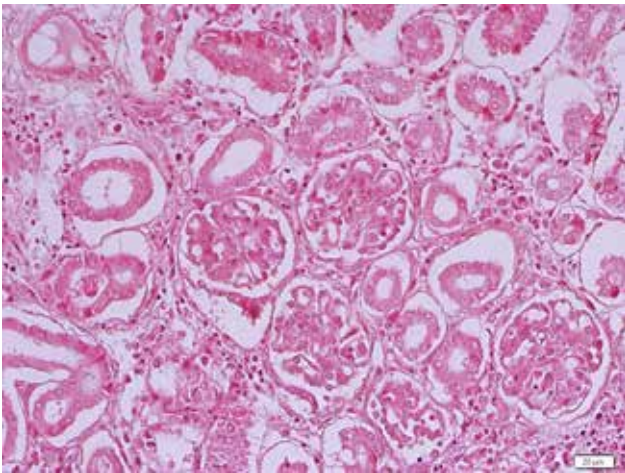


Figure 5. Periglomerular (arrowed), tubular oedema and tubular degeneration (H&E) x400

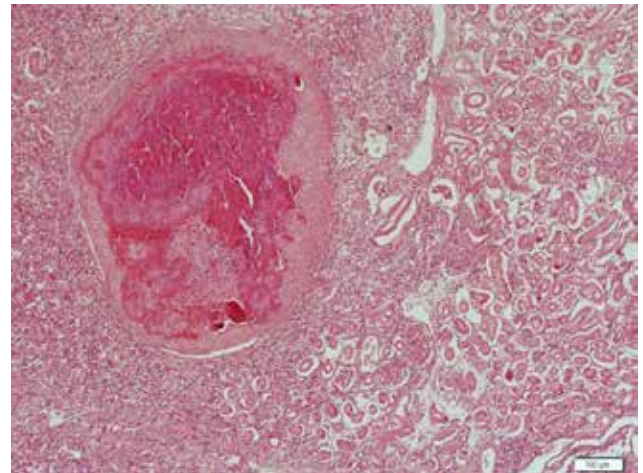


Figure 8. Granuloma in the interrenal haemopoietic tissue of kidney (H&E) X100

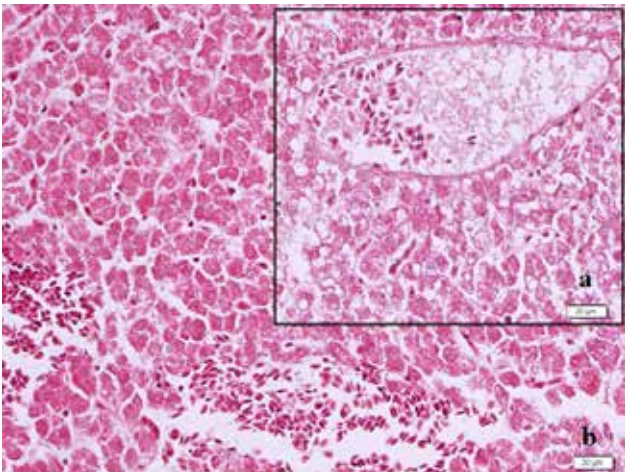


Figure 6. Congestion (a) and hemorrhage (b) among the hepatocyte (H&E) x400

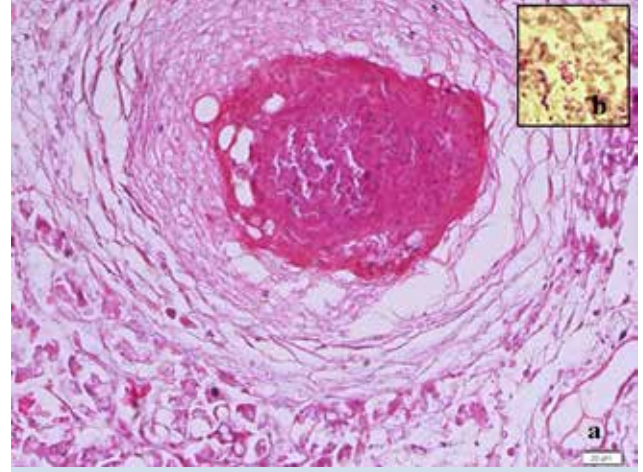


Figure 9. Necrotizing granuloma surrounded by epithelioid in the liver (a) (H&E), Gram negative bacteria in the granuloma (b) (B&H) X400

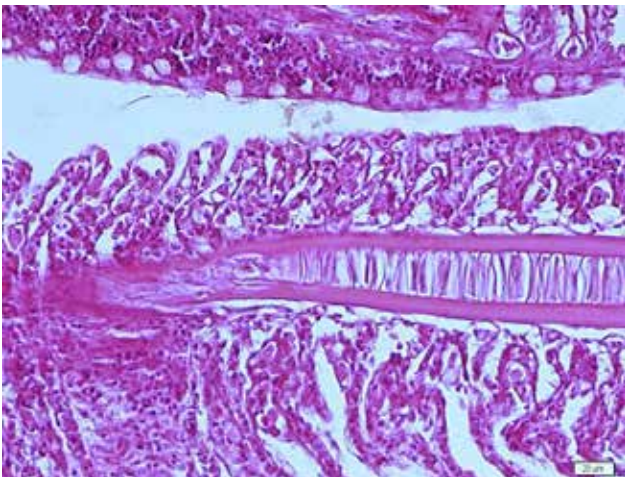


Figure 7. Gills showing hyperplasia of the respiratory epithelium and increased goblet cells (H&E) x400

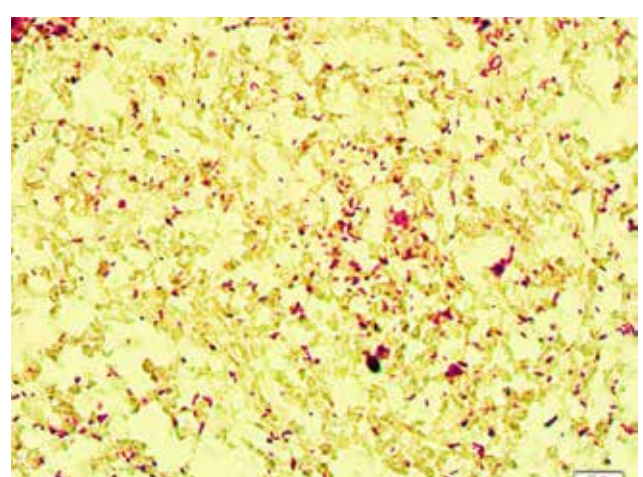


Figure 10. Presence of Gram-negative bacteria caused granuloma in the kidney tissue (B&H) X1000

DISCUSSION

Pseudomonas agents such as *P. fluorescens*, *P. putida*, *P. luteola*, *P. aeruginosa*, 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; Altinok 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 (Altinok 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 fluorescent 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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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. longissimus 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 °C. Humidity averaged $87 \pm 4.60\%$ 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 \times 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 \times 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 ($20 \times 20 \times 10$ 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 lengths 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 (Koochmaraie 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; Koochmaraie 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 varia-

tion 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; Koochmaraie 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.

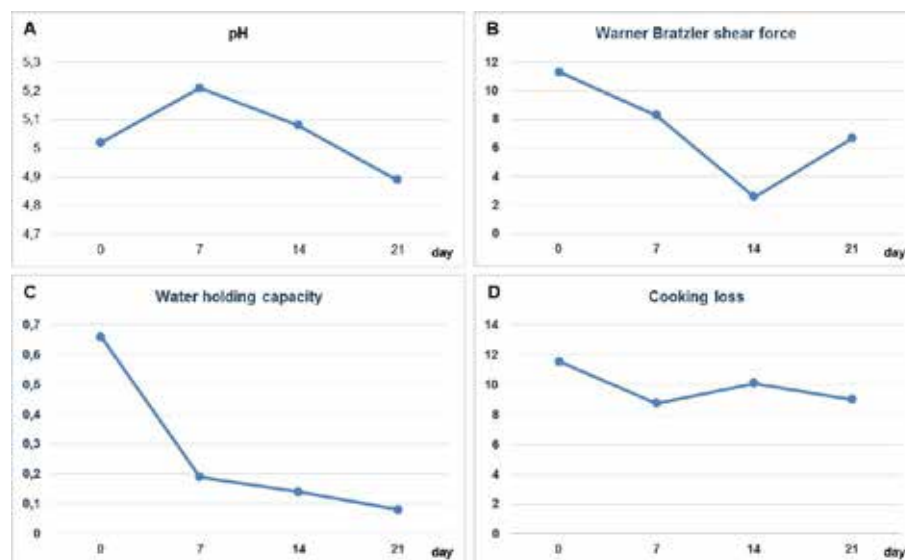


Figure 1. Changes in beef quality parameters including pH, Warner Bratzler shear force (N/mm), water holding capacity, and cooking loss (%) during 21 days of dry-ageing

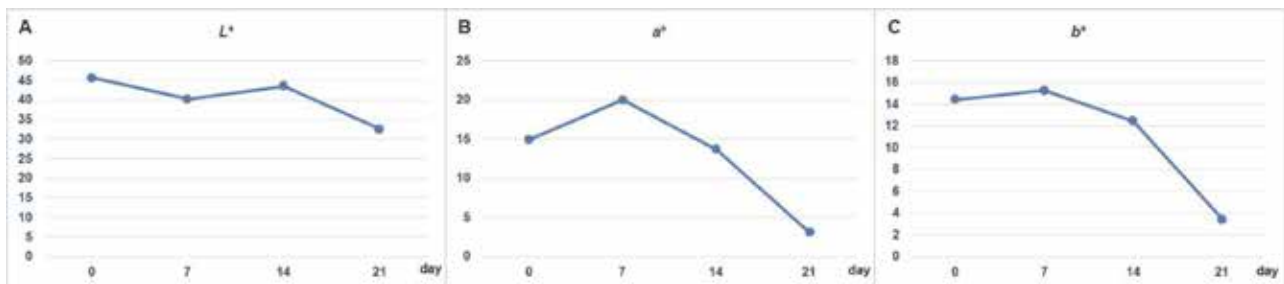


Figure 2. Changes in beef colour parameters during 21 days of dry-ageing

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 (Koochmaraie 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, reduction 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, require-

ments 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.

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Mass mortality of unknown etiology in alpine newts (*Ichthyosaura alpestris 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.

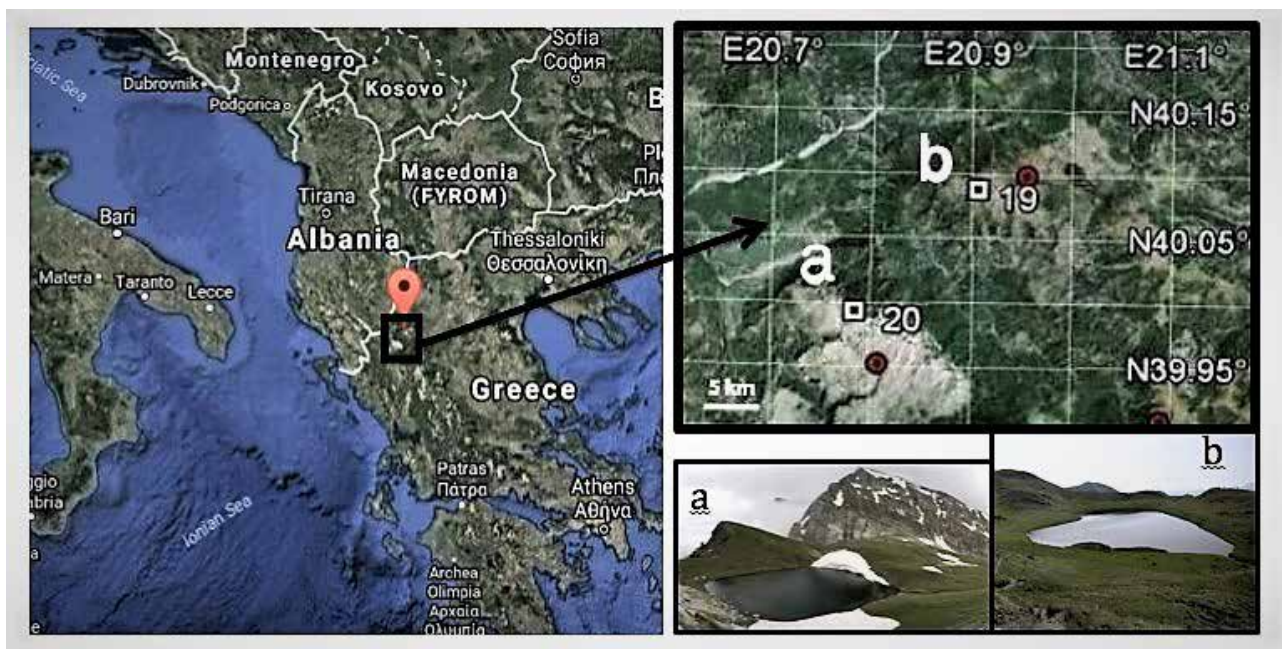


Figure 1. A satellite view of the study area in the mountains of NW Greece. The studied populations are indicated with square shape. Other known newt populations are indicated with red dots. (a: Drakolimni Tymphis, b: Drakolimni Smolika) (Satellite photos by Google-earth)

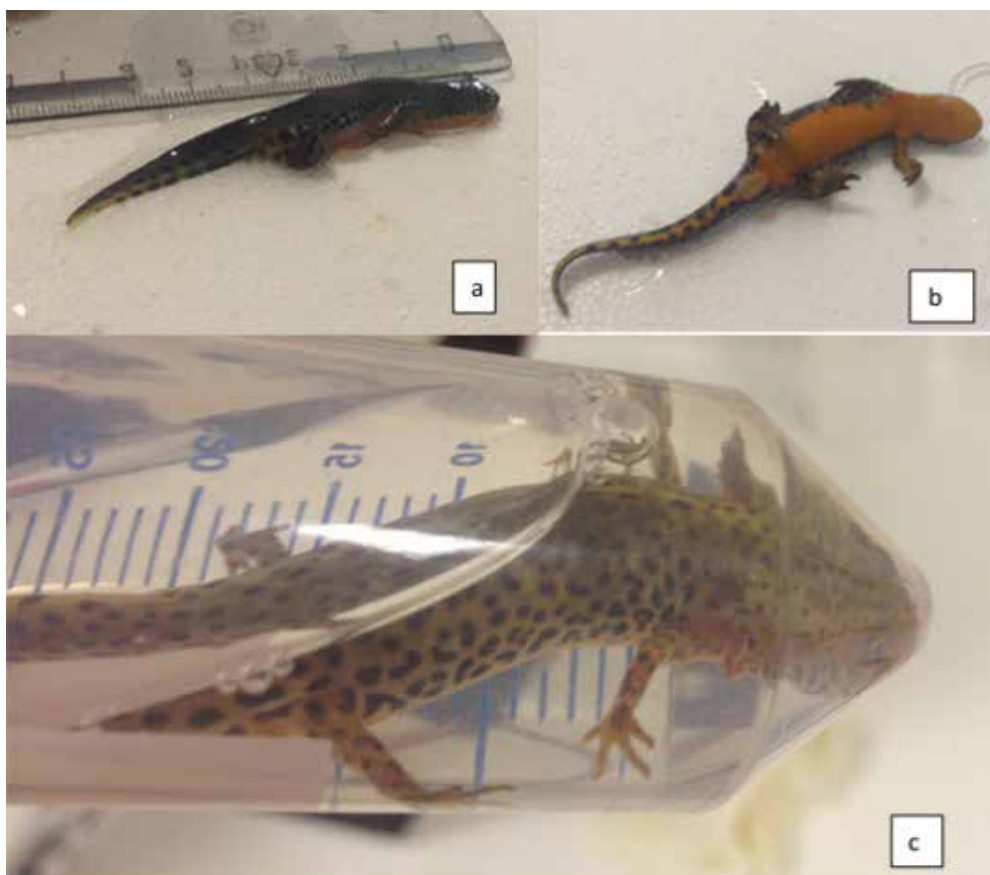


Figure 2. a) Dead male alpine newt under examination (dorsal view) b) male newt abdominal view c) neotenic newt (close up the external gills)

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 IL) 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 con-

trols. 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 (Szatecsny 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 *Ichthyophonus* 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, Perkinsa 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 have triggered 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.

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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; Ikononopoulos 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 overgrows *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 unknown Johne'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 μ l) was transferred to a microcentrifuge tube and centrifuged at 12000 rpm for 5 min. The pellet was resuspended in 200 μ l 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 μ l 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 re-

sults were revealed (Ellingson et al. 1998; Miller et al. 1999; Ellingson et al. 2005). Liver samples that were PCR positive, used for mycobacterial 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

	No of positive PCR results (Total samples)	Liver culture	
		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 PCR of 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 level of *MAP* isolation from the liver of experimentally infected sheep has been reported by Dukkupati 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 livers 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.

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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 $7.00 \pm 1.00 \log_{10}$ 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, O111 were 6.76 ± 0.45 , 6.64 ± 0.53 , 7.12 ± 0.43 , 6.00 ± 1.39 , $5.89 \pm 1.37 \log_{10}$ 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 $1.18 \pm 0.62 \log_{10}$ 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 toxicogenic *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 for a couple of days to weeks (Arocha et al., 1992; Ioanna et al., 2017; Bellio et al., 2018; Oztekin 2019). According to Arican and Andiç (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 con-

sumed 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 thermo-box 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°C by 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) 2x25 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, 2x25 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 dou-

ble 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 samples x time 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 chang-

es 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 $6.00 \pm 1.00 \log_{10}$ 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 $1.18 \pm 0.62 \log_{10}$ 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 *Lactobacillus* spp. and *Lactococcus* spp counts were 5.02 ± 0.22 , $4.93 \pm 0.31 \log_{10}$ cfu/g respectively. The number of mesophilic *Lactobacillus* spp. and *Lactococcus* spp. of experimentally contaminated yogurt samples were 7.04 ± 0.51 , $7.42 \pm 0.21 \log_{10}$ 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 $7.80 \pm 0.38 \log_{10}$ cfu/g, respectively. The number of *Lactococcus* spp. in the same groups were 7.91 ± 0.22 , 7.90 ± 0.19 , 7.88 ± 0.27 , 7.94 ± 0.51 and $7.93 \pm 0.42 \log_{10}$ 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; Miszczycha 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 salivarius* 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 $6.64 \pm 0.53 \log_{10}$ cfu/mL and $5.69 \pm 1.37 \log_{10}$ 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 $1.51 \pm 0.98 \log_{10}$ cfu/g and $1.18 \pm 0.62 \log_{10}$ 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 days of 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 STECs should 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.

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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 hydrochloride, 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-hydroxytryptamin₃ (5-HT₃) receptors and thus can be controlled with the specific 5-HT₃ receptor antagonists (Grunberg 1993; Hsu 2010). Granisetron, ondansetron, and tropisetron, 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; Pinarlı 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 placebo 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 electrocardio-

graphic 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 hours before the 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.

$$JTc = \frac{JT}{\sqrt[3]{RR}} \quad QTc = \frac{QT}{\sqrt[3]{RR}}$$

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 consid-

ered 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	-0.034±0.173	0.028±0.175	0.044±0.151	-0.004±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.

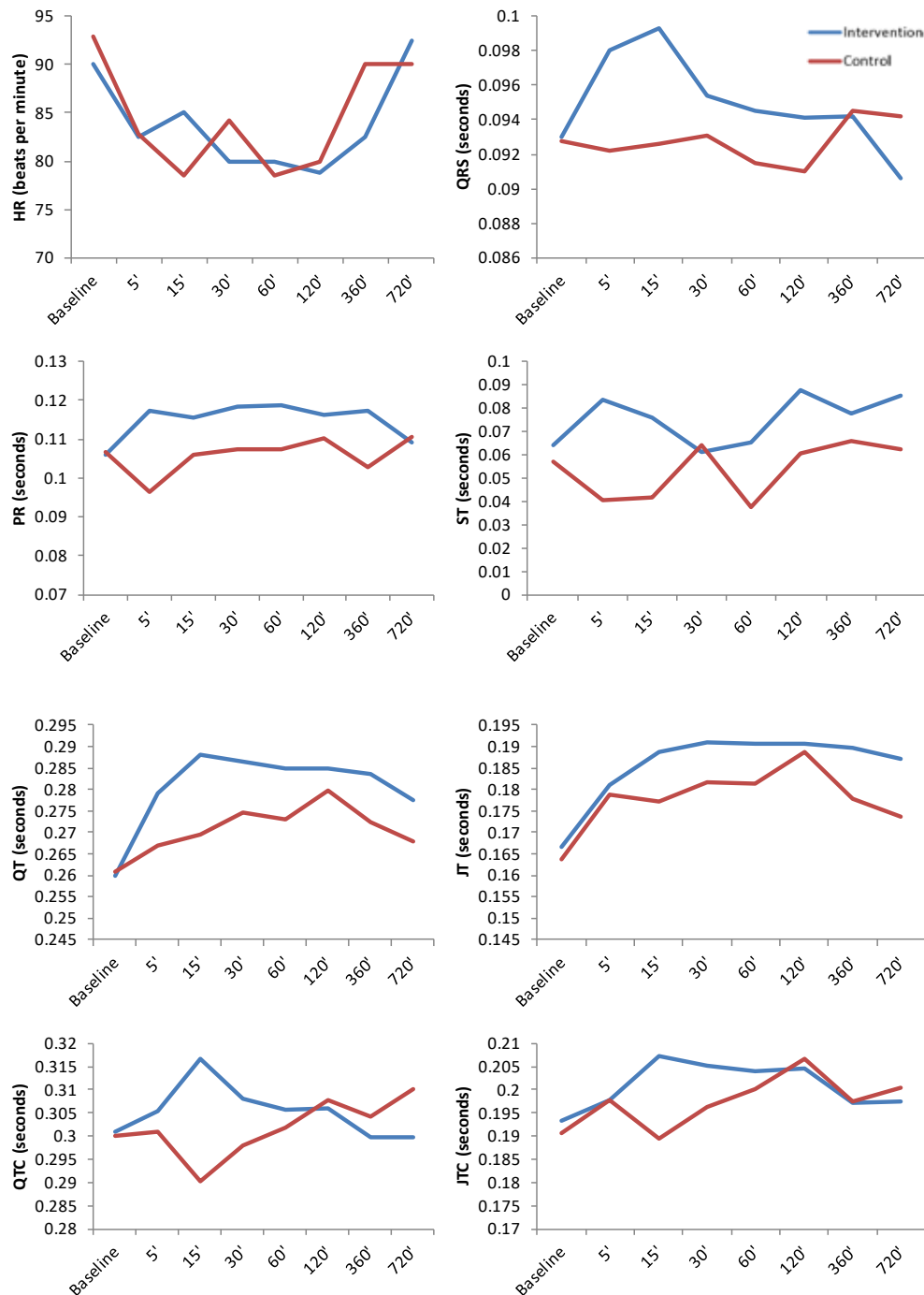


Figure.1. Comparison of the changes in mean values of some measured parameters over the first twelve hours after the intervention between the intervention and the control groups

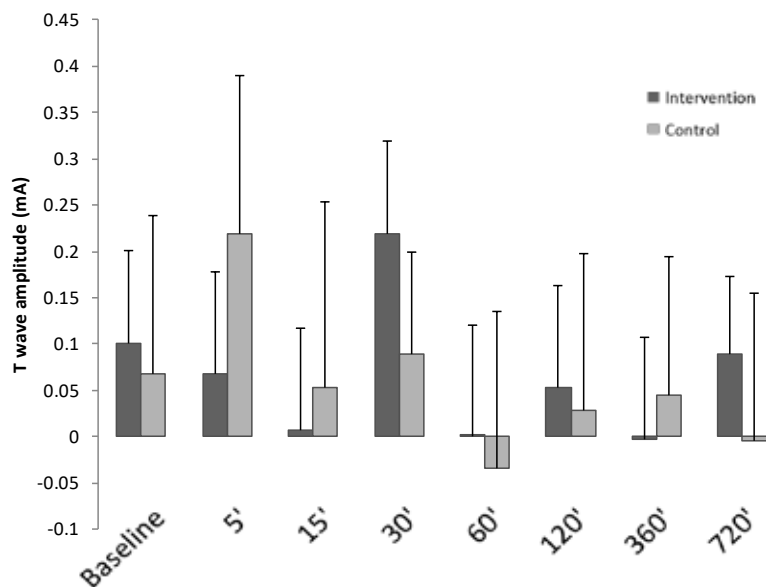


Figure 2. Comparison of the changes in mean T wave amplitude between the intervention and the control groups

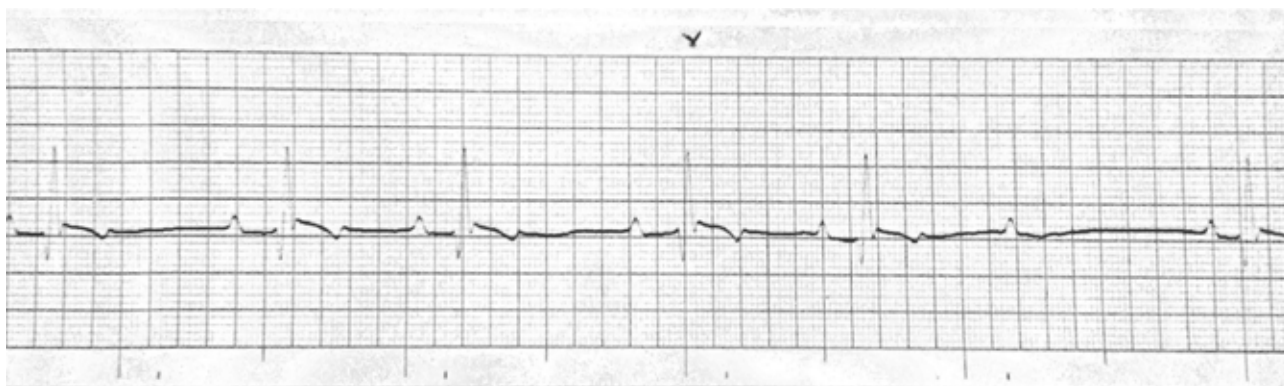


Figure 3. Second-degree ventricular block in one dog in the intervention group

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 possess 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 find-

ing 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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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, *Listeria monocytogenes*, *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 Kislak, 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 (Karabiyikli et al., 2014). Sumac sauce inhibited the growth of *E. coli* O157:H7 and *L. monocytogenes* in vitro conditions (Kunduhoglu 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), Brilliant 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 μ L of supernatant phase was mixed with 100 μ L of Folin-Ciocalteu solution and 1,500 μ L of distilled water for 10 min. Then, 50 μ L 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 (Abdullakassim 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 microorgan-

isms. 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; Tađi, 2010), plums (Shyamala Gowri 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)

	<i>E.coli</i> Type I	<i>S.Typhimurium</i>	<i>E.coli</i> O157:H7	<i>L.monocytogenes</i>	<i>L.ivanovii</i>	<i>S.aureus</i>
Plum	100%	28 ^{ax}	24 ^{abx}	29 ^{axy}	25 ^{abx}	24.2 ^{abx}
	10%	1.0	1.0	2.0	2.0	1.0
	1%	1.0	1.0	1.0	1.0	1.0
Pomegranate	100%	32 ^{ax}	36.4 ^{ay}	26 ^{bx}	1.0 ^{dy}	9.0 ^{ey}
	10%	17	3.0	3.0	1.0	6.0
	1%	5.0	1.0	1.0	1.0	1.0
Seville orange	100%	33.8 ^{ax}	37 ^{ay}	33.8 ^{ay}	24 ^{bx}	25 ^{bx}
	10%	2.0	7.0	2.0	3.0	22
	1%	6.0	2.0	6.0	1.0	2.0
Sumac	100%	35.2 ^{abx}	38.2 ^{aby}	40.2 ^{bz}	40.7 ^{bz}	32.5 ^{az}
	10%	22.5	21.5	20	15	21.2
	1%	1.0	5.0	1.0	9.0	3.7

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.

Table 3 MIC test results (µg/mL)

	<i>E.coli</i> Type I	<i>S.Typhimurium</i>	<i>E.coli</i> O157:H7	<i>L.monocytogenes</i>	<i>L.ivanovii</i>	<i>S.aureus</i>
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.

Table 4 Antimicrobial activity of fruit sauces on chicken breast meat samples

	TVC	Coliform (SMM)	Coliform (MPN)	<i>E.coli</i>
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 Niranjana (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 Niranjana, 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 Kislal, 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 than 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* (Shya-

mala 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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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 various plant additives through rabbit nutrition or partial substitution of feedstuffs 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 et al., 2017; Duda et 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 (Chhay et al., 2013; Peiretti, 2018; Peiretti et al., 2018). The scientists report on effective inclusion of amarant: 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 (Hoptsi 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 Exper-

imental 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 $1,430 \pm 61.3$ g were randomly 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 ($19.4^{\circ}\text{C} \pm 0.07$) and relative air humidity ($63.5 \pm 0.04\%$), 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 of the national standard DSTU 7693:2015 (2016). The digestible energy (DE) was calculated according with Axelson's formula modified by Grigorev and Volkov: $DE = 0.73 \times GE (1 - CF \times 1.05)$, 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, $(1 - CF \times 1, 05)$ - 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°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 - pH₀) and chilled carcass pH (24 hours - pH₂₄) 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 (Tortend 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°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 to National 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 \pm 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 \pm standard deviation)

Indicators	Groups		P value
	I	IIa	
Live weight, g	3543.6 \pm 296.62	3417.5 \pm 212.17	0.07
Hot carcass weight, g	2057.6 \pm 203.96	1991.6 \pm 181.40	0.21
Chilled carcass weight, g	2007.2 \pm 157.62	1959.4 \pm 145.46	0.24
Dressing out percentage, %	58.0 \pm 1.11	58.28 \pm 1.05	0.33
Drip loss percentage, %	2.45 \pm 0.46	1.92 \pm 0.48	0.06
HeP, %	0.45 \pm 0.21	0.46 \pm 0.10	0.08
LvP, %	5.15 \pm 0.49	5.45 \pm 0.54	0.05
LuP, %	0.68 \pm 0.05	0.64 \pm 0.06	0.06
KiP, %	0.84 \pm 0.05	0.87 \pm 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 reported the slow reproduction of 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 fi-

bers were uneven colored, which was due to physical and chemical changes in muscle tissue in the course of maturation.

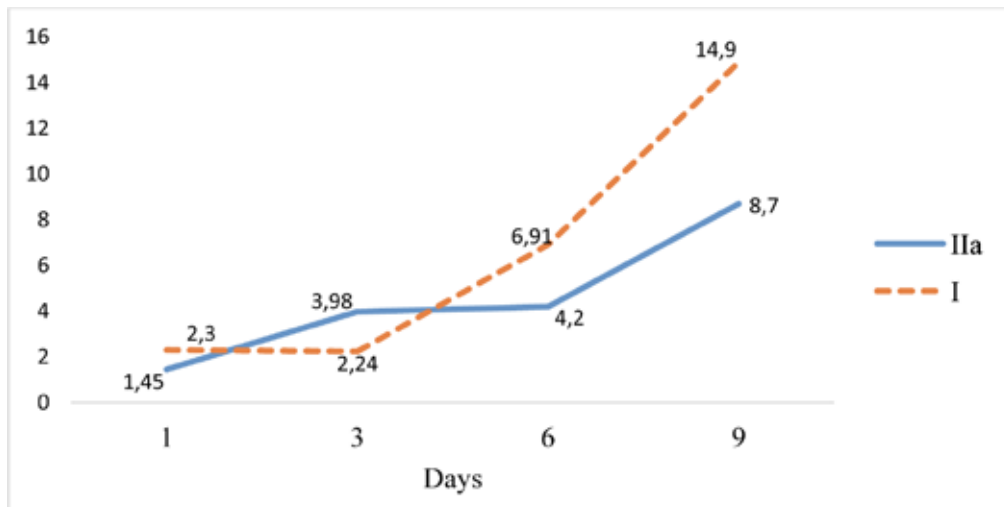


Figure 1. Dynamics of microorganisms count in tissue smears of rabbit meat during shelf-life

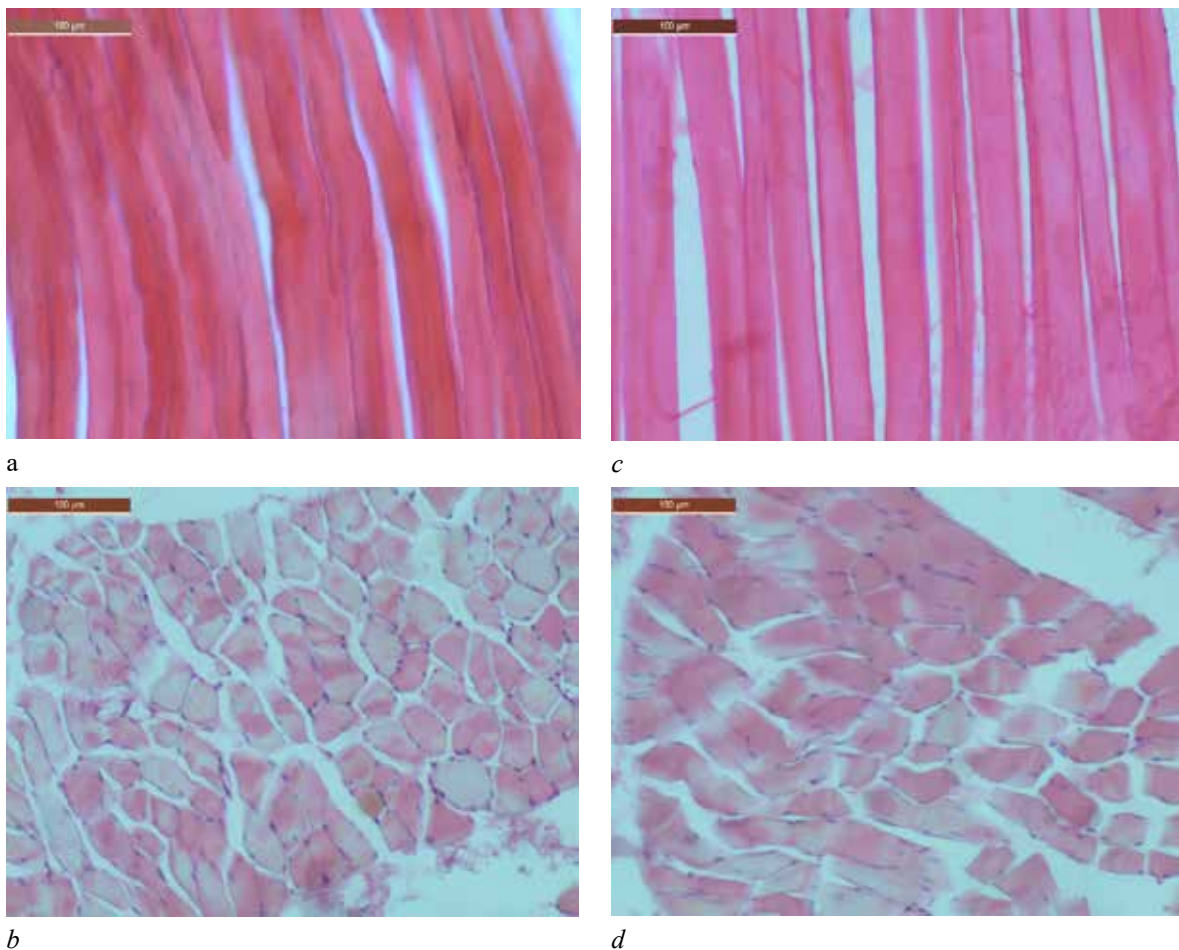


Figure 2. Histology longitude and transverse sections of rabbit muscles *Longissimus dorsi*: a, b - I group; c, d - IIa group. Haematoxylin and eosin, $\times 200$

DISCUSSION

Adding 20% of *Amaranthus hypochondriacus* oil-cake 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 $72.65 \pm 0.98\%$ 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 phytochemicals as squalen, unsaturated fatty acids, protein, tocotrienols, tocopherols (Caselatoand Amaya-Farfan, 2012, Tang and Tsao, 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 (Alagón et al., 2015; Molina et al., 2018) and boiling losses (Kone et 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 microorganisms count 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 meat-needs 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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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 surveil-

lance for foodborne outbreak 2009-2010, *S. Enteritidis* was the most common serotype with 76 outbreaks out of 225 confirmed *Salmonella* outbreaks (Ekateriniadou 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 (Pope 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, Qalqyia 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.

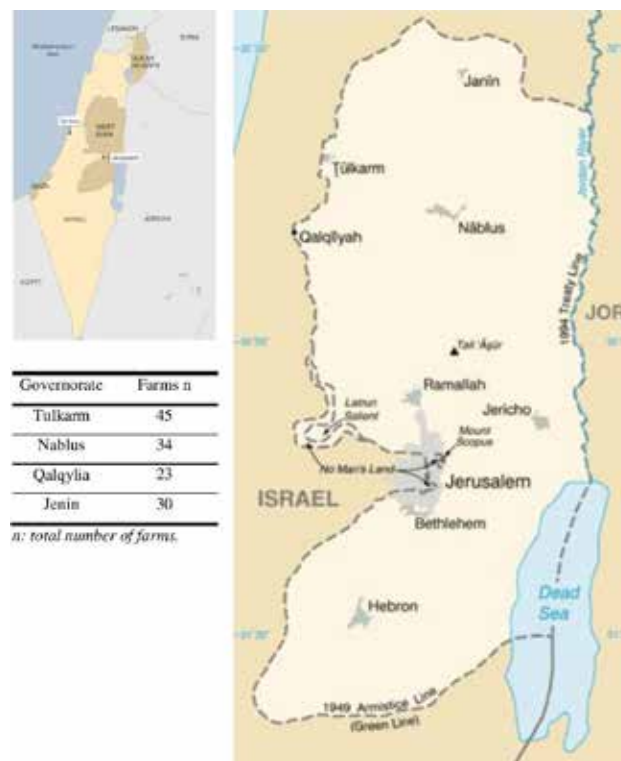


Fig. 1. Palestinian Governorates in the West Bank

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 °C 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)
Qalqyia	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 °C for 24 hours. 1 ml of the result sample was cultured in Tetrathionate broth (Müller-Kauffman) at 37 °C for 24 hours. 100 µl was then streaked on *Salmonella Shigella* agar (SS agar, Oxoid, CM0533) and incubated at 37 °C 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 °C 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 H₂S, 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 H₂O, 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 H₂O, 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 °C.

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 μ l) was performed using 12.5 μ l of PCR master mix (promega), 0.2 μ M of each primer, and 100-125 ng of DNA template. The cycling conditions were: initial denaturation at 94 °C for 2 min; followed by 35 cycles of denaturation at 94 °C for 20 s, annealing at 57 °C for 1 min and extension at 72 °C for 1 min; followed by a single final extension step at 72 °C 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 μ g/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 μ l) was performed using 30 μ l PCR buffer containing 4 μ l 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 μ l. After heating at 95°C for 10 min, 2.5 units Taq DNA polymerase in 10 μ l 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 °C for 2 min; followed by 35 cycles of denaturation at 94 °C for 40 sec, annealing at 55 °C for 50 sec and extension at 72 °C for 50 sec; followed by a single final extension step at 72 °C 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 μ g/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.

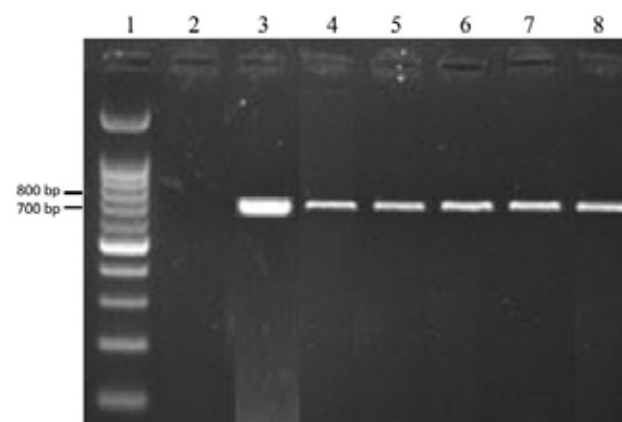


Fig. 2. Gel electrophoresis of PCR amplification product of *invA* gene of *Salmonella* spp.: lane 1: 100-bp ladder, lane 2: Negative control, lane 3: Positive control, lanes 4-8: Positive *Salmonella* isolates

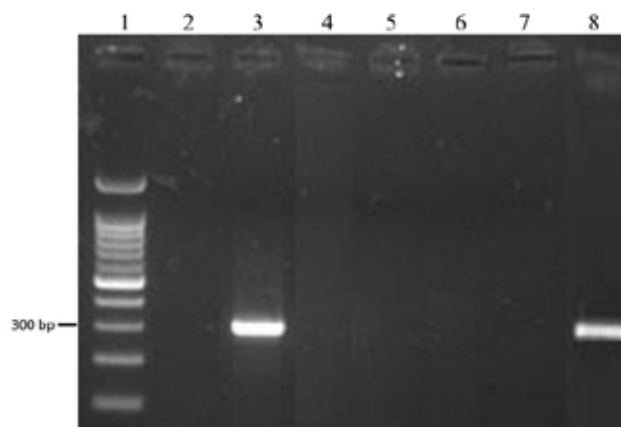


Fig. 3. Gel electrophoresis of the PCR amplification product of the *IE* gene sequence of *S. Enteritidis*: lane 1: 100-bp ladder, lane 2: Negative control, lane 3: Positive control. lanes 4-7: Negative *S. Enteritidis* samples, lane 8: Positive *S. Enteritidis* isolate

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 <i>Salmonella</i> samples (%)	Number of <i>S. Enteritidis</i> isolates among all <i>Salmonella</i> isolates (%)
Tulkarm	215	30 (14.0)	0.0 (0.0)
Nablus	193	13 (6.7)	0.0 (0.0)
Qalqyia	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 <i>Salmonella</i> samples	Number of <i>S. Enteritidis</i> isolates among all <i>Salmonella</i> isolates (%)
Tulkarm	194	12 (6.2)	2 (16.7)
Nablus	165	2 (1.2)	0.0 (0.0)
Qalqyia	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 con-

ducted 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) (Awany 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 *Sal-*

monella 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.

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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). There is 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 lectin as 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-HADYK/2017-078) before starting the study. A total of 15 multiparous Brown Swiss and Simmental dairy cows, average 2-4 years of age, were used in 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 pelleted dairy feed (feeding ingredient: 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* to animals 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 duplicate using the BIO-TEK EPOCH, GEN 5 software.

Biochemical analysis

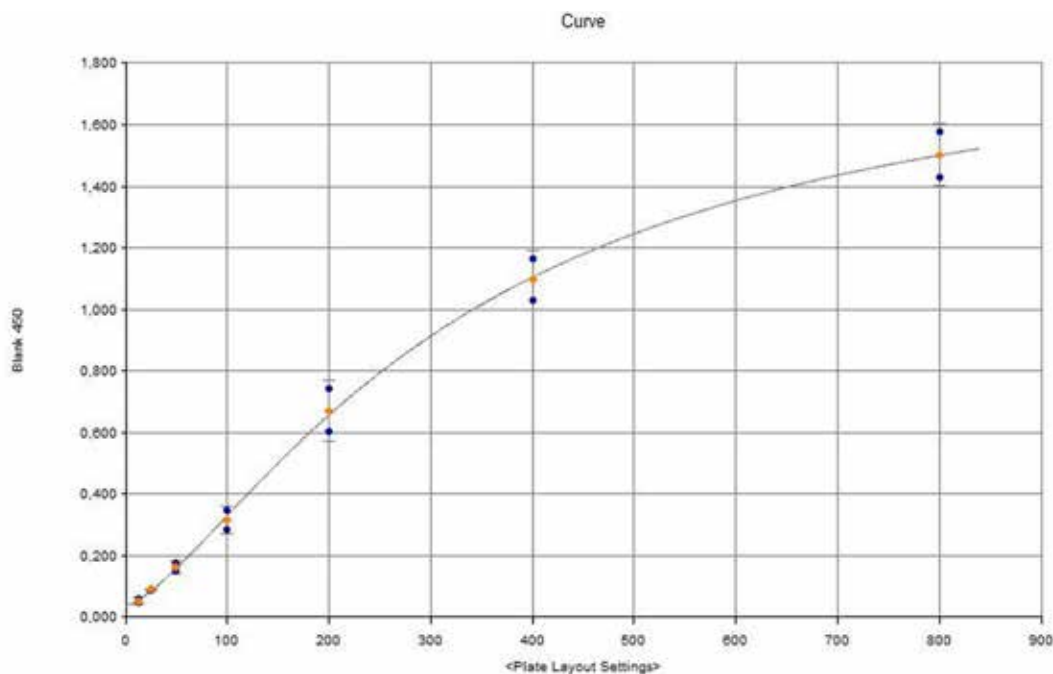
Serum omentin-1 levels were measured using the

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 omen-

tin-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 provided as mean±standard error ($X\pm SEM$). $P<0.05$ 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 than P, 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 than P, 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).

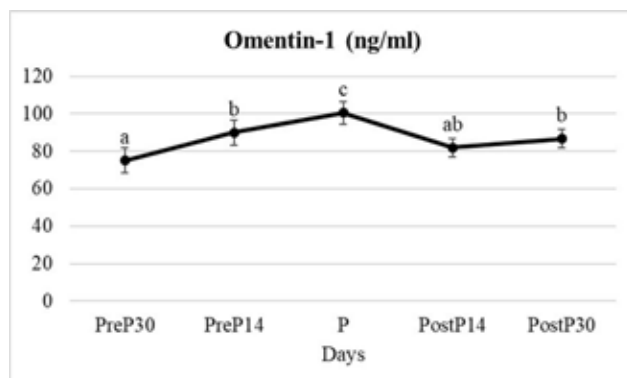


Figure 1. The change in omentin-1 concentration according to periparturient sampling periods (abc: present statistical differences between groups)

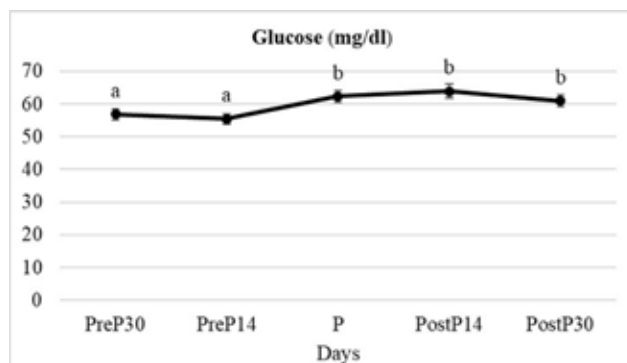


Figure 2. The change in glucose concentration according to periparturient sampling periods (abc: present statistical differences between groups)

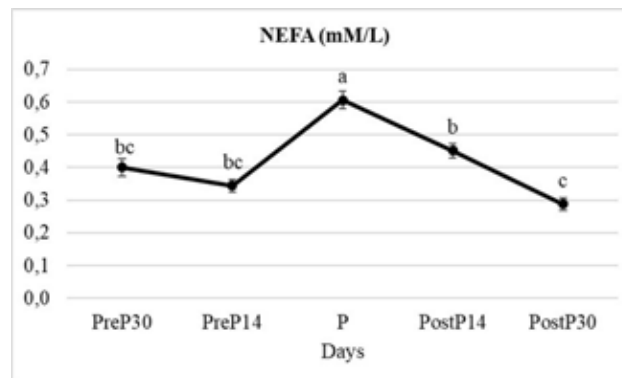


Figure 3. The change in NEFA concentration according to periparturient sampling periods (abc: present statistical differences between groups)

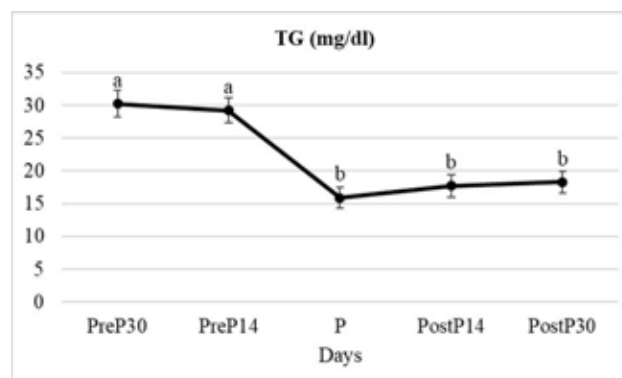


Figure 4. The change in TG concentration according to periparturient sampling periods (abc: present statistical differences between groups)

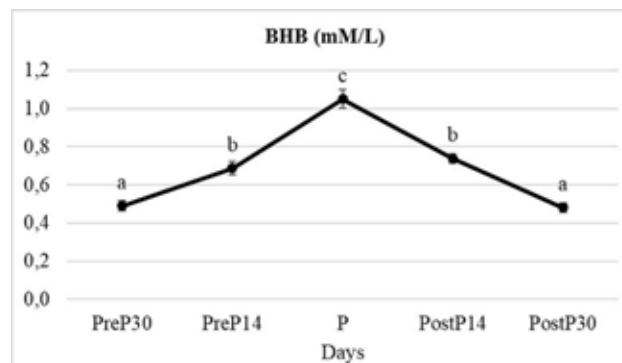


Figure 5. The change in BHB concentration according to periparturient sampling periods (abc: present statistical differences between groups)

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 of high 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 of its 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 other state 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 examine 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; Koryany et al., 2018).

In cows, ketone bodies and especially BHB are known to cause subclinical and clinical ketosis, suppress feed intake, and have a 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, a statistically significant relationship between NEFA levels and TG, glucose, and omentin-1 levels could not be determined. While a significant 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 adipokines. 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.

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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, Fumonisin 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 Fumonisin (92%) were found in direct examination of feed samples. Toxin 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 losses for 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.) (Capriotti *et 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, Fumonisin, 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 10mL of 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 °C) for 3-7 days. After incubation isolated colonies of fungi were sub-cultured on 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) was used by sub-culturing representative 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 red color 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 mL Chloroform: 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 wavelength after 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 was studied for the mycotoxin presence.

Detection of Aflatoxins in Feed by Agra Strip test

The 2nd part of the feed samples was directly used for mycotoxin presence, where Aflatoxin test kits AgraStrip® was used having 4ppb Cut-off value. 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. and used for aflatoxin detection. An amount 50µL diluent assay was added into the micro-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 5 minutes 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 bag and buffer bag was added followed by the addition of 30mL distilled water was added and mixed for 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 °C Bands were observed after 3 minutes of incubation.

Detection of mycotoxins in feed by Enzyme Linked

Immunosorbent Assay (ELISA)

A competitive ELISA was performed with the help of Aflatoxin and Fumonisin ELISA test kit Agra-Quant® as per manufacturer instructions. 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 were thoroughly mixed and 100µL of it was transferred to the 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 again at room temperature for 5 minutes. 100µL of stop solution to each well was added and mixed slightly and the 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 with spotted samples. TLC plate was then 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 the solvent finishing line was recorded. Plate was dried in the air and visualized under UV lamp at 364 nm wavelength. Spots with blue 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-Hernández et 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 highest frequency 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 preliminarily confirmed with the help of ammonium vapors test in YEA medium (Figure 1). Production of mycotoxins (Aflatoxins and Fumonisin) were further confirmed with the help of AgraStrip® for total Aflatoxin and Fumonisin and ELISA. Direct examination of feed samples for mycotoxins by AgraStrip and ELISA revealed that the majority of the feed samples was contaminated with Aflatoxins (77%) and Fumonisin (92%).

Table 1. Prevalence of different fungi in feed samples

Fungal Genera	Growth (%)
<i>Aspergillus flavus</i>	46%
<i>Aspergillus niger</i>	22%
<i>Aspergillus parasiticus</i>	21%
<i>Mucor</i> spp.	6%
<i>Rhizopus</i> spp.	4%
<i>Fusarium proliferatum</i>	1%

The quantitative analysis of mycotoxins through ELISA revealed that the aflatoxin quantities in dif-

ferent samples vary between 0-40 PPB while that of Fumonisin between 0-500 PPB. The concentration values of Aflatoxins and Fumonisin in feed samples were determined from a calibration curve prepared by using known concentrations. Aflatoxin produced by fungal isolates in 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^{-1} for 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 CH_3 , 1228-1216 cm^{-1} for PO_2 asymmetric 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).



Figure 1. (A) Shows aflatoxigenic nature under UV light at 365 nm, (B) Ammonia vapor

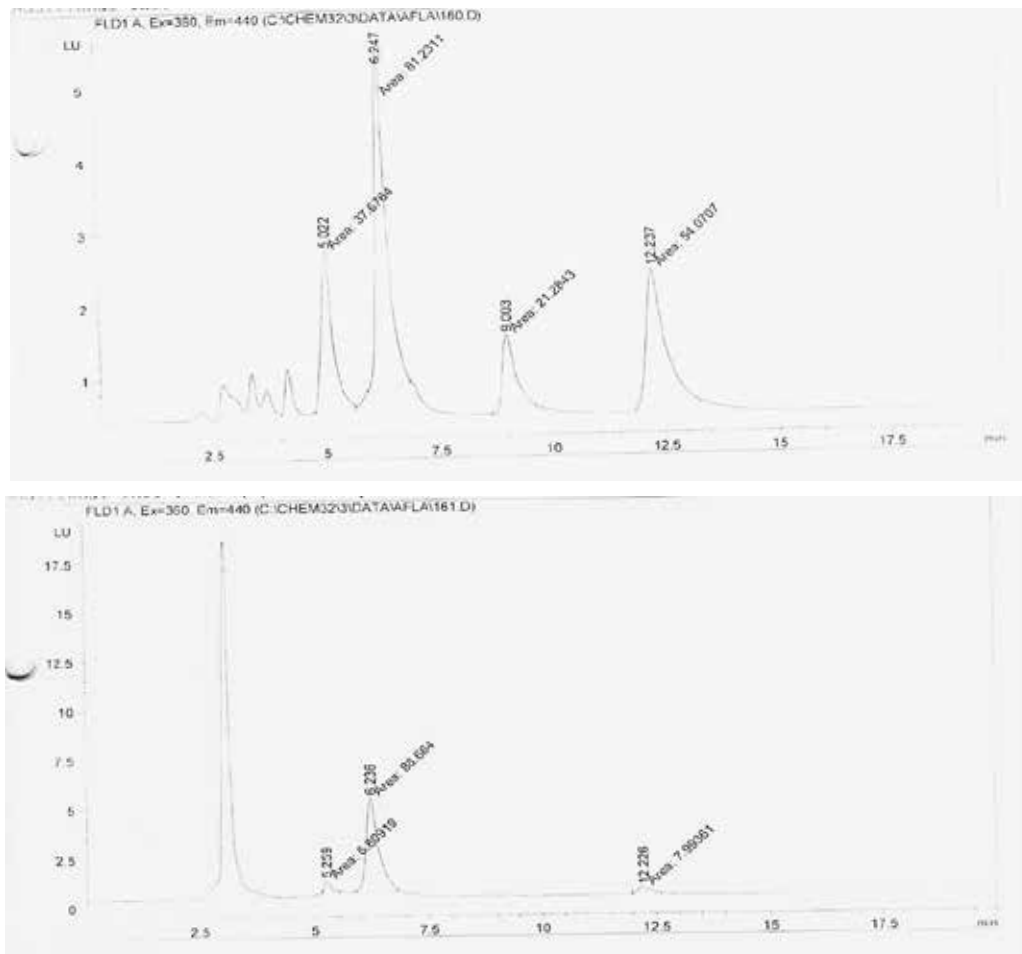


Figure2. Aflatoxins chromatogram of HPLC detection,(A) Standard (B) Sample

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 following the figures

reported by Pacinet al., (2003) from Ecuador. The *A. flavus* was found the most dominant fungi, most likely due to its ability to tolerate harsh environment and high temperature (Battilani et 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 for aflatoxinigenic 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

35.94 $\mu\text{g/g}$ and that of AFB₂ was 2.53 $\mu\text{g/g}$, 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.

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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 depression and somatic cell counts of Honamlı goat during second lactation in 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 120th lactation 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 its 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 CO₂ 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 and Caccese, 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 original 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 depression and somatic 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:

k

$$TMYL = y_1 t_1 + \sum_{i=2}^k (y_i + y_{i+1}) / 2 (t_{i+1} - t_i) + y_{k+1} * 15$$

i=2

Where TMY is total milk yield; y_1 is yield at first milk record, t_1 is interval between lambing and first recording; y_i is yield of the record i and t_i is interval between the record i and the record $(i+1)$, ($i = 1, \dots, k$), 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 Honamli goats. Table 2 shows mean milk yield (g) in different lactation stage. Mean lactation period of Honamli goats was 202.4 days. In this study, the mean lactation milk yield of Honamli goats was 92.6 kg. The daily milk yield of Honamli 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 Honamli goat and kids from the research flock



Figure 2. Does of the Honamli goat, the research flock

Table 1. Mean lactation milk yield of the Honamli 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 theHonamli 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	30 th day	n	60 th day	n	90 th day	n	120 th day	n	150 th day	n	180 th day	n	210 th day	P
Morning	30	238.3 ^c ± 17.4	30	375.0 ^a ± 26.6	30	326.7 ^b ± 27.9	30	324.7 ^b ± 33.1	30	184.7 ^c ± 16.6	24	145.8 ^d ± 18.0	7	257.1 ^c ± 77.5	*
Evening	30	190.0 ^b ± 14.1	30	235.0 ^b ± 18.3	30	351.7 ^a ± 25.7	30	144.0 ^c ± 19.5	30	68.3 ^d ± 8.21	24	275.0 ^b ± 36.7	7	142.9 ^c ± 40.0	*
Total	30	428.3 ^b ± 30.7	30	610.0 ^a ± 41.6	30	678.3 ^a ± 47.2	30	468.0 ^b ± 47.3	30	253.0 ^c ± 20.8	24	420.8 ^b ± 51.3	7	400.0 ^b ± 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	30 th day			60 th day			90 th day			120 th day			P
		Morning	Evening	Mean	Morning	Evening	Mean	Morning	Evening	Mean	Morning	Evening	Mean	
Fat (%)	30	2.2 ± 0.30	3.8 ± 0.28	3.0 ^a ± 0.27	1.5 ± 0.17	3.2 ± 0.19	2.4 ^b ± 0.17	1.8 ± 0.12	1.5 ± 0.10	1.7 ^b ± 0.10	2.4 ± 0.26	3.4 ± 0.16	2.9 ^a ± 0.19	*
Protein (%)	30	4.6 ± 0.09	4.5 ± 0.09	4.6 ^a ± 0.09	4.2 ± 0.06	4.2 ± 0.05	4.2 ^b ± 0.05	4.2 ± 0.06	4.1 ± 0.06	4.1 ^b ± 0.06	4.3 ± 0.06	4.1 ± 0.06	4.2 ^b ± 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 ± 0.34	11.9 ± 0.21	13.5 ± 0.22	12.7 ^a ± 0.21	11.9 ± 0.15	11.5 ± 0.15	11.7 ^b ± 0.15	12.5 ± 0.31	12.9 ± 0.22	12.7 ^a ± 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 ± 41.20	53.8 ± 16.00	111.8 ± 25.30	82.8 ^c ± 19.50	92.3 ± 47.90	138.8 ± 75.40	113.1 ^b ± 61.40	331.0 ± 105.634.0	155483.0 ± 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. Milk composition, FDP, and SSC in different lactation stage (150 - 210thdays) of Honamlıgoats ($\bar{x} \pm S_{\bar{x}}$)

Parameters	n	150 th day			180 th day			210 th day			P
		Morning	Evening	Mean	Morning	Evening	Mean	Morning	Evening	Mean	
Fat (%)	30	3.0 ± 0.21	3.3 ± 0.17	3.2 ^b ± 0.17	4.4 ± 0.26	3.6 ± 0.21	4.0 ^b ± 0.22	5.9 ± 0.85	8.1 ± 0.84	6.6 ^a ± 0.71	*
Protein (%)	30	4.2 ± 0.06	3.9 ± 0.08	4.0 ^c ± 0.07	5.4 ± 0.16	5.2 ± 0.13	5.3 ^b ± 0.12	9.0 ± 0.51	8.7 ± 0.57	8.8 ^a ± 0.43	*
Lactose (%)	30	4.7 ± 0.02	4.7 ± 0.02	4.7 ^a ± 0.02	4.4 ± 0.15	4.5 ± 0.03	4.4 ^a ± 0.07	3.0 ± 0.36	3.4 ± 0.31	3.2 ^b ± 0.25	*
Total Solids (%)	30	12.9 ± 0.26	12.7 ± 0.25	12.8 ^c ± 0.23	14.9 ± 0.35	14.2 ± 0.31	14.6 ^b ± 0.31	16.9 ± 1.02	20.3 ± 0.106	18.6 ^a ± 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 ± 118.0	939.0 ± 232.0	728.0 ± 160.0	834.0 ^a ± 176.0	816.0 ± 315.0	977.0 ± 417.0	896.0 ^a ± 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 3b present 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. Additionally, 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 significant differences between lactation stages ($P > 0.05$).

In the present study, it was determined that SCC was 145.7×10^3 cell/ml, 82.8×10^3 cell/ml, 113.1×10^3 cell/ml, 483.0×10^3 cell/ml, 432.0×10^3 cell/ml, 834.0×10^3 cell/ml, and 896.0×10^3 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. While this value was lower than the mean lactation period observed for goats by Bolacalı and Küçük (2012), and Králíčková et al. (2013); but higher than the mean lactation period determined for goats by some researchers (Tuncel and Okuyan, 1985; Forik, 1995; Sengonca et al., 2003; Simšek et al., 2006; Ata, 2007; El-Tarabany et al., 2016). When compared with values in the literature, lactation period of Honamlı goats may have been associated with genotype and environmental factors such as management 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; Simšek 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 similarity with 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; Simšek 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. Similarly 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 Idamokoro 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). Additionally, it 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. Similarly to this, Králíčková et al. (2013) reported an increase in protein content value only during last periods of lactation. Kuchčík 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 (Kuchčík et al., 2015; El-Tarabany et al., 2016). Additionally, 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%). Kuchčík 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 82.8×10^3 cell/ml and 113.1×10^3 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 270×10^3 to 2.000×10^3 cell/ml. In the present study, together with the increase of SCC, a decrease occurred in 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 400.0×10^3 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 the 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.

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***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 plasmacytes 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 in-

flammatory 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, Poly-science Inc, USA), immersed in isopentane, cooled to its freezing point in liquid nitrogen and stored at -80°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 $5\text{-}\mu\text{m}$ 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 $5\text{-}\mu\text{m}$ tissue sections, which were placed in a 60°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 immersing 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 Retrieval-

al Solution-pH 9 was preheated for 5 min at 450W, before immersing 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 (H₂O₂) 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- μ m 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

<i>antigen</i>	<i>clone</i>	<i>Isotype</i>	<i>specificity</i>	<i>source</i>	<i>sections</i>	<i>dilution</i>	<i>incubation time</i>
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 immunolabelling 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 scabiectic 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).

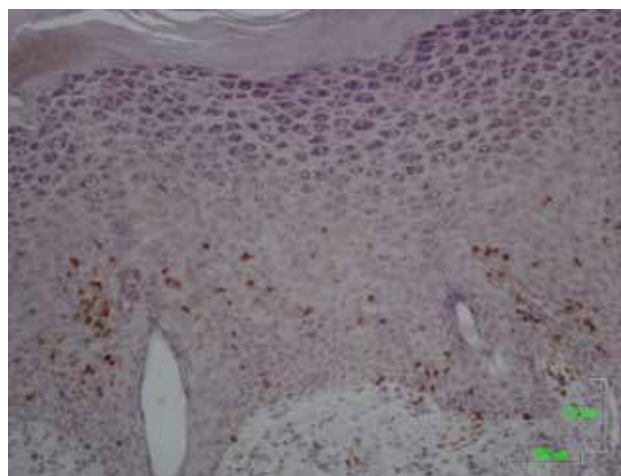


Figure 3. Paraffin section of facial skin from a ewe with sarcoptic mange: exocytosis of CD3+ T-lymphocytes in hyperplastic epidermis. IHC, EnVision™ detection system

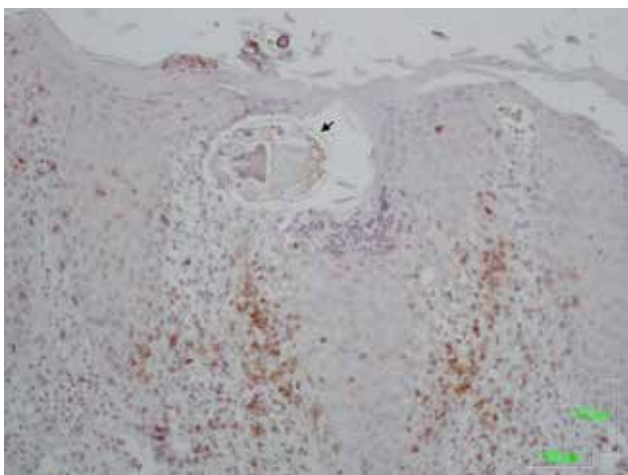


Figure 4. Paraffin section of foot skin from a ewe with sarcoptic mange: infiltration of CD3+ T-lymphocytes in upper dermis (dermoepidermal area) and exocytosis toward a *Sarcoptes scabiei* mite (arrow), burrowing in epidermis. IHC, EnVision™ detection system



Figure 7. Paraffin section of foot skin from a ewe with sarcoptic mange: there are few CD79a+ cells in the dermis. IHC, EnVision™ detection system.

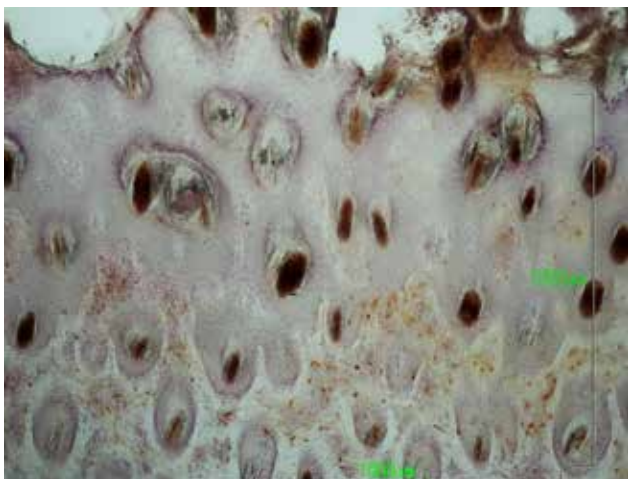


Figure 5. Cryostat section of foot skin from a ewe with sarcoptic mange: infiltration of CD4+ T-lymphocytes in upper dermis. IHC, EnVision™ detection system

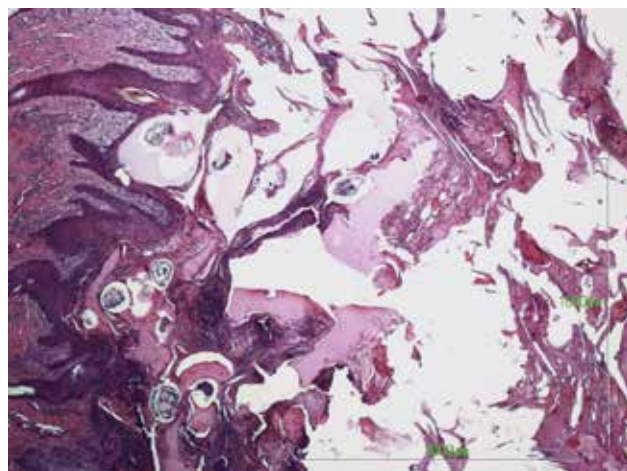


Figure 8. Paraffin section of scrotum skin from a ram with sarcoptic mange: occurrence of many *Sarcoptes scabiei* mites in thick serocellular crusts. Haematoxylin-Eosin stain

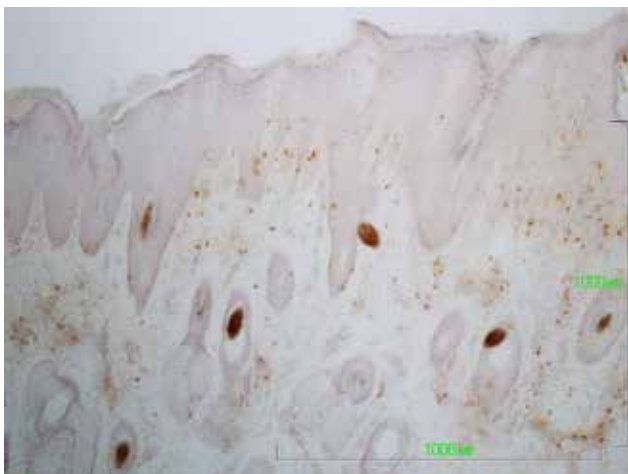


Figure 6. Cryostat section of foot skin from a ewe with sarcoptic mange: mild infiltration of CD8+ T-lymphocytes in upper dermis, as well as focal lymphocytic exocytosis in epidermis. IHC, EnVision™ detection system

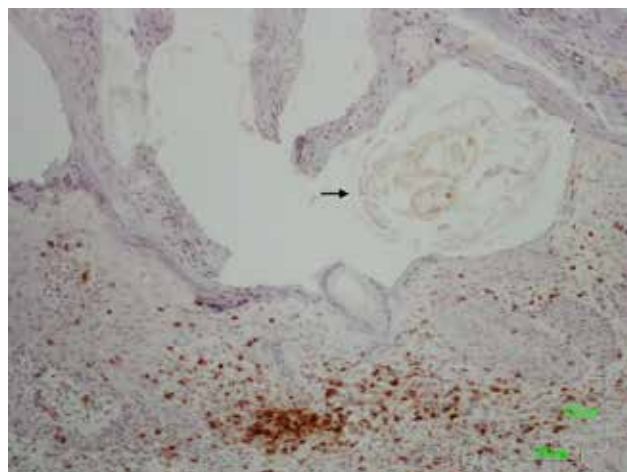


Figure 9. Paraffin section of scrotum skin from a ram with sarcoptic mange: notice the *Sarcoptes scabiei* mite (arrow) burrowing into parakeratotic stratum corneum associated with exocytosis of CD3+ T-lymphocytes in epidermis and severe focal dermoepidermal inflammation. IHC, EnVision™ detection system

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 stain-

ing 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 plasmacytes (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 with 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 $\gamma\delta$ ⁺ subpopulations. The lymphoid system of ruminants contains a large number of $\gamma\delta$ ⁺ 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, $\gamma\delta$ ⁺ T-cells are the predominant lymphocyte subpopulation in woolled 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 $\gamma\delta$ ⁺ 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 $\gamma\delta$ ⁺ 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 $\gamma\delta$ ⁺ 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 $\gamma\delta$ ⁺ 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).

CONCLUSION

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.

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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 *Staphylococcus* 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 (Ceauși 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 of 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 (TUIK, 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 nine-fold 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, TMAAnB, 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. pneumoniae*, *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
TMAAnB ^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 ^s	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 ^s	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 ^s	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
<i>Staphylococcus</i> 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
TMAAnB	18.7 ^a	4 ^a	22.7
Coliform	14.7 ^a	4 ^a	18.7
Yeasts	14.7 ^a	2 ^b	16.7
<i>Staphylococcus</i> 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	
<i>Klebsiella pneumonia</i>	26 (60.4)	3
<i>Enterobacter cloacae</i>	11 (25.6)	3
<i>Klebsiella oxytoca</i>	6 (14)	Not detected
Non-coliform Gram (-) bacteria	y	
<i>Proteus vulgaris</i>	38 (60.3)	14
<i>Serratia marcescens</i>	17 (27)	8
<i>Proteus mirabilis</i>	8 (12.7)	1
<i>Staphylococcus</i> spp.	z	
<i>S. hominis</i>	10 (33.3)	2
<i>S. epidermidis</i>	8 (26.6)	1
<i>S. hemolyticus</i>	6 (20)	1
<i>S. capitis</i>	6 (20)	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).

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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
<i>Proteus vulgaris</i> (n:38)	38(100)	0(0)	NA	0(0)	38(100)	38(100)	38(100)	0(0)	38(100)	0(0)	38(100)
<i>Klebsiella pneumonia</i> (n:26)	0(0)	0(0)	26(100)	10(38)	0(0)	0(0)	0(0)	26(100)	26(100)	26(100)	26(100)
<i>Serratia marcescens</i> (n:17)	NA	0(0)	NA	0(0)	0(0)	0(0)	0(0)	4(24)	17(100)	4(24)	17(100)
<i>Enterobacter cloacae</i> (n: 11)	NA	3(27)	0(0)	0(0)	0(0)	3(27)	0(0)	3(27)	3(27)	3(27)	4(36)
<i>Proteus mirabilis</i> (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)
<i>Klebsiella oxytoca</i> (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 1.9×10^2 cfu/g to 4.6×10^3 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 TMAAnB and the mean TMAAnB 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 (Tyset 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 3.4×10^1 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. (Pucciarelli 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 (Ceauși 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, TMA_nB, 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, TMA_nB, 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.

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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, H₂S 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 (Rasoloflo 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).

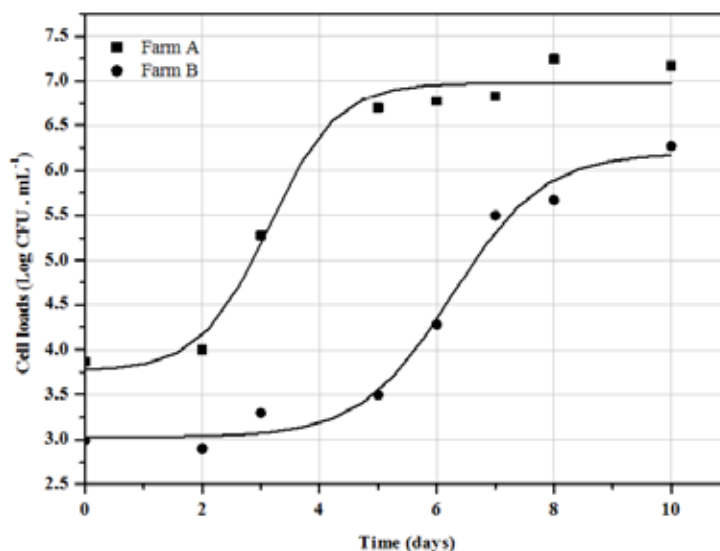


Figure 1. Growth curves of total viable bacteria count obtained by Baranyi and Roberts Model in milk refrigerated during 10 days at 4 °C, from two dairy farms in the basin (A) and mountain (B) in the region of Mila, Algeria

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	μ_{\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 (Δ log 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* (Gay-sinsky 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.

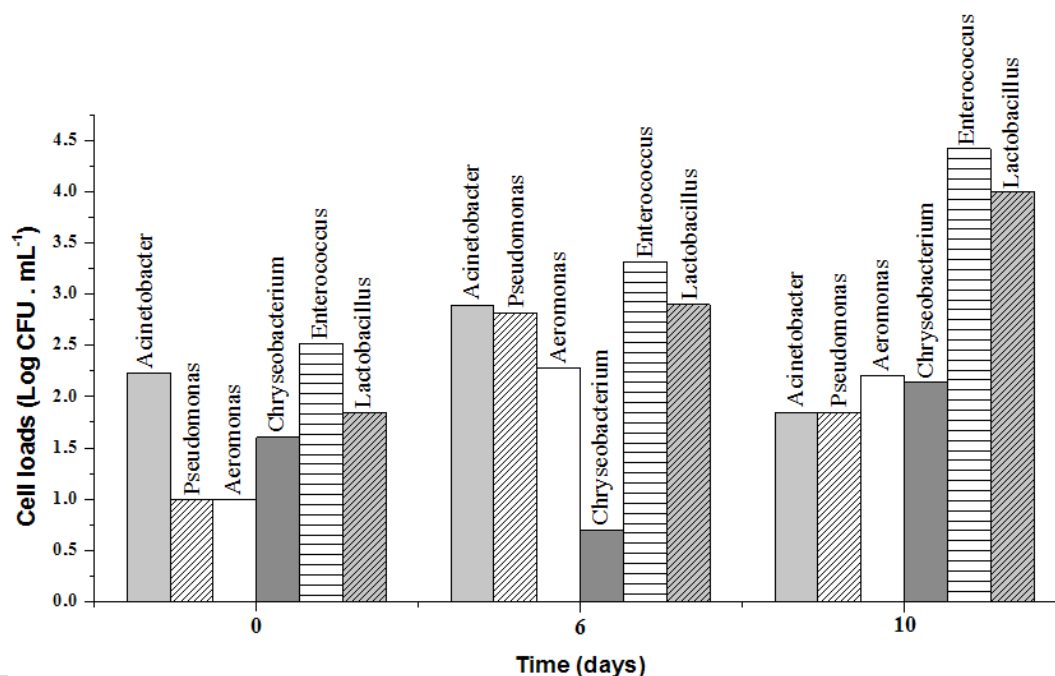


Figure 2. The evolution of psychrotrophic bacterial populations in mountain milk stored at 4°C during 10 days in the region of Mila, Algeria

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 (Rasoloflo 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. Usu-

ally, *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 (H₂O₂), diacetyl (2, 3-butanediona), reuterin, bacteriocins and carbon dioxide (CO₂) (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.

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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 km² situated between latitude 27 to 29 N and longitude 69° to 75° E at height of about 112 m above sea level (Ali *et al.*, 2009).

The Cholistan 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 Cholistan 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*, *Tribulus longipetalus*, *Tribulus terrestris*, *Pulicaria crista*, *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 (\pm SEM) for the hematological 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 \pm 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 $3.41 \pm 0.17 \times 10^6/\mu\text{L}$, $3.73 \pm 0.13 \times 10^6/\mu\text{L}$ and $3.93 \pm 0.25 \times 10^6/\mu\text{L}$ 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 \pm SE values for white blood cells (WBC), lymphocytes (LYM), monocytes

(MON), granulocytes (GRA).

The mean leukocyte (WBC) value was $17.32 \pm 1.70 \times 10^3/\mu\text{L}$ in non-pregnant goats and prominent higher $24.44 \pm 2.08 \times 10^3/\mu\text{L}$ was detected in pregnant goats and significantly lower $20.78 \pm 1.65 \times 10^3/\mu\text{L}$ was recorded in lactating goats. The difference was statistically significant ($P < 0.05$). Lymphocyte (LYM) mean \pm SEM value was $9.04 \pm 1.14 \times 10^3/\mu\text{L}$ in non-pregnant goats. The highest value recorded $13.77 \pm 1.56 \times 10^3/\mu\text{L}$ in pregnant goats and the lactating goats also showed higher values of Lymphocyte as compared to non-pregnant goats. The mean \pm SEM value of monocytes (MON) was $0.84 \pm 0.05 \times 10^3/\mu\text{L}$ in non-pregnant goats which was significantly ($P < 0.05$) lower than lactating and pregnant goats.

Apparently higher $9.67 \pm 0.79 \times 10^3/\mu\text{L}$ 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 \pm 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 ($10^6/\mu\text{L}$)	3.41 ± 0.17	3.73 ± 0.13	3.93 ± 0.25
Hb (g/dl)	7.93 ± 0.20	$7.32 \pm 0.16^{*a}$	$7.05 \pm 0.25^{*b}$
HCT (%)	10.08 ± 0.52	10.69 ± 0.38	11.34 ± 0.72
MCV (μm^3)	29.60 ± 0.16	$28.67 \pm 0.31^{*a}$	$28.83 \pm 0.19^{*b}$
MCH (Pg)	24.14 ± 0.99	20.47 ± 0.86	$18.70 \pm 0.85^{*b}$
MCHC (g/dl)	81.39 ± 3.21	$69.25 \pm 2.29^{*a}$	$64.83 \pm 2.92^{*b}$
RDW (%)	16.86 ± 0.84	$14.94 \pm 0.37^{*a}$	$13.68 \pm 0.48^{*b}$
RDW-SD (μm^3)	24.17 ± 0.31	23.09 ± 0.39	$21.37 \pm 0.67^{*b}$

Table 2. The mean \pm 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 ($10^3/\mu\text{L}$)	17.32 \pm 1.70	24.44 \pm 2.08* ^a	20.78 \pm 1.65
LYM ($10^3/\mu\text{L}$)	9.04 \pm 1.14	13.77 \pm 1.56	10.96 \pm 1.08
MON ($10^3/\mu\text{L}$)	0.84 \pm 0.05	1.00 \pm 0.09	1.04 \pm 0.08
GRA ($10^3/\mu\text{L}$)	7.43 \pm 0.67	9.67 \pm 0.79	8.77 \pm 0.69
LYM (%)	50.04 \pm 2.32	54.00 \pm 2.54	52.33 \pm 2.13
MON (%)	5.30 \pm 0.27	4.48 \pm 0.38	5.13 \pm 0.27
GRA (%)	44.66 \pm 2.14	41.54 \pm 2.32	42.56 \pm 2.07

Table 3. The mean \pm 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 \pm 7.50* ^a	36.43 \pm 4.83* ^a	39.29 \pm 5.13* ^b
ALT(IU/L)	26.43 \pm 4.33* ^a	20.71 \pm 3.15* ^b	23.14 \pm 2.83* ^b
Cholesterol (mg/dL)	56.86 \pm 6.92* ^a	76.14 \pm 6.69* ^b	70.71 \pm 5.44* ^b
Triglyceride (mg/dL)	54.86 \pm 6.01	74.43 \pm 3.91* ^a	63.43 \pm 3.15
Sodium(MEQ/L)	152.9 \pm 12.00* ^a	131.57 \pm 4.27* ^b	128.57 \pm 4.44* ^b
Potassium(MEQ/L)	3.96 \pm 0.21	3.54 \pm 0.23	3.90 \pm 0.31

DISCUSSION

This is the first study of Jattal goats of Colistan which was designed to determine salient hematochemical 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 (Yilmaz, 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, lymphocytes and eosinophiles 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 AbedMaksoud, 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 lactating 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.

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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 trypanosomosis 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 Silva et 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 reported that 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), lachrymation, 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.



Figure 1. 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 haematological 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 (3.5 mg/kg b. w., intramus-

cularly) and other supportive therapy. With a single dose of diminazene aceturate, each dog recovered after follow up of 15 days.

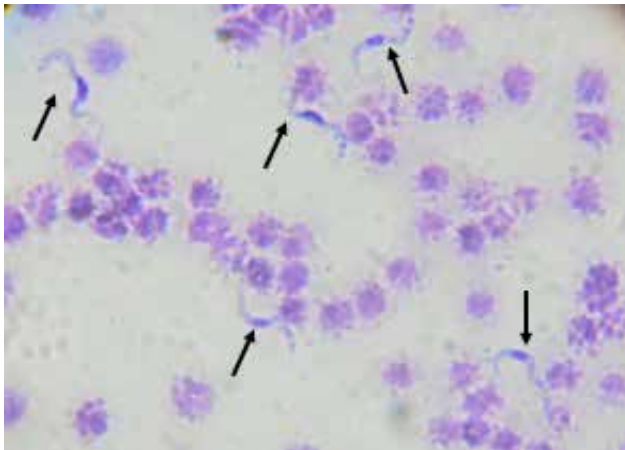


Figure 2. *Trypanosoma* spp., dog 1, Field's-stained thin blood smear (X100)

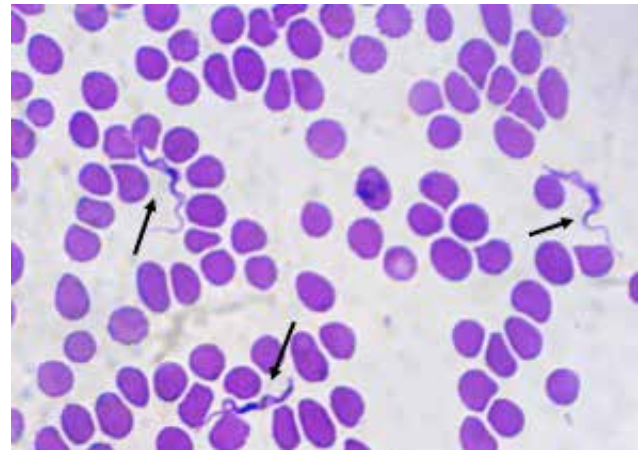


Figure 3. *Trypanosoma* spp., dog 2, Field's-stained thin blood smear (X100)

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; Abenaga 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 adminis-

tration) 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 diacetate (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 Merieux, 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 trypanosomiasis 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 haemoglobin (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 trypanosomiasis but for species confirmation, molecular assays are required and single dose of diminazene aceturate may recover infect-

ed dogs. To our knowledge, it is first case report of trypanosomiasis 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.

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