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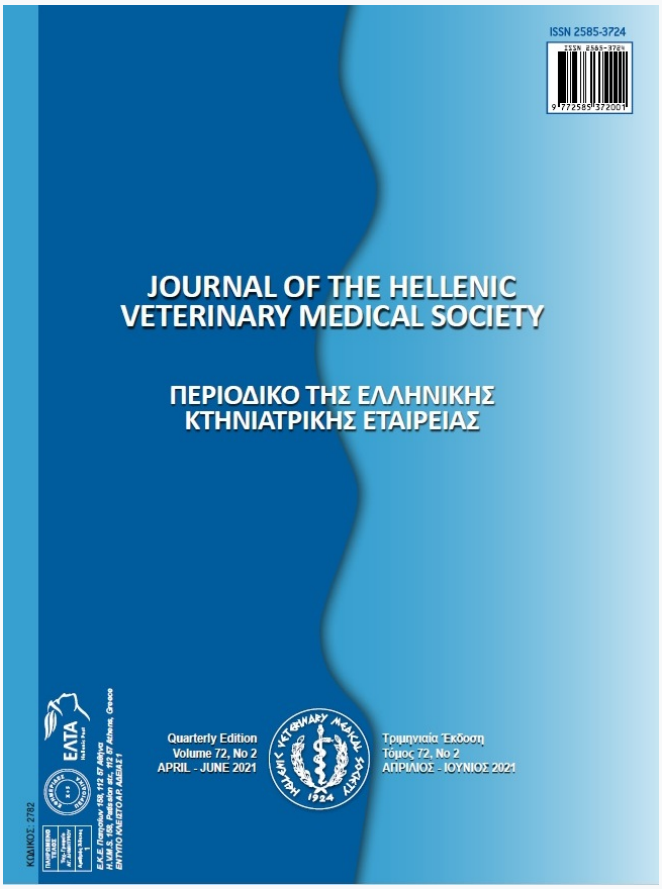

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				Number of specialist veterinarians active in Greece Αριθμός ειδικευμένων κτηνιάτρων εργαζόμενων στην Ελλάδα
1		ECAR	European College of Animal Reproduction	2
2		ECAWBM	European College of Animal Welfare and Behavioural Medicine	2
3		ECAAH	European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

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History of the Hellenic Veterinary Medical Society

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

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

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

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

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

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

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

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

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

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

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

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

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

President:	Theodoros Cl. Ananiadis†
Vice-President:	Veniamin Albalas
General Secretary:	Athanassios E. Tyrpenou
Spec. Secretary:	Konstantinos Chandras
Treasurer:	Olga Sabatakou
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Review article
Ανασκόπηση

Current research and application of stem cells in the dog and cat

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SUMMARY: Stem cells (SCs) are multipotent cells with differentiation and proliferation capacities in many cell lineages. The majority of literature applications are about bone marrow derived stem cells (BMSCs) and adipose derived stem cells (ADSCs). Most clinical trials have been done for the treatment of musculoskeletal and neurological problems in canine patients. Hematopoietic SCs (HSCs), synovium (SDSCs) and cartilage (CSPCs) - derived SCs, umbilical cord blood-derived SCs (UCSCs), muscle, dental, cardiac and hepatic SCs have been used with promising results. Despite the overall progress crucial questions about SCs remain unanswered. It is still unclear if the regeneration mechanism of SCs owed to their differentiation into specific progenitor cells or due to their immunomodulatory and anti-inflammatory secretions. Also, there are questions about the best origin of stem cells, whether they should be delivered in situ or systemically, if they should be embedded into scaffolds or not and which is the suitable transplantation stem cells number. Many of the published studies have limitation regarding to sample size, blind randomization, control groups and homogeneity of population. In addition,the long-term efficacy and safety of MSCs need further evaluation. This review is an update in usage of SCs, mainly focused on BMSCs and ADSCs in small animals and its purpose is to present the late developments in this field. Also, the advantages, the disadvantages and the limitations of the current literature review arediscussed.

Keywords: ADSCs, cats, dogs, MSCs, stem cells, transplantation.

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INTRODUCTION

Stem cells (SCs)are multipotent cells with differentiation and proliferation capacities in many cell lineages. They produce factors with immunomodalityand angiogenesis properties (Takemitsu et al., 2012). They divided intoembryonic SCs(ESCs) and adult mesenchymal SCs(MSCs). The latter can be harvested by mesodermal, endodermal or ectodermal tissues (de Bakker et al., 2013). Depending on their origin they have advantages and limitations. For example, bone marrow - derived mesenchymal SCs (BMSCs) have the higher differentiation capacity in musculoskeletal cells, but their culture is time consuming (Fortier and Travis, 2011). Their osteogenic potential is better than adipose tissue - derived SCs (ADSCs) (Park et al., 2012), while the latterexhibit higher proliferation rate (Webb et al.,2011). Also, allogeneic versus autologous transplantation is still debatable (Zhang et al., 2015). In vitro studies proved that allogeneic transplantation is safe (Ryan et al., 2005), due to a protective mechanism against T and B cells that forms a safe micro-environment (Ryan et al., 2005). Moreover, xenogeneic SCs transplantation was also presented. Human SCs promoted healing in dogs with bone defects (Cruz et al., 2015, Zang et al., 2016), dermatological problems(Ferrer et al., 2015) andneurological diseases (Lee et al., 2009,Chung et al., 2013).

SCs usage in tissue engineering is a progressive technique that continues to expand increasingly in the veterinary world. Many scientific papers have been published over the last 10years (Arinzeh et al., 2003, Yan et al. 2007,Cui et al. 2007, Wang et al. 2014, Nantavisai et al., 2019, Voga et al. 2020).

ADVANCES IN STEM CELLS

Adipose tissue - derived stem cells (ADSCs)

Dogs

Musculoskeletal applications

In 2007, Cui et al. (2007) showed that autologous ADSCsenhanced bone regeneration in critical sized bone defects in canine models. Same conclusions retrievedfrom trials on mandibular (Haghighat et al., 2011) and long bone defects (Bigham-Sadegh et al., 2012). In 2013, Luiet al. repeated the same model with allogeneic ADSCs and presented similar results without adverse reactions (Liu et al., 2013).

The application of autologous ADSCs in coxofemoral (Black et al., 2007) and humeroradial joints with osteoarthritis (Black et al., 2008, Guercio et al., 2012) resulted in clinical improvement. Another study reinforced these results with kinetic force assessments (Vilar et al., 2014). Equally effective was the application of allogeneic ADSCs (Harman et al., 2016). Moreover, the combination of autologous ADSCs with platelet-rich plasma (PRP) (Vilar et al., 2013, Cuervo et al., 2016, Yun et al., 2016) reduced pain in cases of osteoarthritis, but the studies lacked of control groups.

In 2014, an innovative administration of allogeneic ADSCs for hip dysplasia, in acupuncture points, showed clinical improvement (Marx et al., 2014). Also, promising was the administration of allogeneic ADSCs in elbow dysplasia (Kriston-Pal et al., 2017). Nevertheless, these studies did not present arthroscopic findings. Recently, another research demonstrated that the local injection of ADSCs was superior compared to intravenous for degenerative arthritis (Shah et al., 2018). Unfortunately, the outcomes were based only on clinical evaluation.

ADSCs combined with PRP accelerated the reconstruction of partial ruptured anterior cruciate ligament (Canapp et al., 2016a). In contrast, the use of ADSCs during tibial tuberosity advancement procedure did not diminish the healing time (Dos Santos et al., 2018). The results about ADSCs advantages were controversial, but both studies had heterogeneous populations and different rehabilitation protocols. Furthermore, preliminary results proposed the combination of ADSCs with PRP for refractory supraspinatus tendinopathy (Canapp et al. 2016b), semitendinosus myopathy (Gibson et al., 2017) and tracheal cartilage defects (Hashemibeni et al., 2012). Lastly, the combination of ADSCs with growth factor showed promising results in a canine tendon injury model (Sheh et al.,2018).

Neurological applications

ADSCs were used for spinal cord injury (SCI) (Roszek et al., 2016). Canine models investigated ADSCs capacities in situ (Ryu et al., 2009). Interestingly, ADSCs promoted functional improvement after SCI (Park et al., 2012) and accelerated disk regeneration after disk degeneration disease (DDD) (Ganey et al., 2009). However, the results were based on experimental conditions. In clinical field, two studies described the benefits of ADSCs transplantation in dogs with thoracolumbar DDD (Kim et al., 2016)

and peripheral (facial) nerve trauma (Ghoreishian et al., 2013). Both confirmed clinical improvement, but lacked of histopathological confirmation.

Dentistry and ophthalmology applications

ADSCs were used successfully in periodontal trauma (Tobita et al., 2013), atonic oral ulcers (Alamoudi et al., 2014) and as an osseointegration factor for dental implants (Bressan et al., 2015). To the authors' knowledge, no clinical studies are currently available. After the proof of ADSCs safety inperiocular (Wood et al., 2012) and intra-lacrimal (Park et al., 2013) injections, interest was concentrated in ADSCsusage against keratoconjunctivitis sicca (KCS) (Villatoro et al., 2015). Peri- and intra-lacrimal ADSCs transplantation leaded to KCS remission (Bittencourt et al., 2016). However, possible adverse reactions were not studied.

Other applications

The in vitro differentiation of ADSCs into cells similar to hepatocytes (Banas et al. 2007) triggered-further studies. In one preclinical study, ADSCsdecreased hepatic parameters after liver trauma (Teshima et al., 2017), and increased survival time in one dog with hepatocutaneous syndrome (Nam et al., 2017). Also, ADSCs were employed for dogs with inflammatory bowel disease (Perez-Merino et al., 2015). The conclusions are suggestive, as these studies lacked of long term efficacy and safety data. Despite the fact that ADSCs improve the cardiac function after myocardial infarction, clinical trials failed to prove their benefits in dilated cardiomyopathy (Pogue et al., 2013). A current comparison of ADSCs and BMSCs intrarenal injection in a canine renal injury model revealed better level of protection for ADCSs (Osman et al. 2020). Lastly, ADSCs application was beneficial for one dog with an atonic ulcer (Han et al., 2015) and another with pemphigus (Zubin et al., 2014), but was not helpful for allergies (Hall et al., 2010).

Cats

One pilot study showed improvement in renal function in cats with chronic kidney failure after intrarenal administration of autologous ADSCs (Quimby et al., 2011). However, the intravenous administration of allogeneic ADSCs was associated with adverse effects (Quimby et al., 2013). Further research in order to attempt tominimize these reactions failed (Quimby et al., 2016). The intravenous injection of ADSCs in cats with inflammatory bowel disease leaded to (Webb and Webb, 2015) signs remission. Also, the intraperitoneal injection of ADSCs was showed to be safe and possibly effective for various feline diseases (Parys et al., 2016). ADSCs were employed successfully in autoimmune diseases like gingivostomatitis (Arzi et al., 2016), asthma (Trzil et al., 2015) and eosinophilic keratitis (Villatoro et al., 2018). Nevertheless, larger sample sizes are needed to obtain more reliable results.

Bone marrow-derived mesenchymal stem cells (BMSCs)

Dogs

Musculoskeletal applications

The implantation of autologous BMSCs embedded into various scaffolds, encouraged bone formation in alveolar (Kim et al., 2009), mandible (Hu et al., 2014), orbital (Yang et al., 2014) and long bone defects (Ozdal-Kurt et al., 2014). Also, the combination of cell sheet and scaffolds resulted in healing in mandible injuries (Shan and Hu, 2017). All the macroscopic results were co-evaluated with imaging and histological findings. Despite the fact that these were control studies and their results were significant different between groups, their sample sizes were limited. Few experimental works studied the importance of scaffolds and the migration of systemically administrated BMSCs. The intra-osseous injection of allogeneic BMSCs into femurs promoted bone regeneration in mandible defects (Liu et al., 2014). Similar were the results of intra-arterialinfusion of autologous BMSCs in dogs with femoral head necrosis (Jin et al., 2016). Both studies concluded that the technique was not only promising for bone density problems, but it was also safe. Moreover, genetic modification of BMSCs enhanced specific capacities. The induced expression of vascular endothelial growth factor 165 ($VEGF_{165}$) (Hang et al., 2012) or bone morphogenetic protein -2 (BMP-2) (Peng and Wang, 2017) stimulated the quantity and quality of new formed bone in femoral head osteonecrosis. These suggestions resulted from control-based studies with big sample sizes. Additionally, smaller studies concluded that microRNA-31 and dentin matrix protein-1 (DMP-1) manipulated BMSCs enhanced bone formation with superior features compared to simple BMSCs (Deng et al., 2014, Liu et al., 2016). BMSCs have also been

used for cartilage repair (Bornes et al., 2014). In 2005, Wayne et al. proposed that the injection of BMSCs in canine joints with articular defects promoted superior quality of new formed cartilage. Concurrently, the outcomes from intralesional injection of bone marrow into traumatized meniscus, implied healing properties of BMSCs (Abdel- Hamid et al., 2005). Equivalent findings concluded from the intravenous and intrarticular injection of BMSCs, in dogs with partial cruciate ligament rupture, despite the limited sample and volume of synovial fluid available for tests (Muir et al., 2016). Following studies focused on PRP combination with BMSCs, because it improved their proliferation rate (Chen et al., 2014) and chondrogenetic capacity (Kazemi et al., 2017). A large retrospective study suggested that their injection in dogs with supraspinatus tendinopathy resulted in clinical and ultrasonography improvement (McDougall et al., 2018). However, there are no immunohistochemical assessments of these findings. Lastly, BMSCs were used successfully in tendon repair not only in vitro (Ozasa et al., 2014) but also in vivo (Case et al. 2013) and esophagus reconstruction (Tan et al., 2013). Nevertheless, the data were preliminary.

Neurological applications

BMSCs were also helpful for neurological disorders (Kamishina et al., 2006). In 2008, Hiyama et al.(2008) investigated the intralesional autologous BMSCs injection in a canine model of DDD.The histological and imaging analysis showed alteration of the micro-environment and inflammation's inhibition (Hiyama et al., 2008). However, this was an experimental model. Therefore, scientists investigated the safety of the procedure in clinical cases of DDD. Their results were controversial. One study presented improvement of nociception and proprioception after autologous BMSCs transplantation (Besalti et al., 2015). Another study concluded that the procedure may be safe, but not useful (Steffen et al., 2017). Both reports failed to make a statement due to limited samples and heterogenous populations. Furthermore, clinical results highlighted BMSCs efficacy after decompression surgery, in SCI (Nishida et al., 2012, Besaltiet al., 2016) even in chronic cases (Nishida et al., 2011). The improvement was similar after autologous or allogeneic BMSCs implantation (Jung et al., 2009, Sarmento et al., 2014). Concurrently investigation about the most suitable time for BMSCs application (Penha et al., 2014) and the number of implanted cells (Serigano et al., 2010) was made. These parameters affected the outcomes, but no protocols were proposed. Recently, Wu et al. (2018) created a canine model with complete transection of spinal cord and presented that BMSCs could be beneficial even for devastating cases. Moreover, autologous BMSCs combined with scaffolds were used successfully in sciatic (Ding et al., 2010) and ulnar nerve defects (Kaizawa et al., 2016). Degeneration was accelerated functionally and histologically. Lastly, BMSCs were proposed for cases of autoimmune meningoencephalomyelitis. Although the diagnosis was made by clinical examination, there was noted signs remission (Zeira et al., 2015).

Dentistry and ophthalmology applications

One report studied autologous BMSCs implantation into alveolar clefts combined with scaffolds, PRP and bone grafts (Yuanzhneng et al., 2015). The combination of BMSCs with PRP showed the best results. The findings were equivalent to studies that support PRP's proliferation properties in BMSCs (Chen et al., 2014, Kazemi et al., 2017, McDougall et al. 2018). Another study proposed autologous, alveoral BMSCsas a different origin of BMSCs, due to their access and osteogenic potentials (Wang et al., 2018). Nevertheless, the conclusions were preliminary. Additionally, large oral (Aly et al., 2014), vocal fold (Kanemaru et al., 2003) and laryngeal ulcers (Iravani et al., 2017) were favoured by the intra-lesional administration of autologous BMSCs. These results were supported by histological findings, but more research is needed. In veterinary ophthalmology experimental models of corneal ulcer (Tognoli et al., 2008) and retinal degeneration (Tracy et al., 2016), showed that autologous BMSCs were safe and anti- inflammatory.

Other applications

Canine models of cirrhosis (Matsuda et al., 2017), renal trauma (Lim et al., 2016) and chronic enteritis (Xu et al., 2016) were usedinvestigate other effects. Autologous BMSCs improved function in liver failure, inhibited fibrosis in renal injuries and diminished clinical signs in post radiation enteritis (Xu et al., 2016). Also, the usage of allogeneic BMSCs promoted healing in cutaneous wounds without adverse effects (Kim et al., 2013). Furthermore, Memon et al. (2005) presented that the intra-lesional injection of BMSCs accelerated regeneration in ischemic myocardium. Also, their intracoronary injection reduced infractions' areas, confirmed by histology and immunochemistry analysis (Hao et al., 2015). In the same

model, the adjunct of basic fibroblast growth factor augmented the engraftment and the differentiation of BMSCs (Wang et al., 2015). Undoubtedly, the previous studies had preliminary value and more research should be done in dosage, time points and administration routes.

Cats

Feline BMSCs morphology and isolation are similar to human and rodents, (Martin et al., 2002, Zhang et al., 2011,Munoz et al., 2012) with higher neurogenic properties (Zhang et al., 2011). Also, c-kit$^+$ feline BMSCs differentiated into cardiac myocytes with cardiac action potentials (Kubo et al., 2009). However, feline BMSCs had culture limitations referred to the donor health status (Quimby et al., 2011). In vivo applications are few. A randomized clinical trial failed to demonstrate significant effect of BMSCs in acute kidney injury. Also, it left unanswered questions about the ideal routes, doses and frequency of BMSCs administration (Rosselli et al., 2016). Additionally, a case report of a cat with lumbar fracture that gained clinical rehabilitation after intraoperative BMSCs transplantation reinforced their beneficial effects in SCI (Penha et al., 2012).

Different tissue derived mesenchymal stem cells

Synovium (SDSCs) and cartilage (CSPCs) - derived stem cells

SDSCs showed osteogenic capacity similar to BMSCs and rapid proliferation like ADSCs (Bearden et al., 2017). As referred to chondrogenic differentiation, SDSCs were found to be superior to ADSCs and BMSCs (Sasaki et al., 2018). In fact, the intra-articular injection of autologous SDSCs combined with hyaluronic acid in a canine model for cartilage repair, resulted in better macroscopic and histological scores compared to the control group (Miki et al., 2015). However, the studies lacked of statistical analysis. Until now, only autologous CSPCs harvested from ear cartilage showed capacity to regenerate both elastic and hyaline chondral tissue (Mizuno et al., 2014).

Muscle stem cells

Satellite cells become activated after trauma, as muscle precursor cells (MPCs). Satellite cells have high myogenic capacities, but they are no good candidates for transplantation, because of harvesting, survival and migration problems. However, their good expanding and long term survival in vivo (Eberli et al.,2012) promoted their usage for functional regulation of urinary sphincter (Eberli et al., 2012) and re-innervated thyroarytenoid muscle (Paniello et al., 2018) in canine models. Histological analysis of both studies revealed the formation of new innervated muscle fibres. Muscle- derived stem cells (MDSCs) can be differentiated not only in myogenic, but also in other lineages, such as osteogenic, chondrogenic and tenocyte-like cells. In fact, MDSCs were superior compared to BMSCs for tendon repair (Ozasa et al., 2014). Nevertheless, these findings were concluded in vitro. The majority of studies investigated MDSCs efficacy in dystrophic models. In 2011, a study proposed the intra-arterial administration of allogeneic MDSCs in dystrophic dogs to promote muscle regeneration (Rouger et al., 2011). Despite the limitation of small sample size, clinical and histopathological outcomes were favourable. Additionally, MDSCs were studied in molecular lever (Robriquet et al., 2015, Lardenois et al., 2016). Furthermore, a trial to make their usage easier showed that transient immunosuppression of the hosts was sufficient (Lorant et al., 2018). Undoubtedly, more research is needed about the immunology features of MSDSs.

Cardiac derived stem cells (CSCs)

Despite the fact that the majority of cardiac cells have not potentials to further differentiation, there is a population of CSCs with capacities to form myocytes and promote angiogenesis. In contrast to MSDSs, which could differentiate into muscle cells when injected into the myocardium (Yoon et al., 1995), CSCs could enhance both cardiac regeneration and function by producing functional cardiac cells (Welt et al., 2013, Taghavi et al., 2015). Autologous CSCs helped cardiac remodelling in infraction canine model (Welt et al., 2013) and improved cardiac function in feline cardiomyopathy model (Taghavi et al., 2015). However, in both studies the delivery method was invasive and new methods should be tried in order to make CSCs usage a possible clinical approach.

Hepatic stem cells

Hepatic progenitor cells (HPCs) differentiated into hepatocytes and chollangiocytes (Kruitwagen et al., 2014). The activation, reaction and identification of HPCs were evaluated in normal dogs (Ijzer et al., 2010) and cats (Ijzer et al., 2009). Also, HPCs were assessed in dogs with various liver diseases, such as acute and chronic hepatitis, cooper toxicosis, cancer

or biliary problems (Ijzer et al., 2010, Kruitwagen et al. 2019) and in cats with feline cholangitis (Otte et al., 2018) and lipidosis (Valtolina et al., 2018). Despite the fact that HPCs could be an alternative to liver transplant, which is no feasible in veterinary medicine, no current trials of HPCs usage have been reported.

Dental pulp (DSCs), skin and hair follicle (HFSCs) stem cells

DSCs have high proliferation rate, easy accessibility and differentiation potentials into many cell lineage (Ashiry et al., 2018). Autologous DSCs were used successfully in canine models for pulp regeneration (Nakashima and Iohara, 2014, Chen et al., 2015, Ashiry et al., 2018) and periodontal healing (Khorsand et al., 2013). Also, allogeneic DSCs application was efficacious for periodontitis management (Iohara et al., 2018). Moreover, allogeneic DSCs were used indegenerative heart disease (Petchdee and Sompeewong, 2016) and xenogeneic (human) DSCs in SCI in dogs (Feitosa et al., 2017). Despite the promising results, both studies lacked histological and imaging assessments. Also, periodontal stem cells (PDLSCs) were isolated as another tool for periodontitis therapy, but the studies were in vitro (Khoshhal et al., 2017). To the authors' knowledge skin and hair follicle stem cells were identified and characterized in dogs (Brachelente et al., 2013), but they have not been used in vivo.

Umbilical cord blood-derived stem cells (UCSCs)

UCSCs exhibit less immunogenicity, higher plasticity and production of cells per volume compared to MSCs (Jin et al., 2008).UCSCs were used mainly in SCI models (Lim et al., 2007, Park et al. 2011, Ryu et al. 2012) in order to promote clinical improvement and regulate inflammation (Park et al., 2011). However, histopathology and imaging evidence is needed (Lim et al., 2007, Ryu et al., 2012).

Hematopoietic stem cells (HSCs)

HSCs originate from bone marrow,umbilical cord and peripheral blood. HSCs form lymphoid and myeloid progenitor cells (Gomes et al., 2017). In 1962 and 1974, bone marrow transplantation in dogs implied its benefits (Thomas et al.,1962,Epstein et al., 1967, Dale and Graw, 1974). Later, studies used autologous hematopoietic stem cells transplantation (HSCT), against auto-immune diseases (Stolfi et al., 2016). Also, allogeneic HSCT was employed in the modification of host-versus-graft reaction (Zorn et al., 2011, Mathes et al., 2014, Vrecenak et al., 2014, Rosinski et al., 2015). Interestingly, allogeneic HSCT improved hosts' tolerance towards skin allografts, by T-cells regulation (Mathes et al., 2014). The first clinical case of allogeneic HSCT was completed in a dog with lymphoma (Lupu et al., 2006). Then, two studies tried autologous HSCT for lymphoma (Escobar et al., 2012, Willcox et al., 2012) and acute leukemia (Suter et al., 2015). All agreed that HSCT increased the duration of signs remission. However, according to Schaefer et al. (2016) allogeneic HSCT had problems related to HSCs ability to reach bone marrow without being trapped in other tissues. Lange at al. (2017) tried to identify the best route of administration by comparing the intravenous and intraosseous HSCT. However, the study failed to prove superiority of one method over the other. Few are known about feline HSCs, which have similar proliferative capacities to canine (Abkowitz et al., 1993, Abkowitz et al., 1995). Feline HSCs were used after genetical manipulation for inhibition of coronavirus replication and development of feline infectious peritonitis (Anis et al., 2017).

CONCLUSIONS

In conclusion, literature review cannot still answer questions regardingthe best origin ofSC, the most suitable route of administration, whether they should be embedded into scaffolds or notas well as which is the ideal number of transplanted cells. Also, it remains unanswered if the therapeutic potentials of SCs exist due to their differentiation into progenitor cells or due to their immunomodulatory secretions. Many experimental models are used for preliminary data, while the clinical use of SCs is in early stages. Most of these studies focused on the usage of BMSCs and ADSCs, because of their easy collection and culture, their sufficient differentiation capacity in musculoskeletal cells and high proliferation rate. However published data have limitations because of their small sample size, heterogeneity of population, lack of blind randomization and control groups. Therefore, a future research plan should involve methods and techniques capable to collect answers for all these questions.

CONFLICT OF INTEREST

None declared.

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Review article
Ανασκόπηση

Clinical aspects of feline prostate cancer: a literature review

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ABSTRACT: Feline prostate cancer is a rare condition, which means that the literature is very sparse. This makes it difficult to make sound conclusions about the epidemiology and the effectiveness of the treatment strategies that are utilized. The literature suggests that the condition mainly affects older cats and that these tumours are usually (adeno) carcinomas. Feline prostatic cancer patients often suffer from haematuria, dysuria and inappetence. Both surgery and chemotherapy have been used to treat the condition. Unfortunately, most animals still die within a year. Further research is needed to better understand the epidemiology of the disease and to guide treatment decisions.

***Keywords*:** Cats, Feline, Prostate, Cancer, Carcinoma

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INTRODUCTION

Prostatic conditions in the cat are rare *(Caney et al., 1998; Tursi et al., 2008)*. Prostatic abscesses *(Mordecai et al., 2008)*, prostatitis *(Roura et al., 2002)*, paraprostatic cysts *(Newell et al., 1992)* and cancer (*Hubbard et al., 1990)* have all been described. Prostatic carcinoma has been mentioned to be the most common prostatic conditions in cats, but remains a rare occurrence *(Hubbard et al., 1990; Caney et al., 1998)*. A retrospective study investigating lower urinary tract tumours failed to identify any cases of feline prostate cancer (*Sapierzyński et al., 2007*).

Due to its rarity, the epidemiology and risk factors for feline prostatic neoplasms are poorly understood. Additionally, it is difficult to make the distinction between prostatic carcinoma and urothelial carcinoma that invades the prostate but originates elsewhere *(Tursi et al., 2008)*.

The limited experience with the condition means that there are currently no standard of care or specific guidelines to guide treatment decisions *(Griffin et al., 2018)*. Despite this lack of guidelines both surgery and chemotherapy have been used to tackle the disease.

The aim of the current paper is to synthesize the current literature regarding clinical aspects of feline prostatic carcinoma. Additionally, possible directions for future research and their limitations are discussed.

SEARCH STRATEGY

The PubMed, PubMed Central and Web of Science databases were searched for database-specific variants and combinations of the following keywords: “Feline”, “Cat”, “Prostate”, “Prostatic”, “Cancer”, “Carcinoma” and “Adenocarcinoma”. The indices of Veterinary and Comparative Oncology, Journal of Veterinary Internal Medicine, Journal of Feline Medicine and Surgery and Journal of Feline Medicine and Surgery Open Reports were manually searched. The search engine Google was searched using the same keywords. Papers were selected if they described any case of feline prostate cancer, contained clinical information and had at least an English summary. Finally, the references of the selected publications were screened for further relevant literature.

IDENTIFIED LITERATURE

The amount of available literature regarding prostatic neoplasms in cats is very limited. The identified literature consisted exclusively of case reports describing a maximum of two animals. The total number of animals included in this review was ten. Four further cases were identified: a case of prostatic fibroadenoma *(Cotchin, 1984)*, two cases of adenocarcinoma and one of unknown histological subtype (*Barsanti and Finco, 1979; Hornbuckle and Kleine, 1980*). Unfortunately, these publications were unable to be sourced and were therefore not included.

No case-control - studies or cohort studies assessing treatment strategies or risk factors were found. Furthermore, no interventional studies or clinical trials assessing therapeutic strategies for any type of feline prostate cancer were identified.

CASE CHARACTERISTICS

The identified literature was screened for the following characteristics per animal: breed, age, neutering status, histological diagnosis and symptoms. The current review focuses on the characteristics and clinical aspects of the different cases. More information regarding histological, diagnostic and other aspects can be found in the original publications.

Hawe described a ten-year old, neutered cat with adenocarcinoma. The breed was not specified. The animal suffered for two weeks from haematuria, dysuria and pollakiuria *(Hawe, 1983)*.

In 1990, Hubbard and colleagues *(Hubbard et al., 1990)* described the case of an eleven-year old, neutered cat that suffered from haematuria. The breed was not specified. The animal was diagnosed with prostatic adenocarcinoma.

Caney and colleagues *(Caney et al., 1998)* described two cases of prostatic carcinoma in 1998. The first case concerned a nine-year old domestic longhair that was neutered. The animal suffered seven months from dysuria, haematuria, and increased urinary frequency. Additionally, it was mentioned that there were two instances of urethral obstruction in the two months before admission. The second animal was a seven-year old domestic shorthair that was also neutered. In addition to presenting dysuria and a single instance of haematuria, this animal also showed inappetence, weight loss, lethargy in the seven weeks before admission. Other symptoms included dyschezia and constipation. After exploratory surgery, this cat suffered from urinary incontinence and haematuria.

A case report of a nine-year old domestic shorthair

was published in 2004 *(LeRoy and Lech, 2004)*. When the animal presented, it was suffering from diarrhoea. Three months later, the animal was seen again when it suffered from inappetence, vocalization during urination and unusually frequent licking of the perineal region. Eleven months later, the cat was suffering from lethargy, vomiting, weight loss, tenesmus, haematuria and pyuria. The animal was diagnosed with adenocarcinoma.

In a case report, Tursi and colleagues *(Tursi et al., 2008)* described a twelve-year old, neutered domestic shorthair. The cat suffered from inappetence, weight loss, weakness, dysuria, haematuria and dyspnoea and was diagnosed with prostatic adenocarcinoma.

A twelve-year old mixed-breed, neutered cat suffered from dysuria, haematuria, dyschezia and constipation for three weeks *(Zambelli et al., 2010)*. The animal showed urinary incontinence two months after having surgery for his sarcomatoid carcinoma.

Lapshin and Kondratova presented a case report of prostatic adenocarcinoma in a cat in 2015 *(Lapshin and Kondratova, 2015)*. The animal was a nine-year old, intact Siberian that suffered from acute urinary retention, stranguria, general depression, drop-by-drop urination, anorexia and haematuria during the course of its disease.

In a book by Villalobos and Kaplan *(Villalobos and Kaplan, 2017)*, two cases were mentioned. A thirteen-year old domestic short-hair suffered from prostatic cancer of unknown histological type. Whether the animal was neutered and what its symptoms were, was not specified. A second cat was sixteen years old and was diagnosed with carcinoma. It suffered from urinary obstruction. Whether it was neutered, was not specified.

In the most recent case report, de Oliveira and colleagues *(de Oliveira et al., 2019)* present the case of a six-year old, intact domestic shorthair that was diagnosed with prostatic carcinoma. The cat presented with dysuria, haematuria, inappetence, constipation, lethargy and prostration.

Due to the small number of reports, it is difficult to make generalisations about the case characteristics to the whole population of feline prostate cancer patients. However, a few observations can be made. The median age of the animals diagnosed with prostate cancer in the reported cases was 10.5 years (range 6 – 16 years). This supports the hypothesis that prostate cancer occurs mainly in older cats. It is also apparent from the literature that most diagnosed tumours are carcinomas, more specifically adenocarcinomas. Several breeds were described, mainly domestic shorthairs. Prostate cancer was diagnosed in both neutered and intact animals. Finally, several symptoms can be identified that are often present in cats diagnosed with prostate cancer. Haematuria, dysuria and inappetence occur often in animals suffering from the disease.

TREATMENT AND OUTCOMES

From the identified case reports, the treatment strategy, clinical outcome and the location of possible metastases were extracted per animal. Treatment for alleviating symptoms (e.g. catheterisation for restoring urinary outflow) and diagnostic procedures (e.g. retrograde contrast urethrocystogram) were performed in most animals and are not specifically described in this review.

The treatment strategy of the cat in the case report of Hawe *(Hawe, 1983)* was not specified. The animal was euthanized, and lung metastases were identified.

The cat in the 1990 case report *(Hubbard et al., 1990)* was initially treated with a prostatectomy. Afterwards, doxorubicin (30mg/m^2) and cyclophosphamide (300 mg/m^2) were given. After four treatments, the chemotherapy was stopped due to proteinuria. The animal was euthanized ten months after the prostatectomy due to a recurrence of the tumour. Metastasis to the lungs and pancreas was identified.

The nine-year old domestic longhair in the case report of Caney and colleagues *(Caney et al., 1998)* was treated with ampicillin (11 mg/kg daily) and betamethasone (0.03 mg/kg daily) for two weeks when a provisional diagnosis of urethritis was made. A little over two weeks, later, a provision transitional cell carcinoma diagnosis was made, and ampicillin and betamethasone treatment was continued. Three months later, the animal was euthanised due to quickly worsening symptoms. No sign of metastasis to medial iliac lymph nodes was discovered. Microscopic metastases to the lungs where however identified. The seven-year old domestic shorthair in the same case report *(Caney et al., 1998)* was treated with antibiotics and glucocorticoids which did not resolve the symptoms. A prostatic -, urethral mass or urethritis was suspected after retrograde positive contract urethrography. Four days later, the cat was euthanised due to a worsening of its condition. No sign of metastasis to the medial iliac lymph nodes or lungs was discovered.

Three months after first presentation, the cat in the publication of Leroy and Lech *(LeRoy and Lech, 2004)* underwent an orchiectomy. Eight months later, it underwent surgery again for a urethral transection and removal of the mass. At the same time, chemotherapy was initiated (enrofloxacin 15 mg daily and cephalexin 100 mg every 12 hours).

No treatment strategy was explicitly mentioned in the article of Tursi and colleagues *(Tursi et al., 2008)*. The animal was euthanized when its condition deteriorated. The time at which this occurred was not specified. Metastasis to the lungs and myocardium was identified. There was also infiltration of the mediastinal lymph nodes.

Before being referred, the cat described by Zambelli and colleagues *(Zambelli et al., 2010)* was treated with antibiotics and urinary acidification. After referral, a prostatectomy was performed and amoxicillin 15 mg/kg every 48h was given for 7 days postoperatively. Two years after the surgery, this animal was not showing any problems related to recurrence of the disease or metastasis. This is currently the only case where it seems possible that the animal was cured.

The treatment of the Siberian cat in Lapshin and Kondratova's article (Lapshin and Kondratova, 2015) was aimed to alleviate symptoms. It was euthanized when the owner was unable to continue therapy. Whether or not metastasis was present, was not specified.

The first cat in the text of Villalobos and Kaplan *(Villalobos and Kaplan, 2017)* underwent debulking surgery and open cystotomy. Afterwards, it received mitoxantrone and piroxicam for recurrent disease. The animal survived for one year. The presence of metastases was not discussed. The second cat was treated with mitoxantrone every 21 days and piroxicam 1 mg daily After six of these cycles, carboplatin was initiated. The cat lived for one more year and presented with cutaneous metastases in the last six months.

The cat in the case report of Oliveira *(Oliveira et al., 2019)* underwent a laparotomy for tumour excision. Because of the involvement of the urethra, a urethral anastomosis was performed to allow for a continued urine flow. Four hours after surgery, the animal died. The authors state that the death was not related to complications related to the surgical procedure. There was no sign of pulmonary metastasis in this animal.

Due to the lack of research investigating a causal relationship between treatment strategies and survival outcome, no definitive conclusions can be made regarding the effectiveness of the different treatments. Both the use of surgery, mainly prostatectomy and chemotherapy have been described. Currently there has only been a single case where the animal was alive at the time of the publication of the article. In all other cases, the animal died within a year, often due to euthanasia. Metastasis was often identified at the time of necropsy, usually to the lungs, but metastasis to other regions such as the pancreas and myocardium was also described. Some animals were also free of metastasis. On several occasions, there was infiltration of mediastinal lymph nodes.

FUTURE RESEARCH

The currently literature regarding feline prostatic neoplasms is very limited and suffers from a lack of internal validity. This precludes any conclusion regarding the general effectiveness of a specific treatment strategy. A first step in improving the knowledge of this condition is to collect information about cases of feline prostate cancer in a more systematic way. A larger collection of case reports and case series could provide better information about the epidemiology of the disease and current treatment practices.

In parallel, a multi-institutional retrospective study could also provide more insight into the epidemiology of the condition but could also investigate associations between treatment strategies utilized for feline prostatic neoplasms and survival outcomes. With a large retrospective study, the associations between the utilization of treatment strategies and post-treatment complications could be investigated. For example, it could be interesting to assess the association between prostatectomy and post-operative urinary incontinence in feline prostatic cancer patients.

Based on the current, limited evidence for any treatment strategy, it may not be justified to perform a prospective clinical trial at this point. It is currently not clear what treatment strategy should be tested in such a trial. Furthermore, due to the very limited incidence of the condition, a clinical trial may not be feasible due to the difficult of reaching a sufficient sample size.

CONCLUSION

The literature regarding feline prostate cancer is very sparse and consists mainly of single case reports.

This makes it difficult to make sound conclusions about the epidemiology and the effectiveness of the treatments strategies that are utilized. The literature suggests that the condition mainly affects older cats and that these tumours are usually (adeno)carcinomas. Feline prostatic cancer patients often suffer from haematuria, dysuria and inappetence. The current treatment strategies, surgery and/or chemotherapy, often fail to cure the condition. Further research is needed to establish causal links between treatments strategies and survival outcomes.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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Review article
Ανασκόπηση

Acute non-compressive nucleus pulposus extrusion in dogs and cats: An overview

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ABSTRACT: Acute non-compressive nucleus pulposus extrusion (ANNPE) is a common neurologic emergency and is characterized by a sudden extrusion of hydrated nondegenerated nucleus pulposus without or with minimal remaining spinal cord compression. It causes primarily spinal cord contusion and is often the consequence of intensive exercise or trauma. It is accompanied by a peracute onset and often but not always, by lateralization of spinal cord dysfunction. The T3-L3 spinal cord segments are mostly affected, resulting in paraparesis or paraplegia. Urinary and fecal incontinence can also be present. Neurologic manifestations do not deteriorate after the first 24 hours and then there is a progressive improvement or they remain static; that depends on the severity of the spinal cord injury. It has been mainly diagnosed in older non-chondrodystrophic large breed dogs, mostly in males but any sex and canine breed can be affected. On rare occasions cats can be affected as well. It concerns usually middle-aged, non-purebred and mostly male cats, that have experienced external spinal trauma. The onset is also peracute, spinal pain can be evident and the T3-L3 spinal cord segments are mostly affected, resulting in non-progressive paraparesis or paraplegia. Urinary and fecal incontinence are also possible. The diagnostic procedure and the treatment are similar in dogs and cats. The diagnosis is usually presumptive and is based on the medical history, the clinical presentation and the magnetic resonance (MRI) findings, which is the diagnostic modality of choice. There are several MRI criteria such as: an hyperintense lesion overlying an intervertebral disk, reduced volume of nucleus pulposus, extradural material or signal change and intervertebral disk space narrowing in T2 weighted images, that helps us to differentiate ANNPE from other myelopathies. The extruded nucleus pulposus can, rarely have an intradural, extra-/intramedullary detection. The ANNPE should be differentiated from other causes of acute myelopathy such as: ischemic myelopathy and fibrocartilagenous embolism, Hansen type I intervertebral disc disease (the compressive or the non-compressive type), vertebral fracture/luxation. Aortic thromboembolism, ischaemic myelopathy, fibrocartilaginous embolism, intervertebral disc extrusion, vertebral fractures/luxations are the main differentials in cats. A definitive diagnosis can only be achieved through histological examination, postmortem or after surgery. The treatment includes usually conservative management (cage rest, nursing care and physiotherapy), so a surgical exploration takes rarely place. The outcome of ANNPE in both dogs and cats is favorable, except the cases with loss of nociception and extended spinal cord injury.

***Keywords*:** Contusion, nucleus pulposus, extrusion, non-compressive myelopathy, MRI

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INTRODUCTION

Traumatic intervertebral disc extrusion (TIVDE) is a primary type of spinal cord injury that is typically occurred to dogs with underlying degeneration of the dorsal aspect of the annulus fibrosus.(Dewey and da Costa, 2016, Hansen, et al., 2017,) An external force causes the focal rupture of annulus fibrosus and the entrance with high velocity of nucleus pulposus material (either degenerative or hydrated) into the spinal canal. (Dewey and da Costa, 2016) In an interesting study, less than one third of the dogs (29%) with traumatic disc extrusion had spinal cord compression and that, was positively associated with chondrodystrophic breeds, older individuals and generalized intervertebral disc (IVD) degeneration. The authors proposed that preexisting IVD disease may predispose dogs to spinal cord compression subsequent to traumatic IVD extrusion. But, in contrast to that, in the majority of the dogs in the study (15 out of 22) that experienced non compressive nucleus extrusion, the latter was not degenerate. (Henke, et al., 2013) It is evident that in non-chondrodystrophic breeds of dog and in cats, that suffer from a traumatic disc extrusion, the herniated nucleus pulposus is often not mineralized but hydrated and therefore causes a primarily concussive injury, with little or no compression, the nowadays so called "acute non-compressive nucleus pulposus extrusion" (ANNPE) (Platt and Olby, 2004) It is also known as traumatic disc extrusion, high velocity low volume disc extrusion, dorsolateral intervertebral disc explosion, traumatic disc prolapse and Hansen type III intervertebral disc (IVD) disease. Although from all the terms used, the one that is best to be avoided, is Hansen type III, as Hansen described intervertebral disc degenerations (type I and type II) and this particular type of disc disease is non-degenerated. Moreover, Hansen was not the one that described it. (De Risio, 2015, Beltran, 2017) Information about this entity is scarce. A research in Pubmed based on words like: ANNPE (6 results for dogs and 4 for cats), traumatic disc extrusion (8 results for dogs and 1 for cats), high velocity low volume disc extrusion (only 2 results for dogs) and traumatic disc prolapse (12 results for dogs, 2 for cats) in dogs and cats, retrieves limited number of papers.

BIOMECHANISM

The combination of the healthy hydrated nucleus pulposus surrounded by a dense and fibrous annulus fibrosus enables the normal IVD to withstand marked variations of physiologic loading and biomechanical stress without resulting in structural failure. However, under specific circumstances, where the vertebral segment and IVD are subjected to supraphysiologic forces, for example during intense exercise or trauma, structural integrity may be disturbed. In such a case, a small cleft may occur in the complex lamellar structure of the annulus fibrosus, which has as a consequence, that the nondegenerate nucleus pulposus material will be extruded dorsally into the vertebral canal. (De Decker and Fenn, 2017) The fact that dogs with ANNPE tend to be older may be associated with age-related changes in the microstructure and biomechanics of the anulus fibrosus, such as alterations in collagen fiber cross-linking, decreases in water and proteoglycan content, and changes in interfiber cohesiveness. These changes may contribute to the separation of annular fibers and the development of annular clefts, providing potential pathways for extrusion of nuclear material when mechanical stress is applied to the spine. (Fenn, et al.,2016b, Hansen, et al., 2017) Dewey W. C., and da Costa C. R support, that the degeneration of the thinner dorsal aspect of the annulus fibrosus, that may be occurred through the years, make the fibrous lamellae less resistant to a sudden increase of intradiscal pressure. (Dewey and da Costa, 2016) The nuclear material is hypothesized to extrude with great force, causing a focal contusive injury and sometimes significant bleeding to the ventral or lateral portion of the spinal cord. As the extradural material may be nondegenerate and, highly hydrated (consists of 80% to 88% water, Henke, et al., 2013), it typically rapidly dissipates or is resorbed, leaving minimal to no spinal cord compression. Postmortem findings of small clefts in the dorsal annulus, as well as extradural nondegenerate nucleus pulposus material in the vertebral canal support this scenario. The nearby region of the spinal cord may present evidence of focal contusive injury, hemorrhage, and necrosis. (Bergknut, et al., 2013, De Decker and Fenn, 2017) The initial mechanical injury (primary spinal cord injury) may cause relatively little damage in itself, but it triggers the secondary mechanism of injury which augments the severity of damage to the spinal cord. (Jeffery and Blakemore, 1999, Dewey and da Costa , 2016) A spontaneous recovery is possible. It is more often observed in animals with moderate-to-severe rather than 'complete' injuries and it can be noticed in conservatively or placebo-treated clinical spinal cord injuries. It occurs, either as the result of the reversal of reversible lesions, such as the restoration of normal intra- and extracellular concentrations of ions

or the removal of compression, if existed, allowing the restoration of normal white matter conduction, and usually starts quite soon after the injury occurred, that is within about 14 days, or through the process of 'plasticity', which consists of the reorganization of surviving CNS neurons and can continue over a period of weeks or months. (Jeffery and Blakemore, 1999, Freeman and Jeffery, 2016)

CLINICAL PRESENTATION

ANNPE is characterized by peracute onset, often lateralized (60% up to 90% of the affected dogs) paresis or paralysis of one to four limbs, depending on the location of the affected spinal cord segment and the extent of the lesion. The clinical signs usually occur suddenly after vigorous exercise or traumatic injury. After an initial short period of deterioration (within the first 24 hours), the clinical status of affected dogs may stabilize or even improve, without specific treatment. The clinical findings are pretty similar to those of ischemic myelopathy (IM). Recent studies had shown that the most common detection of the lesion is located at T3-L3 spinal cord segments, and more specifically the T12-T13, T13-L1 and L1-L2 IVD spaces are most commonly affected, whereas IM is more often at L4-S3 spinal cord segments.(Feen, et al.,2016b, De Decker and Fenn, 2017). Nociception may be present or not and spinal shock is also possible. (Mari, et al., 2019, Cardy, et al., 2015, Feen, et al.,2016 b, Mari, et al., 2017, Full, et al., 2015) There are also cases, where a lesion at C6-T2 spinal cord segments has been reported or in other studies a lesion at C1-C5 spinal cord segments is reported. In addition, despite the fact that any breed can be affected, older large breed dogs are mostly concerned. A breed predisposition has been also referred, with Border Collies and sighthounds (Greyhounds and Whippets) being overrepresented. (Feen, et al.,2016b, Beltran, 2017, De Decker and Fenn, 2017) The exact reason for those predispositions is unknown. It is noteworthy though, that these breeds represent very active and athletic dogs. On the contrary, English Staffordshire bull terriers seem to have predisposition to IM. (Fenn J., Drees R., et al., 2016) It is also important to be mentioned that male dogs are more frequently affected than female dogs. (Feen, et al., 2016, Beltran, 2017,Mari, et al., 2017, De Decker, Fenn, 2017, Mari, et al., 2019) Dogs with ANNPE are significantly older (7.0 ± 2.2 years old) compared to those with ischemic myelopathy (5.9 ± 2.8 years old) and were more likely to have a history of vocalization (62% of the affected dogs) at the onset of clinical signs. Moreover, they may have spinal hyperesthesia (48% of the affected dogs) during the initial examination; this condition is not accompanied by severe or sustained spinal pain, though. (Cardy, et al., 2015, Feen, et al., 2016, Beltran, 2017, De Decker and Fenn, 2017) The fecal incontinence was considered to be another typical clinical finding in dogs with ANNPE compared to those with IM. According to another study though, this opinion seems to be controversial. Fenn J. and his colleagues support that loss of inhibitory upper motor neuron pathways to rectal reflexes, and reduced voluntary control of the external anal sphincter, are more common to IM because ischemic lesions typically affect the central and dorsal portions of the spinal cord, potentially damaging the rectal sensory tracts, whereas ANNPE lesions generally cause contusive damage to the ventral or lateral portions of the spinal cord. They mention as well, that the presence of a lower motor neuron lesion at L4-S3 spinal cord segments that is more frequently met in IM, was not associated with an increased risk of fecal incontinence. (Fenn, et al., 2016b) Some other symptoms that may also be present, are urinary incontinence, and in severe cervical myelopathies, although rare, dysfunction of the respiratory system. (Fenn, et al., 2016b, De Decker, Fenn, 2017)

It is also possible that the extruded hydrated nucleus pulposus has an intradural-extramedullary or intramedullary detection. It is a rare type of intervertebral disk herniation and the number of the published studies in the veterinary literature is limited. (McKee and Downes, 2008, Kent, et al., 2010, Tamura, et al. 2014) The intramedullary nuclear material can be non-degenerated, as it is in the case of ANNPE, or degenerated, as a result of Hansen type I IVD related disease. According to some recent studies, the affected dogs were severely paraparetic or paraplegic and there was lateralization of the signs, that was notable with small differences in motor function or nociception between the two pelvic limbs. (De Risio, 2015)

DIAGNOSTIC PROCEDURE AND DIFFERENTIAL DIAGNOSIS

Differential diagnosis of a paresis/paralysis, following an acute-peracute onset, either after an external trauma or not, includes (Cardy, etal., 2015, De Decker and Fenn, 2017).:1) Acute non compressive nucleus pulposus extrusion (ANNPE), 2) Ischemic myelopathy (IM) or Fibrocartilagenous Embolism(FCE), 3) Hasnen type I IVD (compressive or non-compressive) disease, 4) Vertebral fracture or luxation, 5) Hydrated

nucleus pulposus extrusion (a kind of IVD herniation with predilection for the cervical region) (HNPE).

The MRI is considered to be the gold standard for the diagnosis of ANNPE. (De Decker and Fenn, 2017, Ros, et al., 2017) If MRI is not available or if the owners cannot support this type of examination due to financial reasons, myelography can be proved quiet helpful as well, especially for excluding, acute, compressive mostly, myelopathies. (Ros, et al., 2017) According to several surveys, there are some MRI findings that support a ANNPE diagnosis: (Henke, et al., 2013, Mari, 2017, Fenn, et al., 2016a, De Decker, Fenn, 2017, Trampus, et al., 2018)1) a focal T2-weighted (T2W) intramedullary hyperintensity (IH) overlying a narrowed intervertebral disc. The area of IH on T2-weighted images is often lateralized and it is generally less than one vertebra (2nd lumbar) in length. This has also prognostic role. (Mari, et al., 2019), 2) reduced volume of nucleus pulposus, 3) extradural material compatible with hydrated nucleus pulposus, causing no spinal cord compression (SCC) or compression less than 10 per cent (Ros, et al., 2017), 4) a non-longitudinal directional pattern of the T2W IH originating from an intervertebral disc, often lateralized. We can measure the area of maximal IH (cross-sectional area) and compare it to the area of the normal spinal cord at the same level, on transverse T2W images. If this ratio is greater than 40%, it has a positive association with fecal and urinary incontinence, according to a study. (Mari, et al., 2019), 5) a cleft in the dorsal part of the annulus fibrosus and 6) meningeal/epidural contrast enhancement in post-contrast T1-weighted (T1W) fat-suppressed images. (Mari, et al., 2017)

According to a study of Fenn J and his colleagues, the following from the aforementioned criteria are those with the strongest inter and intraobserver agreement: (Fenn, et al., 2016a)

- a hyperintense lesion overlying an intervertebral disk,
- reduced volume of nucleus puplosus,
- extradural material or signal change and
- intervertebral disk space narrowing.

On the other side, MRI diagnostic criteria for IM include a focal, well demarcated intramedullary T2W hyperintense lesion, mainly affecting grey matter, with the absence of the above mentioned criteria used for ANNPE diagnosis. (Fenn, et al., 2016a, Mari, et al., 2017)

In the case of intradural and extra-/intramedullar intervertebral disk herniation, the MRI pattern is pretty similar to the one in extramedullar ANNPE.(De Risio L., 2015) De Risio L. and her colleagues noticed additionally, a linear tract (predominantly hyperintense on T2-weighted images, iso- to hypointense on T1-weighted images and hypointense on T2*-weighted images) extending from the intervertebral disk into the spinal cord parenchyma. This finding has been suggested from the authors to be specific for intramedullary nucleus pulposus extrusion. The sample though was small, as it has been reported only in two dogs and two cats, to be considered as a gold standard. CT myelography may be more sensitive for diagnosing the intradural, extra-/intramedullar nucleus extrusion, particularly when the nucleus pulposus is detected intradurally extra- /intramedullary, as it shows focal accumulation of iodinated contrast medium within the subarachnoid space and/or within the spinal cord parenchyma and maybe a filling deficit in the center of it, the so called “golf tee sign”. (Tamura, et al. 2014). Furthermore, it helps with the identification of nucleal material in the dilated subarachnoid space, it allows differentiation between hydrated and calcified nucleus pulposus material and differentiation from hemorrhage. In CT myelography or myelography, it is also possible to detect an extradural leakage of iodinated contrast medium, suggestive of a dural tear. (De Risio L., 2015) That was also supported by a report, where communication between subarachnoid space and intervertebral disk space after traction views in myelography, was detected. (McKee, Downes, 2008)

Myelography may reveal a small, focal extradural lesion overlying an intervertebral disk, with an adjacent intramedullary pattern because of focal spinal cord swelling. It can also be used to exclude compressive spinal conditions, such as Hansen type I IVD extrusion. However, it will not allow accurate differentiation between ANNPE and other causes of intramedullary lesions such as IM. (De Decker and Fenn, 2017)

Plain radiography and computed tomography do not allow an accurate diagnosis of ANNPE. They can only exclude the causes of the differential diagnosis that demand urgent surgical intervention. More precisely, CT is a good option for excluding Hansen type I intervertebral disc extrusion and is the diagnostic imaging modality of choice for excluding vertebral fractures and subluxations. (De Decker and Fenn , 2017)

ANNPE is differentiated from Hansen type I IVD disease, according to the following clinical findings: Hansen type I IVD disease has usually an acute instead of peracute onset and the clinical signs often present a deterioration after the first 24 hours of the onset. The spinal hyperesthesia is more common to these animals and the clinical findings are usually bilateral and not so obviously lateralized as in ANNPE. The diagnostic imaging plays the most important role to the differentiation. (Cardy, et al., 2015, De Decker, Fenn, 2017) When cervical myelopathy is the presenting complain, the clinician should include in the differential diagnosis the compressive hydrated nucleus pulposus extrusion (HNPE), a type of minimal to non-degenerate nucleus pulposus extrusion, with acute onset (<24h), which can be in various degrees compressive. In the contrary to ANNPE, the onset of the clinical signs is rarely associated with intense physical exercise or trauma. The neurological deficits are usually symmetric. Some MRI criteria that help to distinguish this disease: (De Decker and Fenn, 2017) 1) Ventral, midline, extradural compressive material, homogenous, hyperintense on T2W images and the nucleus pulposus is isointense to normal in all sequences, nondegenerate and lies immediately dorsal to the affected IVD. 2) The characteristic bilobed or "seagull" appearance of the compressive material could be explained by the location of the compressive material ventral to the apparent intact dorsal longitudinal ligament. 3) The affected IVD space is narrowed, the volume of nucleus pulposus is reduced and the dorsal aspect of annulus fibrosus is ill-defined. 4) Focal intraparenchymal hyperintensity can be detected in the overlying spinal cord, suggestive of spinal cord contusion, and the extruded material can demonstrate variable degrees of contrast enhancement. Post contrast enchanced CT can also provide some useful information regarding differentiation of Hansen type I IVD extrusion and HNPE. (De Decker and Fenn, 2017, Nessler, et al., 2018)

All the above mentioned parameters can help us to form a presumptive diagnosis. However a definitive diagnosis can only be achieved through histologic examination of extruded non-degenerate nucleus pulposus material from the vertebral canal, either postmortem or during surgery. (Feen, et al., 2016b, De Decker and Fenn, 2017,)

TREATMENT

The treatment of ANNPE involves usually conservative management (nursing care and activity restriction) as there are no efficient neuroprotective treatments That includes: Restricted activity (cage rest or walk with short lead), for a 4 to 6 week period. (De Risio, 2015, De Decker and Fenn, 2017) Nursing care, which consists of: 1) Manual bladder expression or urinary catheterization, if urinary incontinence is suspected (Henke, et al., 2013, De Decker and Fenn, 2017) In some clinics catheterization is a standard procedure in nonambulatory, severely paraparetic or paraplegic dogs independent of their urinary function. (Mari, et al., 2019). 2) Monitoring and management of respiratory dysfunction in severe cervical myelopathies, which includes turning recumbent patients every 4 hours to avoid lung atelectasis or accumulations of secretions (De Decker and Fenn, 2017). 3) Due to prolonged recumbency we can have the following consequences: urine scald, pressure sores, and decubital ulcers. To prevent this from happening we could turn the recumbent patient every 4 hours and keep him/her on a dry and soft place. (De Decker and Fenn, 2017). 4) Nutritional support to conserve body condition and sustain physical rehabilitation (De Decker and Fenn, 2017). 5) Physical rehabilitation. That is an important supportive method for the recovery of patients with spinal cord injuries that enjoys increasingly recognition. The severity of neurologic dysfunction will will determine the requirements of physical therapy. Its purpose is practically to retain joint range of motion, to eliminate muscle atrophy, and to prevent patient´s discomfort. (Henke, Gorgas, et al., 2013, De Risio L., 2015, De Decker and Fenn, 2017) The physiotherapy program must fit to the restrictive activity if the latter is indicated.(Thomas et al., 2014) 6) Underwater treadmill training or swimming can be also helpful during the recovery period. (Henke, et al., 2013)

In case of spinal hyperesthesia (48% to 57% of the patients), analgesia may be needed for the first few days. (De Decker and Fenn, 2017) Anti-inflammatory medications, such as steroids at anti-inflammatory doses or nonsteroidal anti-inflammatory drugs, can be administered initially for pain relief, but it should be mentioned that these medications do not have any significant protective effect on the spinal cord. (Dewey and da Costa, 2016, Mari, et al., 2019)

When the patient has difficulties with micturition, medication including (diazepam, prazosin, and phenoxybenzamine) may also be usefull, aiming to reduction of the internal/ external urethral sphincter tone. (Sharp and Wheeler 2005, Mari, et al., 2019).

Surgical procedure may be considered in some

cases, especially when the presumptive diagnosis is intradural/extramedullary nucleus pulposus extrusion and may result in good outcome and prognosis. (Sanders, et al., 2002, Liptak, et all., 2002, Kent, et al., 2010) Moreover, in some cases, surgery is performed as exploratory procedure as there is not yet a diagnostic procedure capable to estimate the volume of intradural/extramedullary or intramedullary extruded intervertebral disk material and to associate it with the degree of spinal cord compression. (De Risio, 2015) Further investigation should take place, in order evidence based guidelines, that would suggest in which cases the surgical procedure would be profitable, to be developed. (De Risio, 2015) Obviously, in case of compressive TIVDE a surgical decompression, through hemilaminectomy or ventral slot, according to the detection of the extrusion, is applied. (Henke, et al., 2013)

OUTCOME

The prognosis for ANNPE is generally favorable if the nociception is present. (Platt and Olby, 2004, Dewey and da Costa, 2016,) Almost the three quarters of the affected dogs recover successfully. Severity of neurologic dysfunction, which can be presented as non-ambulatory para-/tetraparesis or para-/tetraplegia and especially when combined with absent nociception, are negative prognostic factors for both the ambulation and incontinence. (Platt and Olby, 2004) There are several studies, for example the one by Lorenzo Mari and his colleagues that support it. To this study the successful outcome was defined as the ability of unassisted ambulation and complete urinary and fecal continence. It's worth saying that plegic dogs it's hard to regain complete normal gait. (Mari, et al., 2017, De Decker and Fenn, 2017) Although there is a debate to if patients with ANNPE or FCI are more prone to develop fecal incontinence, the vast majority regain continence in the long-term. (Fenn, et al., 2016, Mari, et al., 2017) Some MRI findings such as a lesion affecting a large area of the spinal cord, a large cross-sectional area of the lesion or a hypointense intramedullary signal are negative prognostic factors. (Mari, et al., 2017). Moreover, intramedullary lesions accompany often a poor outcome. There are some limited case reports though, supporting that with the appropriate decompression and management, a successful outcome may be achieved. (Sanders, et al., 2002) There are also three case reports by T. G. Yarrow and N. D. Jeffery, which support that the prognosis after dural laceration following a peracute spinal cord injury appears reasonably good, but some persistent loss of function usually remains. Therefore, diagnosis of dural laceration should not automatically lead us to a poor diagnosis. (Yarrow and Jeffery,2000)

AND NOW WHAT ABOUT CATS?

INTRODUCTION-CLINICAL PRESENTATION

The ANNPE is not well described in cats. However, it is tended to be considered that the pathophysiology, the clinical presentation and the case management is similar to the one in dogs. (Dewey and da Costa, 2016) There are only four case reports (Lu, et al., 2001, McConnel and Garosi, 2004, Chow, et al., 2012, Adams, et al., 2014) and a retrospective study (Taylor-Brown, De Decker, 2015) to our knowledge. The study of Taylor-Brown and De Decker, (2015) analyzes the management and the outcome of 11 cats with presumptive ANNPE. Seven were domestic shorthair cats, followed by three domestic longhair, an Egyptian Mau and a British Shorthair. The median age was 7 years old and the most of them were male neutered. All of them had a peracute onset of clinical signs, with the three quarters experiencing witnessed or suspected external trauma, such as a road-traffic accident or fall from height. The affected spinal cord segments were mostly the T3-L3 (7/11).The clinical signs were non-progressive. Three of the cats in this study had no deep pain sensation. Some of them presented urinary and fecal incontinence. Interesting is that on the contrary to dogs, cats most often are presented with symmetric instead of lateralized clinical signs. Five out of the 11 cats presented spinal hyperesthesia in this study. (Taylor-Brown and De Decker, 2015). A 90% of them were presented with paraplegia or non ambulatory paraparesis. (Taylor-Brown, De Decker, 2015). According to another report, obesity may predispose to ANNPE in cats. (Chow, et al., 2012)

DIAGNOSTIC PROCEDURE AND DIFFERENTIAL DIAGNOSIS

The differential diagnoses for cats presented with an acute or peracute onset of paresis or plegia includes: aortic thromboembolism, ischemic myelopathy, fibrocartilaginous embolism (FCE), intervertebral disc extrusion, and vertebral fractures and luxations. (Taylor-Brown and De Decker, 2015, Mella, et al., 2019) Vertebral fractures and luxations are generally considered the most important differential diagnosis for cats presenting with a peracute onset of spinal cord dysfunction after a witnessed or suspect-

ed traumatic event. (Taylor-Brown and De Decker, 2015) According to signalment, ischemic myelopathy occurs most often in older cats with a stable or improving, non-painful, lateralizing, C6–T2 myelopathy, IVD disease most often occurs in middle aged, purebred cats, with a normal general physical examination and an acute onset of painful and progressive clinical signs, spinal fracture/luxation occurred mostly in younger cats, leading most often in a peracute onset, of a painful, non-ambulatory neurological status. (Mella, et al., 2019). MRI revealed ANNPE findings as defined for the dog as well (Chow, et al., 2012, Taylor-Brown and De Decker, 2015): 1) a reduction in volume of the T2W hyperintensity of the nucleus pulposus signal, 2) very focal, T2W hyperintense intramedullary lesions involving both grey and white matter regions, within the spinal cord overlying an intervertebral disc space (Taylor-Brown and De Decker, 2015), which may help us to differentiate it from FCE, because MRI findings suggesting FCE: affect grey matter mainly, (Lesion‘ s Length: L2) ratio is greater, and the lesion is often lateralized to correspond with the vascular supply to the spinal cord, (Chow, et al., 2012) 3) mild narrowing of the intervertebral disc space, and (Taylor-Brown and De Decker, 2015), 4) extraneous material or signal change within the vertebral canal with absent or minimal spinal cord compression. (Taylor-Brown and De Decker, 2015) Moreover, in five of the 11 cats from the study above mentioned (Taylor-Brown and De Decker, 2015), there was also evidence of ill-defined T2-weighted hyperintensity within the epaxial musculature compared with surrounding muscle suggestive of contusion, hemorrhage or oedema. Definitive diagnosis, as also referred for dogs requires histopathological identification of nucleus pulposus material within the spinal canal, which can be achieved either post mortem or during surgery. (Chow, et al., 2012)

TREATMENT

The treatment is based on supportive care, which includes: 1) Cage rest (Chow, et al., 2012). 2) Physiotherapy (massage, passive range of motion exercises, assisted standing and exercises to develop strength and coordination). 3) In case of spinal hyperesthesia analgetic medication for pain relief can be profitable. That includes opioids, non-steroidal anti-inflammatory drugs and gabapentin. 4) Bladder management: manual bladder expression, indwelling catheter placement or intermittent catheterization. 5) A sympatholytic medication (here prazosin, an a1-blocker) can also be proved quite helpful in bladder management. (Taylor-Brown and De Decker, 2015)

OUTCOME

The outcome is generally good, with almost 90% of the affected cats returning to ambulation with urinary and fecal continence, except one cat that in the long term had permanent urinary and intermittent fecal incontinence according to Taylor-Brown and her colleagues. All the cats that were initially non-ambulatory regained ambulation within a median time of 17 days (range 6–21 days). However none of them had become completely neurologically normal. There is also a case report which supports this outcome. (Chow, et al., 2012) There was no sign of further improvement after the first 6 months. (Taylor-Brown and De Decker, 2015)

CONFLICT OF INTEREST

None declared by the authors.

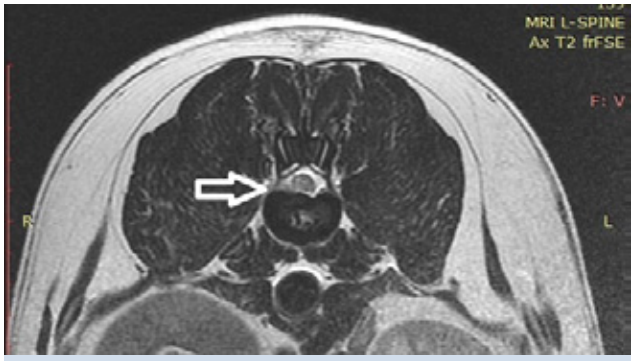

Fig. 1: Sagittal T2W image of the spinal cord of a 7yo acutely paralyzed Cane Corso dog. There is reduced nucleus volume of the L1-L2 intervertebral disc and focal intramedullary hyperintensity (arrow) over the L1-L2 disc space

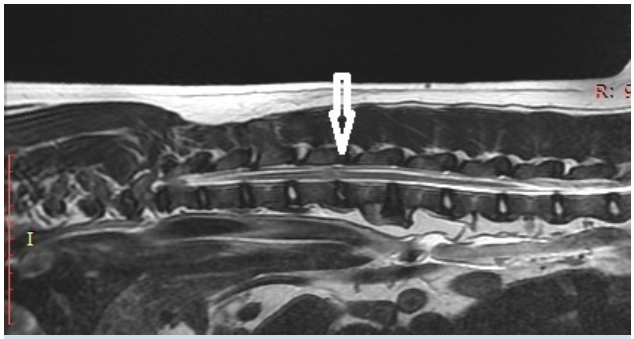

Fig 2: Axial T2W image of the spinal cord of the same dog. The axial image shows mild spinal cord compression by extradural material (arrow) compatible with hydrated nucleus pulposus at the same level L1-L2

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Research article
Ερευνητικό άρθρο

Phenotypic and Molecular Characterization of *Vibrio* Species Isolated from Fish markets in Egypt

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ABSTRACT: Vibriosis is considered a worldwide, threatening bacterial disease that affects mariculture, with high mortalities and severe economic losses**.** Some *Vibrio* species have been frequently involved in outbreaks of food-borne diseases worldwide. The Genus *Vibrio* includes over eighty-five species present in marine and natural habitats of seawater, and the species are widely distributed throughout the world. This work aimed to isolate *Vibrio* species from different markets in Kafr EL-Sheikh Governorate, representing 40% of Egypt's fish production. Samples of Nile tilapia, (*Oreochromis niloticus*), grey mullet (*Mugil cephalus*) and African sharptooth catfish (*Clarias gariepinus*) were collected alive and examined for *Vibrio* species. Isolation and identification of *Vibrio* species were made using colonial morphology and biochemical characteristics, then confirmed using 16S rRNA gene-specific for the genus *Vibrio* and multiplex PCR using species-specific primers. 52 (34. 6%) *Vibrio* isolates were obtained from examined fishes. The highest incidence of *Vibrio* species was detected in *C. gariepinus* (64%), followed by *M. cephalus* (36%) and then *O. niloticus* (24%). In the case of *C. gariepinus*, *V. alginolyticus* was the most predominant species (32%), followed by *V. fluvialis* (12%), *V. cholerae*, *V. parahaemolyticus* (8%), and *V. splendidus* (4%). In the case of *O. niloticus*, the predominant *Vibrio* species were *V. alginolyticus* (12%), followed by *V. parahaemolyticus* (5. 33%), *V. cholerae* (4%), and then *V. splendidus* and *anguillarum* (1. 33%). In *M. cephalus*, *V. alginolyticus* also wasthe predominant species (14%), followed by *V. cholerae* (12%), *V. parahaemolyticus, V. fluvialis* (2%), and *V. splendidus* (2 %). *V. alginolyticus*, *V. cholerae,* and *V. parahaemolyticus* were found to produce PCR products of 737, 304, and 897 bp, respectively. This study highlights the incidence of *Vibrio* species in fish in Egypt.

Keywords: fish, PCR, *Vibrio* species

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INTRODUCTION

Mariculture represents a significant investment for fishers in Egypt; however, the diseases and the high cost of feeding are the main obstacles affectingthis sector's sustainability and profitability (Abdelaziz *et al.,* 2017). Aquaculture considers the fastest growing sector of high protein resources and contributes to economic development and social stability worldwide, promoting nutritional standards and relieving poverty in some developing countries (Béné *et al.,* 2015; FAO 2015). The genus *Vibrio* comprises aquatic microbes that usually live in coastal and estuarine water bodies (Ghenem *et al*., 2017). *Vibrios* are Gram-negative, halophilic, pathogenic bacteria that are a straight or curved rod shape, motile via a single polar flagellum, and negatively impact aquatic ecosystems and human health (Morris and Black 2015; Luan *et al.,* 2007; Lee *et al.,* 2015). *Vibrio* is strongly correlated with high salinity (30-35 ppt), parasitic infestation, high temperature, and mechanical injuries. These factors suppress the immunity and increase fish's susceptibility to vibriosis (Nagasawa and Cruz-Lacierda 2004; Haenen *et al.,* 2014; El-Bouhy *et al.,* 2016). These bacteria are ubiquitous in these environments as they have been isolated from seawater, fish, and shellfish (Alonzo *et al.,* 2017). *Vibrio* species in the aquaculture industry are serious opportunistic pathogens to cultured hosts such as finfish, shrimp, and shellfish (Liu *et al.,* 2016). Most of the aquatic diseases are caused by *Vibrio* species, which leads to a significant problem for the development of aquaculture with great economic losses worldwide because of its high morbidity and mortality rates (mortality ≥ 50%) (Al-Taee1 *et al.,* 2017). Several *Vibrio* species have been concerned with the health problems in marine animals. A recent report showed that *V. alginolyticus, V. harveyi, V. parahaemolyticus, V. owensii,* and *V. campbelli* are the most common species infecting farmed aquatic animals (Nor-Amalina *et al.,* 2017). Among *Vibrio* species, *V. anguillarum*, *V. parahaemolyticus,* and *V. vulnificus* are the main pathogenic species found in saltwater, while *V. cholerae* and *V. mimicus* are the main ones found in freshwater culture (Fouz*et al.,* 2002). Identification of *Vibrio* species is based mainly on their morphological, physiological, and biochemical characteristics (Alsina and Blanch 1994a, b). Traditional food analysis for microorganism's presence relies on the growth of bacteria in artificial media; also, culture techniques are often time-consuming and unreliable. Polymerase chain reaction (PCR) procedures are rapid and highly specific for detecting many pathogens (Anzar and Alarcon 2008).

In Egypt, fish are popular seafood; therefore, they are consumed in high quantities. This study was carried out to investigate *Vibrio* species 's presence in *Oreochromis niloticus, Mugil cephalus,* and *Clarias gariepinus* during the winter and spring seasons. It causes high economic losses, may threaten seafood safety, and increase the risk of illness in people who consume raw fish. This study also shows how to differentiate between different *Vibrio* species using conventional (cultivation and biochemical identification) and molecular methods. 16SrRNA gene was used as a housekeeping gene for the detection of genus *Vibrio*. Multiplex PCR using species-specific primers adopted further molecular identification.

MATERIALS AND METHODS

Samples

Samples of seventy-five Nile tilapia (*Oreochromis niloticus*), fifty grey mullet (*Mugil cephalus*) and twenty-five African sharptooth catfish (*Clarias gariepinus*) were collected from different markets in the Kafr El-Sheikh Governorate, Egypt, from January to May 2018 and transported immediately to the Department of Microbiology, Animal Health Research Institute in Kafr El-Sheikh Governorate. Fish were examined clinically for any abnormalities, including hemorrhages, skin ulceration, fin erosion, and abdominal distention. Bacteriological isolation of *Vibrio* species was performed from samples of the kidney, heart, liver, gills, and skin (Noga, 2010).

Ethical approval

All fish handling was conducted under the guidelines for the care and use of animals for scientific purposes established by the Ethics Committee of the Faculty of Veterinary Medicine, Kafr El-Sheikh University, Egypt.

Isolation and identification of *Vibrio* species

Primary isolation was made from internal organs (Alapide-Tendencia *et al.,* 1997) on trypticase soya broth with 3% NaCL that was incubated at 30°C for 24 hours. Then a loopful streaked on the TCBS media. Plates were incubated at 30°C for 24 hours and examined for the presence of typical colonies of *Vibrio* species. The colonies were examined for morphological characterizations, such as shape, Gram stain, and motility. Biochemical characterization was carried out us-

ing the following tests; oxidase, string test, triple sugar iron, arginine hydrolysis, indole production, methyl red, Voges-Proskauer, citrate utilization, urease, hydrogen sulfide production, nitrate reduction, gelatin liquefaction, ornithine decarboxylase, L-lysine decarboxylase, arginine decarboxylase, β-galactosidase (ONPG), salt tolerance and sensitivity to vibriostatic agent O/129 (Elliot *et al.,* 1995; Austin and Austin 1987).

Molecular identification

Molecular identification was applied to confirm 10 *Vibrio* isolates previously identified by phenotypic and biochemical characteristics.

The material used for DNA extraction

1. QIAamp DNA Mini Kit Catalog no. 51304

The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification for different types of samples. The spin-column procedure does not require mechanical homogenization; therefore, the total hands-on preparation time is only 20 minutes.

2. Ethanol 96% (Applichem)

Equipment and apparatuses used for the extraction of nucleic acids

Extraction of nucleic acids was performed using 1. 5 ml Eppendorf tubes, 20-200 µl mono- channel micropipettes, 100--1000 µl (Biohit), 200 µl and 1000 µl sterile filter tips, a centrifuge (Sigma sartorius), and a type II-A biosafety cabinet (Thermo).

PCR Master Mix used for cPCR

Emerald Amp GT PCR mastermix (Takara) Code No. RR310A

It contains Emerald Amp GT PCR mastermix (2x premix) and PCR grade water.

DNA extraction

DNA extraction from samples was performed using the QIAamp DNA Mini Kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of a lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. Then the sample was washed and centrifuged, as suggested by the manufacturer. Nucleic acid was eluted with 100 µl of the elution buffer provided in the kit.

PCR amplification

Primers were used in a 25-µl reaction containing:

12. 5 µl of EmeraldAmp Max PCR Master Mix **(Takara, Japan)**

1 µl of each primer of 20 pmol

4. 5 µl of water

6 µl of the DNA template

The reaction was carried out in an applied biosystem 2720 thermal cycler.

Detection of *Vibrio* 16srRNA gene (universal primers) and species-specific primers

Ten isolates suspected to be *Vibrio* species were molecularly confirmed, using primers targeting 663 bp of the 16S rRNA gene-specific for genus *Vibrio*. Then, multiplex PCR targeting 737 bp of *Collagenase* gene-specific for *V. alginolyticus*, 304 bp of the *omp*W gene-specific for *V. cholerae*, 410 bp of the *Hsp60* gene-specific for *V. vulnificus*, 121 bp of the *sodB* gene-specific for *V. mimicus*, and 897 bp of the *flaE* gene-specific for *V. parahaemolyticus* as shown in Table (1).

The amplification conditions were; 5 min of primary denaturation at 94ºC, 35 cycles of secondary denaturation for 30 sec at 94ºC, annealing (16S rRNA PCR: 40 sec at 56ºC; typing PCR: 1 min at 57ºC), and extension at 72ºC for 45 sec (16S rRNA and 1 min for typing PCR. A final extension was adjusted for 10 min. The Biotechnology Unit supplied the positive controls, Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt. The amplified products were resolved by electrophoresis in 1. 5% agarose gel (Applichem, Germany, GmbH), and a gene ruler 1 Kb plus DNA Ladder (Fermentas, Thermo Scientific, Germany) determined the sizes of the amplified product. Photographing was done bythe gel documentation system (Alpha Innotech, Biomedia).

Table 1: Oligonucleotide primers sequences
Source: Metabion (Germany).

Target	Gene	Sequence	Amplified product	Reference
Vibrio spp.	*16SrRNA*	CGGTGAAATGCGTAGAGAT TTACTAGCGATTCCGAGTTC	663 bp	**Tarr*et al*., 2007**
V. mimicus	*sodB*	CAT TCG GTT CTT TCG CTG AT GAA GTG TTA GTG ATT GCT AGA GAT	121 bp	
V. parahaemolyticus	*flaE*	GCA GCT GAT CAA AAC GTT GAG T ATT ATC GAT CGT GCC ACT CAC	897 bp	
V. vulnificus	*Hsp60*	GTC TTA AAG CGG TTG CTG C CGC TTC AAG TGC TGG TAG AAG	410 bp	
V. alginolyticus	*Collagenase*	CGAGTACAGTCACTTGAAAGCC CACAACAGAACTCGCGTTACC	737 bp	**Abu-Elala*et al*., 2016**
V. cholerae	*ompW*	caccaagaaggtgactttattgtg ggtttgtcgaat tag cttcac c	304 bp	**De Menezes et al., 2014**

Table 2: Prevalence of *Vibrio* species isolated from the examined fishes.

Recovered isolates	***Oreochromis niloticus* (75)**		***Mugil cephalus* (50)**		***Clarias gariepinus* (25)**		**Total (150)**	
	NO	%	NO	%	NO	%	NO	%
V. alginolyticus	9	12	7	14	8	32	24	16
V. cholerae	3	4	6	12	2	8	11	7.33
V. parahaemolyticus	4	5.33	2	4	2	8	8	5.33
V. fluvialis	0	0	2	4	3	12	5	3.33
V. splendidus	1	1.33	1	2	1	4	3	2
V. anguillarum	1	1.33	0	0	0	0	1	0.66
Total	18	24	18	36	16	64	52	34.6

Analysis of the PCR Products.

The PCR products were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using 5V/cm. For gel analysis, 40 µl of the products were loaded in each gel slot. A generuler 100 bp ladder (Fermentas, Thermo, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and the data were analyzed through computer software.

RESULTS

In this study, one hundred and fifty samples of fish (75 *O. niloticus*, 50 *M. cephalus*, and 25 *C. gariepinus*) were collected and subjected to a full bacteriological investigation. Samples were identified by conventional microbiological methods using selective media and specific biochemical reactions. As shown in Table 2, the highest incidence of *Vibrio* species was detected in *Clarias gariepinus* (64%), followed by *Mugil cephalus* (36%) and then *Oreochromis niloticus* (24%). *V. alginolyticus* was the most predominant species in *Oreochromis niloticus, Mugil cephalus, and Clarias gariepinus*. The result of *Clarias gariepinus* revealed that *V. alginolyticus* was the most predominant species (32%), followed by *V. fluvialis* (12%), *V. cholera*, *V. parahaemolyticus* (8%), and *V. splendidus* (4%). In the case of *Oreochromis niloticus*, the predominant *Vibrio* species were *V. alginolyticus* (12%), followed by V. parahaemolyticus (5. 33%), *V. cholerae* (4%), and then *V. splendidus* and *anguillarum* (1. 33%). In *Mugil cephalus*, *V. alginolyticus* also was the predominant species (14%), followed by *V. cholerae* (12%), *V. parahaemolyticus*, *V. fluvialis* (2%), and *V. splendidus* (2%).

There were two typical morphologies for colonies of *Vibrio* species on TCBS agar. Typical colonies of *V. alginolyticus, V. cholerae, V. anguillarum,* and *V. fluvialis* were smooth, yellow (sucrose positive), and 2-3mm in diameter, while *V. parahaemolyticus* and *V. splendidus* were smooth, green (sucrose negative). On the microscopic examination, all the selected colonies revealed Gram-negative comma-shaped (curved) bacilli, motile by single polar flagella, non-spore former, and non-capsulated.

Table 3: Biochemical characteristics of different *Vibrio* strains

	V. alginolyticus	*V. cholerae*	*V. parahaemolyticus*	*V. fluvialis*	*V. splendidus*	*V. anguillarum*
TCBS agar	Y	Y	G	Y	G	Y
Growth in						
0% Nacl	-	+	-	-	-	-
3% Nacl	+	+	+	+	+	+
6% Nacl	+	-	+	+	+	+
8% Nacl	+	-	+	v	+	-
10% Nacl	+	-	-	-	-	-
Arginine dihydrolase	-	-	-	+	+	+
Ornithine decarboxylase	+	+	+	-	-	-
Lysine decarboxylase	+	+	+	-	-	-
Catalase	+	+	+	+	+	+
Citrate	+	+	+	+	+	+
Indole	+	+	+	+	+	+
Urease	-	-	-	-	-	-
Gelatine liquefaction	+	+	+	+	+	+
Voges proskauer	+	v	-	-	-	+
Methyl red	-	+	+	+	+	-
ONPG	-	+	-	+	V	+

TCBS, thiosulfate-citrate-bile salts, + =positive, - =negative, v =variable, Y=Yellow, G=Green

The biochemical characters of different isolated *Vibrio* strains were shown in Table (3). All the isolated *Vibrio* species were oxidase positive, catalase positive, gelatin liquefaction positive, citrate positive, indole positive, urease negative, H_2S negative, sensitive to O/129 (150 mg), and string test positive to most of *Vibrio* species (figure 1). At the same time, the other biochemical characters differ from one strain to another.

Figure 1:. positive string test for *Vibrio***.**

Vibrio specie's genotypic characters were assessed by examining ten isolates of *Vibrio* species for the genus gene and the species genes. All the isolates were positive for 16s rRNA gene. *Vibrio alginolyticus* (7 isolates) were positive to the species primer (*Collagenase*), *Vibrio cholerae* (2 isolates) were positive to the species primer (*ompW*), and *Vibrio parahaemolyticus* (1 isolate) was positive to the species primer (*flaE*). None of the isolates were positive to *sodB* and *Hsp60* primers of *Vibrio mimicus*and *Vibrio vulnificus,* respectively, as shown in Table 4 and figures2 and 3.

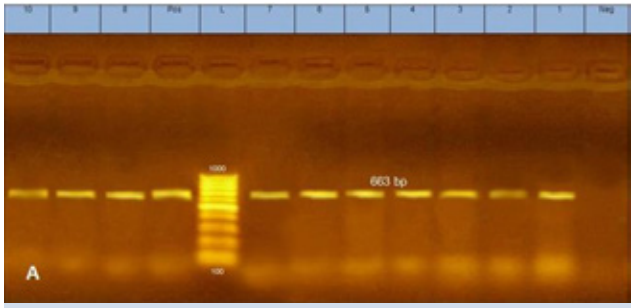

Figure 2: Agarose gel electrophoresis of 16S rRNA gene amplification for the molecular identification of *Vibrio* isolates with amplicon size of 663 bp, Ladder: 100 bp. .

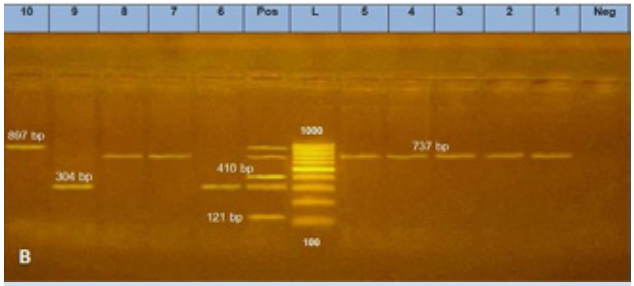

Figure 3: Agarose gel electrophoresis of DNA fragments generated by multiplex PCR with *Vibrio* species. Lanes 1, 2, 3, 4, 5, 7, 8 were *Vibrio alginolyticus* (at 737 bp), lanes 6, 9 were *Vibrio cholerae* (at 304 bp) and lane 10 was *Vibrio parahaemolyticus* (at 897 bp).

Table 4: Molecular identification of the 10 *Vibrio* isolates

Sample	Vibrio 16S rRNA	V. cholerae	V. mimicus	V. parahaemolyticus	V. vulnificus	V. alginolyticus
1	+	-	-	-	-	+
2	+	-	-	-	-	+
3	+	-	-	-	-	+
4	+	-	-	-	-	+
5	+	-	-	-	-	+
6	+	+	-	-	-	-
7	+	-	-	-	-	+
8	+	-	-	-	-	+
9	+	+	-	-	-	-
10	+	-	-	+	-	-

+ =positive, - =negative

DISCUSSION

Bacterial diseases represent one of the most significant problems currently affecting the productivity, development, and expansion of the aquaculture sector. Control of these diseases is difficult because fish are often farmed in systems in which production is dependent on natural environmental conditions. Most bacterial diseases are associated with changes or deterioration of the aquatic environment (Shyne *et al.,* 2008). *Vibrio* spp. have attracted the global interest of the microbiology community and zoonotic diseases experts for being a pathogen of public health concern (Austin and Austin, 2016).

Vibriosis is an economically dangerous infectious disease of cultured freshwater fish, and it is one of the significant diseases occurring in marine and brackish water fishes. *Vibrio* infections are characterized by hemorrhagic septicemia, red necrotic lesions in the abdominal musculature, exophthalmia, as well as erythema of the fin's bases and around the vent (Shahat and Mehana 2000).

Several factors have been recommended to influence *Vibrio*'s survival, persistence, and ability to cause infection. These factors include water temperature, ultraviolet (UV), and salinity (Lipp *et al*., 2002).

This study's limitation is the need to convince the fish farm owners and the fish vendors to take the fish samples necessary for our investigation.

V. alginolyticus causes human illness at significant morbidity and mortality rates, and it was the principal causative agent of marine vibriosis. It was frequently isolated from many outbreaks in Gilthead Sea bream and European Sea bass populations (Zorrilla*et al.,* 2003). *V. cholerae* was first identified as the causative agent of cholera, while *V. parahaemolyticus* is now the predominant etiology of human seafood-borne infections in developing countries (Percival *et al*., 2014).

In most cases, vibriosis outbreaks were attributed to immune suppression because of stress factors. For this particular disease, high water temperature and sudden water temperature fluctuation were among the main triggering factors for *Vibrio* invasion and outbreaks. Historically, this problem was related to spring syndrome (a fall syndrome) (Winfield, 2018).

This study was done during the winter and spring seasons where the most predominant *Vibrio* species isolated from *O. niloticus, M. cephalus, C. gariepinus* was *V. Alginolyticus* followed by *V. cholerae* and *V. parahaemolyticus,* respectively. The high isolation rate of *V. alginolyticus* could be attributed to that; it was present year-round, while the other *Vibrio* species were periodically detectable in summer but less common in winter (Di *et al.,* 2016). Moreover, Chen *et al*. (2010) and Yang *et al.,* (2008) considered that water temperature is the most critical factor affecting *Vibrio* distribution.

The present investigation indicates the total prevalence of vibriosis among examined fish was (34. 6%). This result is similar to that obtained by Moustafa *et al.,* (2010), who found that the prevalence of vibriosis in fish samples from Qarun Lake and Suez Gulf was (34%) and Eissa *et al.,* (2013), who detected vibriosis among naturally infected marine fishes (36%). Likewise, this result is lower than that obtained by Abd El-Gaber *et al.,* (1997), who isolated *Vibrio* species from 40 % of the examined *O. niloticus* and*M. cephalus* fish in both Qarun and Manzala Lakes. Also, Adebayo-Tayo *et al*., (2011) recorded that vibriosis was found in about (44. 2%) of examined seafood samples

obtained from Oron creek infected with *Vibrio* bacteria. The high isolation rate *Vibrio* could be attributed to environmental stresses, significantly organically polluted water, high salinity, and poor hygiene. In addition, Moustafa *et al*. (1990) supported these findings as he found that water pollution and high salinity were the major stress factors for vibriosis among fish.

This high incidence reflects the nature of *Vibrio* spp., known as a halophilic waterborne bacterium, which commonly inhabits worldwide environmental water sources.

On the other hand, this result is higher than the result obtained by Radwan(1995), who detected *Vibrio* species *in O. niloticus* with an incidence of 25%. Also, Levican *et al.,* (2020) isolated *Vibrio* species from farmed *Genypterus chilensis* (21. 6%).

This difference in prevalence percentages may be related to the differences in area, fish species, change in the fish immune system and time, methods of sampling, salinity level, sample sizes, different climate, and water quality characters.

In this study, the highest incidence of *Vibrio* species was detected *in Clarias gariepinus* (64%) followed by *Mugil cephalus* (36%) and then *Oreochromis niloticus* (24%).

The presence of *Vibrio* spp. in the freshwater fish samples suggests increasing in food-borne illness if these fish are consumed in undercooked form. They also could cross-contaminate ready-to-eat foods that are in the same environment.

The causative agent of vibriosis is the genus *Vibrio*. Traditional detection methods, based on cultivation using selective media and characterization of suspected colonies by biochemical reactions, are time-consuming, as they can take 3-4 days. Therefore, in this study, we used a simple, more rapid, sensitive, specific, and reliable method for detecting and characterizing bacteria. Such rapid methods include the polymerase chain reaction (PCR) technique, an in vitro technique used to amplify specific DNA fragments using two specific oligonucleotide primers (Sambrook *et al.,* 1989).

In all the *Vibrio* species, the 16S rRNA gene fragment (663bp) was amplified, confirming the genus in all the isolates while the species-specific genes could differentiate the species of *Vibrio* from each other. PCR confirmed *V. alginolyticus* at a 737 bp chromosomal locus-specific to this species, PCR confirmed *V. cholerae* at a 304 bp chromosomal locus-specific to this species, and PCR confirmed *V. parahaemolyticus* atan 897 bp chromosomal locus-specific to this species. Neither *V. vulnificus* or *V. mimicus* was detected in all examined fish samples using the culture and PCR methods.

CONCLUSION

The results show that the incidence of *Vibrio* spp. *in Oreochromis niloticus*, *Mugil cephalus, a*nd *Clarias gariepinus* collected in Kafr EL-Sheikh Governorate was34. 6%. The highest incidence of *Vibrio* species was detected in *C. gariepinus (*64%), followed by *M. cephalus* (36%) and then *O. niloticus*(24%).

In *C. gariepinus* n=16 (64%) with frequencies of n=8 (32%), n=2 (8%), n=2 (8%), n=3 (12%), n=1 (4%), n=0 (0%) for *V. alginolyticus, V. cholerae, V. parahaemolyticus, V. fluvialis*, *V. splendidus,* and *V. anguillarum* respectively. Likewise, the incidence of *Vibrio* spp. in *M. cephalus*, was n=18 (36%) with frequencies of n=7 (14%), n=6 (12%), n=2 (4%), n=2 (4%), n=1 (2%), n=0 (0%) for *V. alginolyticus, V. cholerae, V. parahaemolyticus, V. fluvialis*, *V. splendidus,* and *V. anguillarum* respectively. On the contrary, the incidence of *Vibrio* spp. In *O. niloticus* was n=18 (24%), with frequencies of n=9 (12%), n=3 (4%), n=4 (5. 33%), n=0 (0%), n=1 (1. 33%), n=1 (1. 33%) for *V. alginolyticus, V. cholerae, V. parahaemolyticus, V. fluvialis*, *V. splendidus,* and *V. anguillarum* respectively. In this study, the bacterial identifications described were based on culturing the strain on TCBS agar media, followed by morphological and biochemical identification. The confirmation was made by using PCR. The molecular identification showed that the most predominant strains were *V. alginolyticus, V. cholerae,* and *V. parahaemolyticus*. The early detection of the bacterial pathogens using the PCR technique before the onset of clinical symptoms offers the possibility of early action and treatment.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research article
Ερευνητικό άρθρο

Microbiological evaluation of ready-to-eat foods by conventional methods and MALDI-TOF MS

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ABSTRACT: The consumption of ready-to-eat foods in daily life is increasing rapidly. Food poisoning occurs as a result of the preparation and preservation of these foods under inappropriate hygienic conditions. There is also an increased risk of getting food poisoning during the summer heat. The purpose of this study was to evaluate the microbiological properties of ready-to-eat foods by conventional methods and investigate the applicability of using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) for the identification of microorganisms in the bacteria isolates from these foods. Eighteen ready-to-eat food samples (tomato soup-G1, lentil soup-G2, tarhana soup-G3, yogurt soup-G4, ezo gelin soup-G5, chicken stew-G6, eggplant ragout-G7, green beans with meat-G8, bulgur pilaf-G9, rice pilaf-G10, macaroni-G11, rice pudding-G12, raspberry fruit dessert-G13, tomato salad G14, cucumber salad-G15, lettuce-G16, shepherd's salad-G17, and yogurt-G18) on sale in restaurants in Ankara-Turkey were evaluated. For this evaluation, the samples were analyzed in terms of staphylococcal enterotoxin (SET-RPLA), *Bacillus cereus* (EN ISO 7932), *Salmonella* spp. (EN ISO 6579-1), *Listeria monocytogenes* (EN ISO 11290-1) and *Escherichia coli* (ISO 16649-2) using conventional microbiological methods. The use of MALDI-TOF MS for the identification of bacteria isolates was also evaluated in this study. Only bulgur pilaf-G9, raspberry fruit dessert-G13, and lettuce-G16 samples exhibited microbial growth on agar plates. *Klebsiella pneumoniae* was detected following the identification of the suspected *Salmonella* spp. in the bulgur pilaf-G9 sample. *B. cereus* (50 cfu /g) and *E. coli* (80 cfu /g) were detected in raspberry fruit dessert-G13 and lettuce-G16, respectively. These bacteria, which were isolated and identified by conventional methods were also rapidly confirmed by MALDI-TOF. In conclusion, these sampled foods, which were available for public consumption, met the general hygiene criteria. Therefore, the foods complied with the Turkish national legislation. The MALDI-TOF MS method has advantages over conventional methods employed in the microbiological evaluation of these foods, in terms of shorter application time, rapidity, and greater simplicity in identifying the causes of poisoning and causative agents involved in food poisoning.

***Keywords*:** Conventional microbiology; MALDI-TOF MS; ready-to-eat foods

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INTRODUCTION

The consumption of ready-to-eat foods is currently growing rapidly with today's fast and busy lifestyle. Home-cooked family recipes such as soups, stews, meat dishes, vegetable dishes, olive oil dishes, pilaf, macaroni, pastries, appetizers, desserts, salads and etc. are presented as ready-to-eat foods. Food is defined as any other substance or product, including water, processed, partially processed, or unprocessed, which is intended to be consumed by humans (5996 Law on Veterinary Services Plant Health Food and Feed, 2010). The precautionary measures taken to neutralize all potential physical, chemical, biological, and other hazards in these foods are collectively known as food safety (Karabal, 2019). During the preparation of food and considering the food safety, conditions must be monitored using hazard analysis and critical control point (HACCP) procedures, and the requisite precautions must be taken to ensure that these are appropriate for consumption (Ceyhun and Artık, 2015). Contaminated foods, particularly in microbiological terms may result if these precautions and conditions are not taken and met at all stages, from food preparation to ready-to-eat food (Hachemi et al., 2019). Microbiologically hazardous foods represent health risks for babies, the elderly, and the sick individuals (Lawrence et al., 2007). Various studies have investigated the microbiological food safety (Iacumin and Comi, 2019). Pathogens play an important role in food poisoning, and *Bacillus cereus*, *Salmonella* spp., and *Listeria monocytogenes* have been the subject of thoroughly investigation (Sapkota et al., 2019). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a relative new generation method employed in the identification of micro-organisms in addition to conventional techniques, relies on the extraction of protein profiles from cells and consequently subjected to ionization by exposing them to an electric field (Yilmaz et al., 2014). This new generation technique has also begun being used for the identification of microorganisms present in food (Kwak et al., 2015). The purpose of this study was to evaluate the microbiological properties of ready-to-eat foods by conventional microbiological methods and investigate the applicability of MALDI-TOF MS for the identification of microorganisms in the bacteria isolates from these foods. Also, these foods were subjected in parallel to microbiological evaluation based on national legislation (Regulation on Turkish Food Codex Microbiological Criteria, 2011).

MATERIAL AND METHODS

Food Sampling

Eighteen ready-to-eat food samples (tomato soup-G1, lentil soup-G2, tarhana soup-G3, yogurt soup-G4, ezo gelin soup-G5, chicken stew-G6, eggplant ragout-G7, green beans with meat-G8, bulgur pilaf-G9, rice pilaf-G10, macaroni-G11, rice pudding-G12, raspberry fruit dessert-G13, tomato salad G14, cucumber salad-G15, lettuce-G16, shepherd's salad-G17, and yogurt-G18) on sale in restaurants in Çankaya-Ankara, Turkey, were obtained and coded. These samples were placed into sterile sample containers. They were then transported to the laboratory under cold chain and aseptic conditions for microbiological analysis.

Weighing the food samples and preparation of dilutions

For initial suspensions of *E. coli* and *B. cereus,* 10 g of each sample was placed into sterile stomacher bags and homogenized by the addition of 90 mL of sterile peptone saline (8.5 g NaCl (Merck), and 1.0 g peptone (Merck), 1000 mL distilled water) (EN ISO 6887-1, 2017). For determination of *Salmonella* spp. and *L. monocytogenes*, 25 g of each sample was placed into sterile stomacher bags for pre-enrichment and homogenized with 225 mL sterile buffered peptone water (Merck) for *Salmonella* spp., and with 225 mL Half Fraser Broth (LabM) medium for *L. monocytogenes*. After homogenization, the mixtures were incubated at 34-38 °C for 18±2 h, and at 30±1°C for 24-26 h for *Salmonella* spp. and *L. monocytogenes* detection, respectively.

Determination of the presence of staphylococcal enterotoxin in samples

For staphylococcal enterotoxin determination, 10 g of each sample was homogenized with 10 mL 0.85% sterile peptone salt solution (Merck) and centrifuged at 4 °C for 30 min. After centrifugation, the supernatant was filtered through a 0.20 μm-diameter sterile filter (Sartorius). After filtration, the staphylococcal enterotoxin kit procedure (SET-RPLA, Oxoid) was applied according to the manufacturer's instruction.

Detection and enumeration of *B. cereus* in the samples

The EN ISO 7932 (2004) method was used for the detection and enumeration of *B. cereus* in the food samples. Briefly, 1.0 mL of the initial suspension prepared was distributed on three dishes with MYP Agar

medium (Oxoid), prepared in duplicates by using six plates and then incubated at 30±1 °C for 21±3 h. After incubation, pink colonies surrounded by an opaque zone due to high lecithinase production in the medium were analyzed further by the conventional method as suspicious colonies. The hemolysis test was performed on Sheep Blood Agar (Merck) for confirmation of the colonies, and incubation was performed at 30±2 °C for 24±2 h. After incubation, the presence of a hemolysis zone on the Sheep Blood Agar (Merck) medium was interpreted as a positive reaction.

Determination of *Salmonella* spp. in the samples

The EN ISO 6579-1 method (2017) was used to determine *Salmonella* spp. in the samples. After the pre-enrichment, the samples were placed into incubation at 41.5±1 °C for 24±3 h in Rappaport Vassilliadis Broth (Biokar) and at 37±1 °C for 24±3 h in Muller-Kauffmann Tetrathionate-Novobiocin Broth (MKTTn broth) (Biokar) media. At the end of incubation, they were incubated in Xylose Lysine Deoxycholate (XLD) Agar (Merck) and Brilliant Green Agar Modified (Condalab) at 37±1 °C for 24±3 h. After the incubation, in order to confirm the colonies identified, black-centered and colorless suspicious colonies growing in the medium were placed into Nutrient Broth (Merck) medium and allowed to incubate at 34-38 °C for 24±3 h. After incubation, culturing was performed in Triple Sugar Iron agar (TSI) (Merck) followed by incubation at 37±1 °C for 24±3 h. The medium was examined for the usage of glucose, non-usage of lactose, non-usage of sucrose, gas formation from glucose, and formation of H_2S. Suspicious colonies also were examined for urea hydrolysis, L-lysine decarboxylase activity and the indole test. For these identification tests, EnteroPluri-Test (Liofilchem) was used, and the manufacturer's instructions were followed. Agglutination control, O antigens, Vi antigens, H antigens were applied for serological confirmation analysis. These antigens were obtained from the Turkish Ministry of Health.

Determination of *L. monocytogenes* in the samples

The EN ISO 11290-1 method (2017) was used for determination of *L. monocytogenes*. After the pre-enrichment process, the sample was placed into Fraser Broth (Biokar) medium and enrichment was performed at 37±1 °C for 24±2 h. After the enrichment process, the samples were inoculated on Agar Listeria according to Ottaviani and Agosti (Merck) and Oxford Listeria Selective Agar medium (Merck), and then incubated at 37±1 °C for 48±2 h. Presumptive *L. monocytogenes* colonies were considered as blue-green colonies surrounded by an opaque halo on Agar Listeria, and grayish-green colonies with collapsed centers, surrounded by a black halo on Oxford Listeria Selective Agar medium. For confirmation of presumptive *L. monocytogenes* colonies, biochemical analyses such as "beta-hemolysis, L-rhamnose, D-xylose, the catalase test, Gram staining" and Microbact *L. monocytogenes* were employed using an identification kit (Oxoid).

Determination of *E. coli* in the samples

The ISO 16649-2 method (2012) was used to determine *E. coli* in the samples. One milliliter of the initial suspension prepared was inoculated on duplicate plates, poured into each Petri dish of the TBX Agar medium (Himedia) and incubated at 44±1 °C for 18-24 h. After incubation, typical blue colony β- glucuronidase-positive *E. coli* colonies on the medium were counted.

Identification of suspected colonies in the samples using MALDI-TOF MS

Suspected *Salmonella* spp. colonies detected on the XLD Agar medium from the bulgur pilaf-G9 sample, and speculated *B. cereus* colonies on MYP Agar medium from the raspberry fruit dessert-G13 sample were also analyzed using MALDI-TOF MS and compared to the conventional methods. *E. coli* colonies in the lettuce-G16 sample were also identified using MALDI-TOF MS.

A MALDI-TOF MS spectrometer (Microflex LT, Bruker Daltonics, Germany) operating by the Flexcontrol software (v.3.0, Bruker Daltonics) for acquiring mass spectra was employed. HCCA (α-cyano-4-hydroxy cinnamic acid) (Bruker) was used as a MALDI-TOF MS matrix. ACN (acetonitrile, HPLC grade) (Sigma-Aldrich), TFA (trifluoroacetic acid) (Sigma-Aldrich), ultra-pure water with a 0.1 μm filter without DNAse and RNAse (Sigma-Aldrich) and a Bruker BTS (bacterial test solution) containing (Sigma-Aldrich) *E. coli*, RNAase and myoglobin protein profiles were used. For microbial biomass analysis with MALDI TOF MS, culture from a single colony with sterile toothpick tip was applied to a special steel 96 MTP-MALDI (Bruker Daltonics) plate (direct transfer method), to which was added 1.0 μL of HCCA matrix solution (12.5 mg/mL HCCA in a mixture of 50% ACN and 2.5% TFA) and the mixture was allowed to dry completely at room temperature. The

MALDI steel plate was loaded onto the MALDI TOF MS. Three studies were performed for each colony and the highest score was taken into consideration by repeated reading. Calibration with BTS was performed simultaneously with the suspected colony.

MALDI-TOF MS was operated with a linear positive ion mode (60 Hz nitrogen laser at 337 nm as ion source) and the method for identifying microorganisms in the mass range of 2,000-20,000 Da. Identification was performed by comparing the mass spectra of suspicious colonies consisting of proteins that were positively ionized by the laser beam (λ = 337 nm) with the most compatible mass profiles in the device library (MALDI Biotyper 3.1; 8500 entires; Bruker Daltonics). In order to obtain the spectra, laser pulses consisting of 40 packets of 240 were performed in the measurement of each colony. The MALDI-TOF MS microbial identification threshold value was set at a 1.70 score value. For identification of micro-organisms, score values between 1.70 to 1.99 were considered as possible genus identification, score values 2.00 between 2.30 indicated secure genus, probable species identification, and score values between 2.30 to 3.00 indicated a higher possible secure species identification (Szabados et al., 2012).

RESULTS

Eighteen ready-to-eat food samples were examined in this study. No growth of atypical or typical *L. monocytogenes* colonies was observed on medium. No staphylococcal enterotoxin was also detected. However, four yellow blackening suspicious *Salmonella* spp. colonies growing on the XLD Agar medium from the bulgur pilaf-G9 sample were subjected to biochemical and serological tests according to EN ISO 6579-1 (2017). The colonies were also identified using MALDI-TOF MS and thus rapidly confirmed. No *Salmonella* spp. were detected in 25 g of bulgur pilaf-G9 sample by either method. Nevertheless, *K. pneumoniae* had already been detected in this sample by MALDI-TOF MS (Figure 1) while using the conventional method. *K. pneumoniae* was reconfirmed as a result of this conventional method.

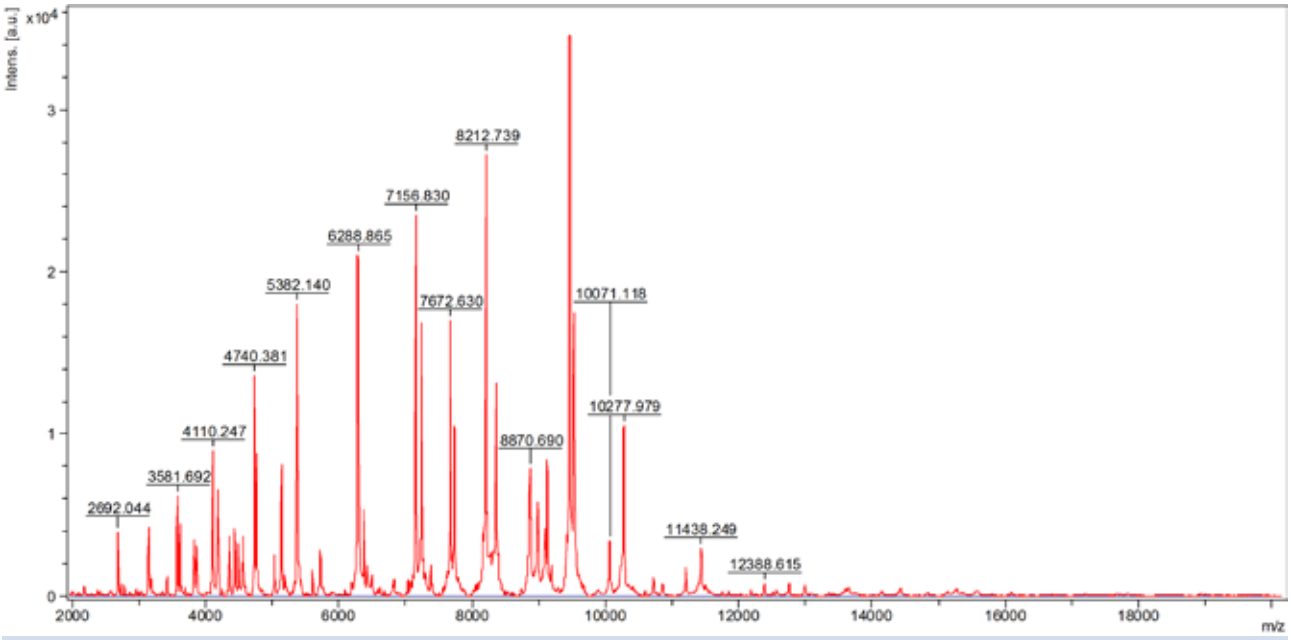

Figure 1. Mass spectrum of *K. pneumoniae* in the bulgur pilaf-G9 (score value=2.322)

Five pink suspicious *B. cereus* colonies surrounded by an opaque zone due to high lecithinase production in MYP Agar medium in the raspberry fruit dessert-G13 sample were confirmed using a conventional method (EN ISO 7932, 2004) and were also identified with MALDI-TOF MS (Figure 2). After these analyses, the number of *B. cereus* colonies per gram of raspberry fruit dessert-G13 sample was 50 cfu. Additionally, typical blue positive β-glucuronidase *E. coli* colonies were observed on TBX Agar from the lettuce-G16 sample, and the number of *E. coli* colonies detected was 80 cfu/g. *E. coli* colonies were also identified by MALDI-TOF MS (Figure 3). In our study, no microbiological growth was observed in the other ready-to-eat food samples, apart from bulgur pilaf-G9, raspberry fruit dessert-G13 and lettuce-G16 samples.

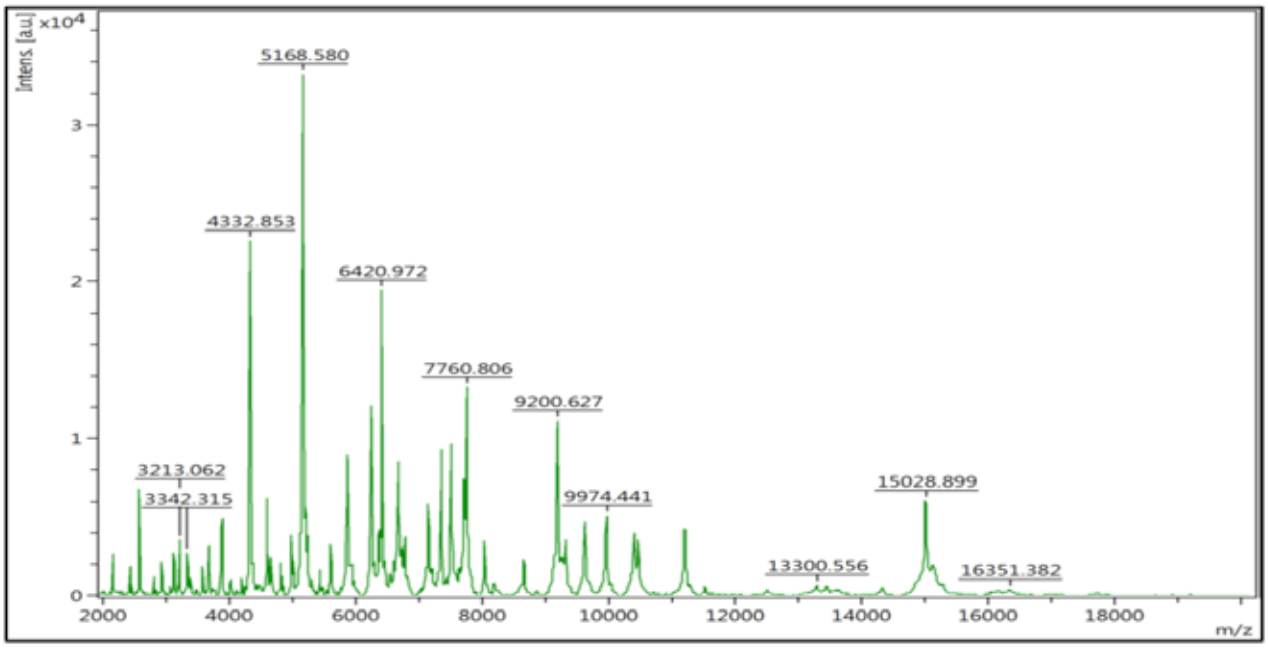

Figure 2. Mass spectrum of *B. cereus* in raspberry fruit dessert-G13 (score value= 2.242)

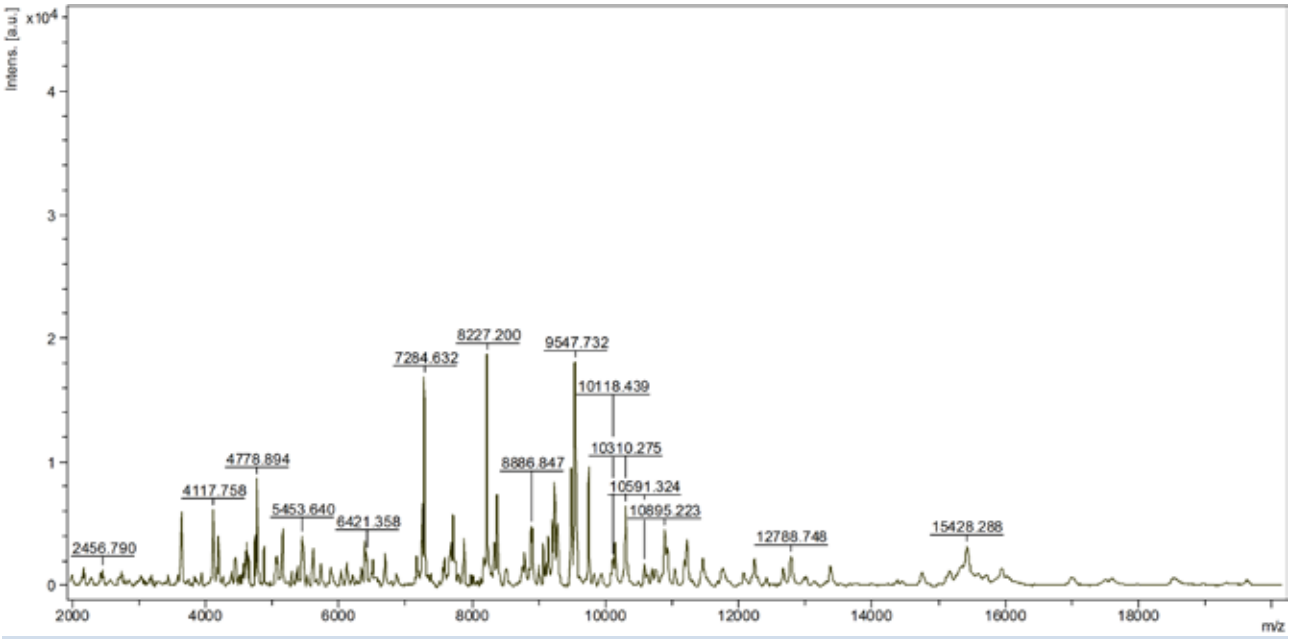

Figure 3. Mass spectrum of *E. coli* in lettuce-G16 (score value= 2.261)

DISCUSSION

Food safety is incredibly important for public health. Cases and/or outbreaks of gastroenteritis may occur due to the consumption of unsafe foods (Aijuka et al., 2018). Microbial agents of food poisoning have therefore been the subject of considerable research (Polański and Sadkowska-Todys, 2018). In this study, the microbiological properties of ready-to-eat food samples available for public consumption in restaurants in Çankaya-Ankara were therefore evaluated. The results of this evaluation were then examined in terms of their compatibility with the Regulation on Turkish Food Codex Microbiological Criteria (2011). In our study, conventional, standard microbiological methods were applied for detecting *Salmonella* spp. (EN ISO 6579-1, 2017), *B. cereus* (EN ISO 7932,

2004), *L. monocytogenes* (EN ISO 11290-1, 2017) and *E. coli* (ISO 16649-2, 2012). Bacteria isolates were also analyzed by MALDI-TOF MS and compared with the results from the conventional microbiological methods. MALDI-TOF MS has previously been used to identify microbiological hazards in foods (Pavlovic et al., 2013; Singhal et al., 2015; Koster and Stanley, 2016; Jadhav et al., 2018). This method has been described as a reliable and rapid technique for the identification and classification of microorganisms (Stahl and Schröder, 2017). The results of this study showed that no *L. monocytogenes* was isolated from any of the samples examined. Moreover, no staphylococcal enterotoxin was also detected. Various studies have investigated the presence of staphylococcal enterotoxin and *L. monocytogenes* in different foods (Leong et al., 2017; Mahfoozi et al., 2019).

Outbreaks caused by *Salmonella* spp. agents have occurred following the consumption of ready-to-eat foods (Donachie et al., 2018). In the present study, suspected *Salmonella* spp. colonies in the bulgur pilaf-G9 sample were isolated and subjected to confirmation tests. *Salmonella* spp. was not detected in any of the ready-to-eat food samples, examined in this study.

However, *K. pneumoniae* was detected in the bulgur pilaf-G9 sample by MALDI-TOF MS (Figure 1). *K. pneumoniae* detection in these foods is not compulsory under the Regulation on Turkish Food Codex Microbiological Criteria (2011). The bulgur pilaf G9 sample was thus compliant with the food safety criteria defined in this regulation, which adopted the Codex microbiological criteria into the national system for use. Nevertheless, it may be hypothesized that hygiene rules were neglected at some stages during the production, preparation, storage, and distribution of the bulgur pilaf-G9 sample. Wang et al. (2019) found that *Salmonella* was commonly present in pickled ready-to-eat meats, a meat product frequently consumed in Shaanxi, China. They recommended that greater attention should be paid to the processing and storage of this ready-to-eat food to prevent bacterial contamination and foodborne outbreaks. Another study indicated that *Salmonella* spp. were found in collected ready-to-eat food samples in middle Thailand (Ananchaipattana, et al., 2016). It was reported that the risk of foodborne illnesses caused by the consumption of these foods after applying suitable hygienic practices in the small food businesses could be reduced (Paul et al., 2017).

Fiedler et al. (2019) investigated food-poisoning by toxin-producing from *B. cereus* strains in ready-to-eat mixed salads in German retail markets. They emphasized the need for monitoring of the presence of these bacteria to ensure that the salads are safe to eat. In the present study, suspected *B. cereus* colonies were identified only in the raspberry fruit dessert-G13 sample and analyzed using conventional methods (EN ISO 7932, 2004) and reconfirmed by MALDI-TOF MS (Figure 2). *B. cereus* was detected at 50 cfu/g in the raspberry fruit dessert-G13 sample. However, *B. cereus* in ready-to-eat foods is not specified in the Regulation on Turkish Food Codex Microbiological Criteria (2011), which adopted the Codex microbiological criteria into the national system for use. *B. cereus* is found in raw and cooked foods, and especially in puddings, cakes, creams, and milk desserts, and has caused significant health problems in public consumption areas serving ready-to-eat foods (Forero et al., 2018). Yu et al. (2019) evaluated *B. cereus* in ready-to-eat food samples including cooked meats, cold vegetable dishes in sauce and rice/noodles in different regions of China, demonstrating the potential hazards of *B. cereus* isolated from these foods. Based on our study results, we have considered that milk desserts should be subjected to a rapid cooling process at +4 °C after cooking to prevent food poisoning caused by *B. cereus. B. cereus* is found in milk desserts as a resulting of poor hygiene during production, storage, and sale (Kaynar, 2020). Amin (2018) recommended that these desserts be prepared in small batches, cooled rapidly, and stored at 4°C.

In this study, *E. coli* (80 cfu /g) was found in the lettuce-G16 sample. However, the amount detected (80 cfu /g) was lower than the threshold limit value for *E. coli* (10^1 cfu /g) which is permitted in these foods by the Regulation on Turkish Food Codex Microbiological Criteria (2011), which adopted the Codex microbiological criteria into the national system for use. The lettuce-G16 sample thus complied with that regulation. Castro-Rosas et al. (2012) investigated the presence of *E. coli* in ready-to-eat salads purchased from restaurants in Pachuca-City, Hidalgo, Mexico. They reported that the salads analyzed were of poor microbiological quality since diarrheagenic *E. coli* pathotypes (DEPs) were identified in up to 6% of salad samples and that raw vegetables should continue to be screened. In addition, they suggested that the irrigation of raw vegetables with untreated sewage water should also be forbidden by national legislation. Mira Miralles et al. (2019) did not detect the presence of *E.*

coli in salads, one component of ready-to-eat foods. Ready-to-eat raw salad can be easily contaminated by *E. coli* originating from water sources (Temelli et al., 2005). In the present study, we hypothesized that cross-contamination may have occurred due to inadequate washing of the ready-to-eat raw lettuce-G16 sample and due to poor hygiene of personnel and equipment during salad preparation.

CONCLUSION

In conclusion, the tested ready-to-eat foods in this study intended for public consumption complied with general hygiene criteria. These foods complied with the national legislation, which adopted the Codex microbiological criteria into the national system for use. However, it is important that good hygiene rules and practices must be applied in all stages of food supply chain, from preparation to consumption to safeguard the public health. It is also essential that the raw materials used in the preparation of ready-to-eat foods should not pose a health risk. MALDI-TOF MS can be employed in addition to conventional methods used in the microbiological evaluation of these foods, as well as for identifying the source of food poisoning. This method also has the advantages of greater simplicity, rapidity, and shorter application times.

CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Nasal carriage of *Staphylococcus aureus* among healthy veterinary students in Greece, 2017-2018: A cross-sectional cohort study

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ABSTRACT: The epidemiology of *Staphylococcus* spp., *Staphylococcus aureus* and MRSA among veterinary students in Greece during 2017-2018 is reported. Nasal swabs and a standardized questionnaire from 160 healthy veterinary students were used to identify potential risk factors for colonization. Antimicrobial susceptibility testing, *pvl, mecA, mecC*, staphylococcal enterotoxin genes and PFGE were used to characterize *S. aureus* isolates. Overall, 76% and 19% of the students were colonized by *Staphylococcus* spp. and *S. aureus* but none by MRSA. Students with a prior visit to a hospital were 1.33 and 2.25 times more likely to be colonized by *Staphylococcus* spp. and *S. aureus,* respectively while, 94% of the *S. aureus* isolates were resistant to penicillin, 68% to amoxicillin/clavulanic acid and 12% were multidrug-resistant. Staphylococcal enterotoxin genes were detected in 32% of the *S. aureus* isolates, while PFGE showed heterogeneity. Although MRSA was not detected, the high rate of *Staphylococcus* spp. colonization suggests the need of sustained implementation of strict hygiene practices among students and the staff involved in veterinary training. The results of the present study add useful information for the assessment of the risks associated with staphylococcal infection in veterinary students.

Keywords: *Staphylococcus*, colonization, PFGE, antimicrobials, MRSA

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a common bacterium found on the skin and nasal cavities of healthy people. Approximately 25-40% of the population is colonized by *S. aureus*. It is also a common cause of skin and soft tissue infections and sometimes causes severe disease such as pneumonia, bacteraemia, meningitis, sepsis, and pericarditis exhibiting a high burden in terms of morbidity and mortality (Frana et al. 2013; Cassini et al. 2019). *S. aureus* is also responsible for food poisoning due to oral intake of Staphylococcal enteroxins (SEs) present in foods (Zhao et al. 2017; Khemiri et al. 2019) which leads to a significant health and economic in accute gastroenteritis (Papadopoulos et al. 2019a). *S. aureus* bacteria harbouring the *mec*A gene are resistant to methicillin and other β-lactam antimicrobials and are referred to as methicillin-resistant *S. aureus* (MRSA) (Frana et al. 2013). MRSA has been considered a major hospital associated pathogen (HA-MRSA), and has become a serious threat in hospitals worldwide but has been also found associated to community setting (CA-MRSA) and to livestock (LA-MRSA) (Pantosti 2012).In Greece, the prevalence of MRSA in clinical isolates of *S. aureus* has been 36-40% during 2015-2018 and remains one of the highest in Europe; the EU/EEA population-weighted mean percentage of MRSA was 16.4% in 2018 (ECDC 2019).

Many countries have developed and implemented national recommendations for preventing the spread of MRSA, including screening, isolation and decolonization of the MRSA carriers and prudent antimicrobial use (Köck et al. 2014). In order to slow the spread of MRSA in Europe, comprehensive MRSA strategies targeting all healthcare sectors remain essential. On the other hand, the monitoring of MRSA in animals and food is currently voluntary and only performed in a limited number of countries. Recently transmission of LA-MRSA to humans by food-producing animals has been described, especially in persons with close contact to animals and mostly in farmers (Graveland et al. 2011), slaughter and abattoir workers (Papadopoulos et al. 2018; Drougka et al. 2019; Papadopoulos et al. 2019c; Papadopoulos et al. 2019d), companion animals' owners (Ferreira et al. 2011) and veterinarians (Hanselman et al. 2006), demonstrating a severe occupational risk for veterinary professionals.Studies have been also conducted among exclusively veterinary students or personnel in veterinary hospitals; the occupational risk associated to veterinary students has also been addressed and reported by works describing infections by MRSA (Wulf et al. 2006; Aklilu et al. 2013; Frana et al. 2013; Huang and Chou 2019). However, in Greece, the occupational risk for colonization among healthy veterinary students that are in contact with animals is still unknown.

Therefore, the aim of this study was to estimate the prevalence of *Staphylococcus* spp, *S. aureus* and MRSA nasal carriage among healthy veterinary students in Greece, to describe the isolated strains of *S. aureus* and to identify possible risk factors of colonization.

MATERIALS AND METHODS

Sampling frame and sample collection

All the students studying in 5th semester of two consecutive years (2017-2018) in the School of Veterinary Medicine, Aristotle University of Thessaloniki in Greece were asked to participate in the study. All individuals that accepted to participate (174 out of 208; rejection rate of 16%) were previously informed of the screening procedure (Figure1). A standardized questionnaire was completed for each participating student, during sampling, in order to collect data with reference to the lifestyle and habits that the students had, and which would be correlated as potential risk factors. Students that reported symptoms of infectious disease during the last two weeks of the interview/sampling day were excluded from the study. Sampling of nasal cavities was performed by swabbing both nostrils with Sterile Transport Swabs STUART (FL MEDICAL, Torreglia, Italy). Swabs were transported immediately for analysis in the Laboratory of Hygiene of Foods of Animal Origin-Veterinary Public Health, of the School of Veterinary Medicine, Aristotle University of Thessaloniki. An informed consent signed by all the participants was obtained prior to enrollment.

Isolation and identification of *Staphylococcus* spp and *S. aureus*

Swabs were placed for enrichment in tubes containing 5 ml Tryptone Soy broth (TSB, LAMB M, Lancashire, United Kingdom) with 6.5% NaCl and 0.3% yeast extract. After 18-24 h incubation at 35 ^{o}C, 10 μl of the enrichment was plated on Baird-Parker agar with Egg Yolk Tellurite (Oxoid, Unipath, Basingstoke, UK) and incubated at 35 ^{o}C for 24-48 h. After incubation, 3-4 colonies from each plate were transferred on Tryptone Soy agar (LAB M Limited, Lancashire, United Kingdom) and incubated for 24 h at 35 ^{o}C. Colonies were selected according to their appearance, black both with and without opaque ha-

loes from Baird Parker. Identification as *Staphylococcus* spp and presumptive *S. aureus* was based upon Gram-staining, catalase reaction, mannitol fermentation and coagulase test, morphological and cultural characteristics. One *Staphylococcus* spp. (suspected *S. aureus*) per student was stored at -80 ºC in TSB containing 20% glycerol.

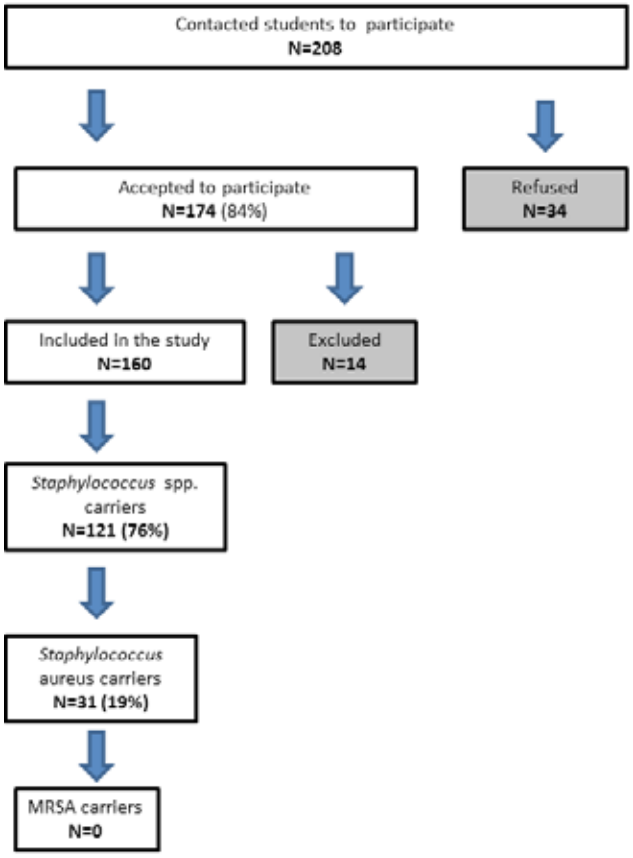

Figure 1: Flow chart describing the populations of students for each particular step of the study, Greece, 2017-18, N=208

One presumptive *S. aureus* isolate from each sample was confirmed by PCR targeting the *S. aureus* species-specific determinants *coa* (coagulase) and *nuc* (nuclease) genes. Extraction of genomic DNA from bacterial cultures was conducted according to the protocol of DNA purification from Gram-positive bacteria by the Pure Link Genomic DNA kit (Invitrogen, Carlsbad CA). A 500- to 650-bp fragment of the *coa* gene and a 416-bp fragment of the *nuc* gene were amplified using previously described primer sets (Hookey et al. 1998; Sudagidan and Aydin 2008) and PCR conditions (Zdragas et al. 2015). The PCR amplicons were separated in 1.5% agarose gels stained with ethidium bromide and visualized under UV illumination (TEX-20 M, Life Technologies, GibcoBRL System). A sample from a healthy student was defined as positive for colonization if it contained at least one *Staphylococcus* spp or *S. aureus* isolate, respectively.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the *S. aureus* isolates was determined by the agar-dilution method in Mueller-Hinton agar (MHA, Merck) according to the Clinical and Laboratory Standard Institute guidelines (CLSI, 2009). Briefly, plates were prepared by incorporating the appropriate amount of antimicrobial agent into MHA. For each bacterial isolate the inoculum was prepared by adjusting the turbidity to 0.5 McFarland and was applied rapidly to the agar surfaces using a multi-channel pipet (Eppendorf, Merck) capable of transferring multiple inocula to each plate. The results were evaluated after incubation at 35 °C for 24 h. Susceptibility towards the following 15 antimicrobials was evaluated with the final concentration in μg/mlin brackets: penicillin, P (0.25); oxacillin, Ox (0.25 and 2); amoxicillin/clavulanic acid, Amc (1/0.125); tetracycline, T (1); erythromycin, E (1); vancomycin, V (2 and 4); chloramphenicol, C (8); ciprofloxacin, Cp (1); trimethoprim/sulfamethoxazole, Sxt (2/38); trimethoprim, Tm (2); gentamicin, G (1); amikacin, Ak (8); kanamycin, K (8); rifampicin (0.5); clindamycin Cl (0.25). Multidrug-resistance (MDR) was defined as previously proposed (Magiorakos et al. 2012). *S. aureus* ATTC 25923, *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as control strains.

Identification of the *mec*A and *mec*C genes

The detection of the *mec*A gene in the phenotypically resistant (OX 0.25) *S. aureus* isolates was achieved by PCR according to Murakami et al. (1991) using the primers (5' AAAATC GATGGTAAAGGTTGGC) corresponded to nucleotides 1282 to 1303 and (5' AGTTCTGCAGTACCGGATTTGC) complementary to nucleotides 1793-1814. The PCR was also performed for the detection of the *mec*C according the method described by Stegger et al. (2012) using the primers 5'-GAAAAAAAGGCTTAGAACGCCTC-3' and 5'-GAAGATCTTTTCCGTTTTCAGC-3'.

Detection of staphylococcal enterotoxin(SEs) genes

Five specific primer sets, previously described by Jarraud et al. 2002), were used for the detection of genes encoding for the five classic SEs (*sea*, *seb*, *sec*, *sed*, *see*). Amplifications of SE-coding genes were performed as single PCR assays.

Table 1: Risk factors for nasal carriage by *Staphylococcus* spp among healthy veterinary students, N=160, Greece 2017-2018.

Characteristics	Colonized by *Staphylococcus* spp(N)	Total	Colonized by *Staphylococcus* spp %	Prevalence ratio (PR)	95% C.I	p value	Adjusted Prevalence ratio (PR)	95% C.I	p value
Sex									
male	61	78	74.39	1.03	0.87-1.23	0.71			
female	60	82	76.92	ref	ref				
Age (years)									
20	39	53	73.58	ref	ref				
21	43	57	75.44	1.03	0.82-1.28	0.82			
22	24	30	80.00	1.09	0.85-1.38	0.49			
23	9	13	69.23	0.94	0.63-1.40	0.76			
24	6	7	85.71	1.16	0.83-1.64	0.38			
Year enter									
2013	10	13	76.92	ref	ref				
2014	24	32	75.00	0.97	0.68-1.40	0.89			
2015	48	62	77.42	1.00	0.73-1.40	0.97			
2016	39	53	73.58	0.96	0.68-1.34	0.80			
Years in clinic									
1	39	53	73.58	ref	ref				
2	76	100	76.00	1.03	0.85-1.26	0.75			
3	6	7	85.71	1.16	0.83-1.64	0.38			
Pet									
yes	92	72	78.26	1.08	0.90-1.30	0.38			
no	68	49	72.06	ref	ref				
Smoking									
yes	44	58	75.86	1.00	0.84-1.21	0.96			
no	77	102	75.49	ref	ref				
Skin infection during last 3 months									
yes	12	14	85.71	1.15	0.91-1.45	0.25			
no	109	146	74.66	ref	ref				
Surgery during last 3 months									
yes	12	13	92.31	1.24	1.04-1.50	0.019			
no	109	147	74.15	ref	ref				
Respiratory disease during last 3 months									
yes	15	17	88.24	1.19	0.98-1.45	0.09			
no	106	143	74.13	ref	ref				
Visited hospital during last 3 months									
yes	43	47	91.49	1.33	1.14-1.54	0.00	1.33	1.14-1.54	0.00
no	78	113	69.03	ref	ref		ref	ref	
Use of antibiotics during last 3 months									
yes	17	24	70.83	0.93	0.70-1.22	0.58			
no	104	136	76.47	ref	ref				
Use of antibiotics during last 6 months									
yes	22	29	75.86	1.00	0.80-1.26	0.97			
no	99	131	75.57	ref	ref				

Table 2: Risk factors for nasal carriage by *S. aureus* among healthy veterinary students, N=160, Greece 2017-2018.

Characteristics	Colonized by *S. aureus* (N)	Total	Colonized by *S. aureus* %	Prevalence ratio (PR)	95% C.I	p value	Adjusted Prevalence ratio (PR)	95% C.I	p value
Sex									
male	13	78	21.95	0.76	0.40-1.44	0.4			
female	18	82	16.67	ref	ref				
Age (years)									
20	11	53	20.75	ref	ref				
21	10	57	17.54	0.85	0.39-1.83	0.67			
22	4	30	13.33	0.64	0.22-1.84	0.41			
23	4	13	30.77	1.48	0.56-3.91	0.43			
24	2	7	28.57	1.38	0.38-4.97	0.63			
Year enter									
2013	3	13	23.08	ref	ref				
2014	6	32	18.75	0.81	0.24-2.77	0.74			
2015	11	62	17.74	0.77	0.25-2.38	0.65			
2016	11	53	20.75	0.90	0.29-2.77	0.85			
Years in clinic									
1	11	53	20.75	ref	ref				
2	18	100	18	0.87	0.44-1.70	0.68			
3	2	7	28.57	1.38	0.38-4.97	0.63			
Pet									
yes	19	92	20.65	1.17	0.61-2.24	0.64			
no	12	68	17.65	ref	ref				
Smoking									
yes	10	58	17.24	0.84	0.42-1.65	0.61			
no	21	102	20.59	ref	ref				
Skin infection during last 3 months									
yes	6	9	42.86	2.5	1.24-5.05	**0.01**			
no	24	151	17.12	ref	ref				
Surgery during last 3 months									
yes	4	13	30.77	1.68	0.69-4.05	0.25			
no	27	147	18.37	ref	ref				
Respiratory disease during last 3 months									
yes	6	16	35.29	2.02	0.97-4.21	0.06			
no	25	144	17.48	ref	ref				
Visited hospital during last 3 months									
yes	15	47	31.91	2.25	1.21-4.18	0.01	2.25	1.21-4.18	0.01
no	16	113	14.16	ref	ref	ref	ref	ref	ref
Use of antibiotics during last 3 months									
yes	7	24	29.17	1.65	0.80-3.40	0.17			
no	24	136	17.65	ref	ref				
Use of antibiotics during last 6 months									
yes	9	29	31.03	1.85	0.95-3.59	0.07			
no	22	131	16.79	ref	ref				

Pulsed Field Gel Electrophoresis (PFGE)

PFGE analysis was conducted following the Pulse-Net protocol (McDougal et al. 2003) using the size standard, electrophoretic conditions, dendrogram construction and comparison criteria previously described (Papadopoulos et al. 2018). Clusters were selected using a cut-off at the 80% level of genetic similarity. The diversity of PFGE type distribution was calculated using the Simpson's diversity Index (D), which ranges from 0 (no diversity) to 1 (extreme diversity) as a measure for PFGE type diversity.

Statistical analysis

Frequencies were obtained and proportions were calculated for categorical variables, age was also treated as categorical variable for comparisons as age ranked from 21 to 28 years old. Categorical variables were compared using the Chi square test or the Fisher exact test. Prevalence ratios (PR), 95% confidence intervals (CI), and P values were calculated. The association between influencing factors and *Staphylococcus* spp. or *S. aureus* nasal colonization was examined using multivariable logistic regression models. Multivariable logistic regression analysis of all variables with a P-value of <0.2 indication significance was also performed. A P value of ≤0.05 was considered statistically significant. All statistical measures were estimated using survey data analysis methods (SVY commands) from STATA package. All analyses were performed using STATA 14 (STATA CORP LP, College Station, Texas, USA).

RESULTS

Prevalence of *Staphylococcus* spp, *S. aureus* and-MRSA in veterinary students

A total of 174 students voluntarily accepted to participate in the study. A total of 14 students were excluded from the study, due to symptoms of illness during the last 15 days before the interview/screening. A total of 160 healthy students were included and sampled with a median age of 21years (range 20-24 years) and 51% (78/160) were females (Figure 1). Table 2 presents in detail the descriptive and the corresponding demographic and lifestyle data of the students participated in this study.

One hundred twenty-one out of 160 (76%) found to be colonized by *Staphylococcus* spp, and 31 (19%) by *S. aureus* (Figure 1). All the 31 confirmed as *S. aureus* isolates carried the *coa* (coagulase) and *nuc* (nuclease) genes. Distributions of *Staphylococcus* spp and *S. aureus* carriers and non-carriers stratified by population characteristics and variables associated with carriage in the univariate analysis are shown in Tables 1 and 2 respectively.

The univariate analysis revealed that students that had visited a hospital (ambulatory consultation and/or visit to an impatient and/or hospital admission and/or surgery) during the last three months were 1.33 times more likely to be colonized by *Staphylococcus* spp (OR 1.33 CI 1.14-1.54). However, students that had a skin infection during the last three months were 2.5 times more likely to be colonized by *S. aureus* (OR 2.5 CI 1.24-5.05) and those who had visited a hospital during the last three months were 2.25 times more likely to be colonized by *S. aureus* (OR 2.25 CI 1.14-1.54). In order to assess the relationship between the potential predictors, taking under consideration potential confounders among the influencing factors, a multivariate logistic regression model was used. This model demonstrated that when controlling for the effects of the other influencing factors, the relationships found in the univariate analyses changed. Only a visit to the hospital during the last three months was found significantly associated with any of the abovementioned characteristics. More specific details are presented in Tables 1 and 2. The adjusted prevalence ratio for colonization by *Staphylococcus* spp was 1.33 (CI 1.14-1.54 95%) and for *S. aureus* 2.25 (CI 1.21-4.18 95%) according to the final regression model. None of the variables was identified as a protective factor for nasal colonization. Correlation of carriage with a specific veterinary-related factor, like having a pet or attending more years in the clinic, could not be established.

Antimicrobial susceptibility testing

The antibiotic resistance patterns of the 31 *S. aureus* isolates are presented in Figure 2. Drug-resistance to at least one antimicrobial agent was observed in all 31 isolates. Resistance to penicillin allocated the highest rate of resistance (94%), following by amoxicillin/clavulanic acid (68%) and erythromycin (23%). Lower resistance rates were observed in ciprofloxacin, clindamycin and tetracycline (6%). Eight isolates showing resistance to low concentration of oxacillin (0.25μg/ml) were identified. However, none of the 31 isolated carried the *mec*A or the *mec*C genes or exhibited resistance to >2 μg/mlof oxacillin thus MRSA strain was not isolated. The *pvl* gene was not detected among all the *S. aureus* isolates in this study; all of them were susceptible to amikacin, kanamycin, gen-

tamicin, rifampicin, vancomycin and trimethoprim with or without sulphamethoxazole (Table 3). Three out of the 31 (10%) isolates showed resistance against antibiotics belonging to three or more antibiotic classes and consequently were characterized as multi-drug resistant. The most common profile was Pe-AC (39%) followed by Pe and Pe-pOx-AC (13%) (Figure 2).

Detection of SEsgenes and *pvl* genes

Detection of SEs genes is shown in Figure 2. Among all the 31 *S. aureus* isolates, at least one of the SEs genes was detected in 10 (32%) of them. The *sec* was the most common detected (19%) followed by *seb* (13%) and *sea* (10%). In two isolates (6.5%), both *seb* and *sec* were detected, while *sea* and *sec*, *sea* and *seb* were both detected in 3.2%. No significant difference (P >0.05) was identified among the risk factors and the detection of any of the tested SEs genes. However, *pvl* gene positive isolate was not identified.

Pulsed Field Gel Electrophoresis

Twenty-eight distinct PFGE types were identified among *S. aureus* isolates with overall similarity 52.6%; 22 of them assigned to five main clusters (80% similarity cut off) consisting from two to seven isolates. The Simpson's index of diversity was calculated as D = 0.974 (Figure 2). Only two cases of students sharing the same PFGE type were identified; the first one with two students no 020 and 069 (same age and same AMR profile). The second case included three students no 101, 107 and 111. This case had different AMR profiles and different enterotoxin profiles. Overall, endemic clones circulating among the students were not identified.

Table 3: Antimicrobial susceptibility testing of *S. aureus* isolates among healthy veterinary students, N=31, Greece 2017-2018.

Antimicrobial	Concentration (mg/l)	N	%
Penicillin	0.25	29	94
Oxacillin	0.25	10	32
Oxacillin	2	0	0
Trimethoprim/ Sulphamethoxazole	2/38	0	0
Gentamycin	1	0	0
Erythromycin	1	7	23
Amikacin	8	0	0
Kanamycin	8	0	0
Tetracycline	1	2	6
Trimethoprim	2	0	0
Amoxicillin/ clavulanic acid	1/0.125	21	68
Ciprofloxacin	1	2	6
Rifampicin	0.5	0	0
Vancomycin	2	0	0
Vancomycin	4	0	0
Clindamycin	0.25	2	6
Chloramphenicol	8	0	0

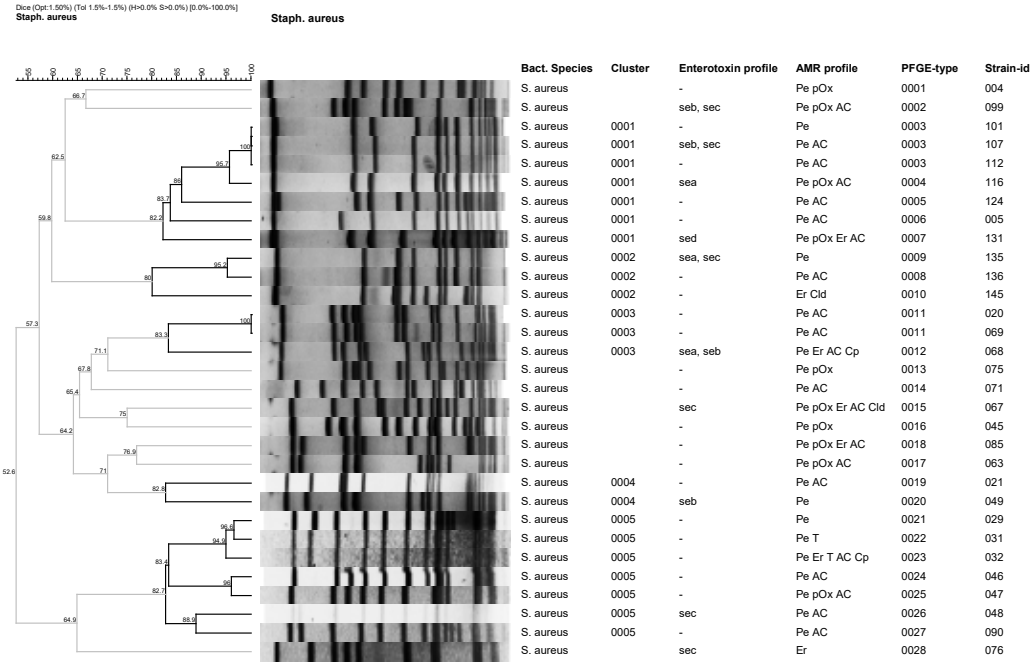

Figure 2: Dendrogram of the 31 *S. aureus* isolates with corresponding antimicrobial resistance profiles, enterotoxin gene profiles and PFGE-types.

DISCUSSION

In this study, *Staphylococcus* spp, *S. aureus* and MRSA nasal carriage among healthy veterinary students was evaluated and the molecular characteristics of the isolated *S. aureus* strains were described. In total, 76% and 19% of the students were colonized by *Staphylococcus* spp and *S. aureus* respectively, while none by MRSA. The present study revealed that only a previous visit to the hospital was independently associated with the carriage of *Staphylococcus* spp and *S. aureus*.

Several studies have been conducted regarding the prevalence of *S. aureus* and MRSA in healthy individuals. Studies in Greece and globally show that about 20% of individuals are persistent *S. aureus* nasal carriers, approximately 30% are intermittent carriers (range 16-70%), and about 50% (range 16-69%) non-carriers (Wertheim et al. 2005; Papadopoulos et al. 2018; Papadopoulos et al. 2019c; Moschou et al. 2020). Results of other studies, targeting medical or university students, are comparable with results from the present study however, most of the studies present a low prevalence of MRSA colonization (<5%) but higher regarding *S. aureus* (29.2-40%) (Rohde et al. 2009; Prates et al. 2010; Roberts et al. 2011; Treesirichod et al. 2013; Petti et al. 2015; Price et al. 2017). Only a few studies have been conducted among exclusively veterinary students or personnel in veterinary hospitals targeting MRSA. The occupational risk associated to veterinary students has also been addressed and reported by others describing infections by MRSA reporting prevalence from 0-23.3% (Wulf et al. 2006; Aklilu et al. 2013; Frana et al. 2013; Youn et al. 2014). Interestingly, studies underscore the importance of occupational exposure mainly to pigs as a risk factor for colonization by MRSA (Frana et al. 2013; Narvaez-Bravo et al. 2016); however, in this study it was not possible to identify students that had contact exclusively with pigs. Overall, the prevalence of *S. aureus* in this study was not high. Nevertheless, there were positive prevalence relationship of *Staphylococcus* spp and *S. aureus* isolates among students visiting hospital.

This study showed that the levels of antimicrobial resistant *S. aureus* were in general low although some strains found to be multidrug resistant. The isolates showed resistance to penicillin and/or amoxicillin/clavulanic acid which are among the most used antibiotics in Greece, country that had the highest rate of non-prescription use of antibiotics during 2016 with nearly 20% or 74.6% of the people using un-prescribed antibiotics (Anonymous 2016). Moreover, these two antimicrobials are also the most over the counter bought antimicrobials by the general population (Anonymous 2017). However, a study in Greece showed that antibiotics are very easy to be asked for and purchased without any justification (Plachouras et al. 2010). Self-medication by 'over the counter' antibiotics appears to be an extensive problem in Greece and this practice may contribute significantly to excess antibiotic use and increased antibiotic resistance.

MRSA has been recognized as a major causative agent of healthcare-associated infections (HA-MRSA) in humans for decades (Sergelidis and Angelidis 2017). In recent years, the isolation of MRSA from livestock (LA-MRSA) and companion animals has also been reported (Nemati et al. 2008); HA-MRSA and CA-MRSA are believed to predominantly affect humans and, in general, are not involved in livestock infections. Surprisingly, the present study demonstrated zero prevalence of MRSA among the Greek Veterinary students despite the fact that a study contacted in Greece during 2014 showed a 5.3% of MRSA prevalence among patients visiting hospitals in Central Greece (Tsiodras et al. 2014). Moreover, according to EARS net the EU/EEA population-weighted mean percentage of MRSA was 16.4% in 2018 but in Greece 36.8%; this practically means that more than one out of three invasive staphylococcal infections in Greece are caused by MRSA (Anonymous 2019).

PFGE has been widely used for the characterization of human or animal isolates, sometimes coupled with other molecular (MLST, *spa* typing or WGS) or phenotypic methods (Güven Gökmen et al. 2018; Lakhundi and Zhang 2018). We found a significant genomic variability among the 31 *S. aureus* isolates; this was demonstrated by the large number -28- of distinct PFGE patterns, as well as from the high values of Simpson's index of diversity. Endemic clones circulating among the students were not identified; an explanation could be that colonized students are "persistent" carriers colonized by a single strain for a long time (Wertheim et al. 2005).

Panton-Valentine leucocidin encoded by *pvl* gene is a toxin that lyses leukocytes and strongly associated with skin infections (Lina et al. 1999; Vandenesch et al. 2003). Our results are in line with the study by Roberts et al. (2011) who collected 24 MRSA strains from a university campus in USA and did not identify any positive for *pvl* gene. Similarly, Heller et al. (2009) did not isolate *pvl* positive MRSA strain

among 64 workers in a veterinary clinic in the UK. In Greece, other studies in healthy workers in dairy industry, dairy animals did not confirm the presence of the *pvl* gene (Papadopoulos et al. 2018; Papadopoulos et al. 2019c; Papadopoulos et al. 2019d).

S. aureus enterotoxins (SEs) are major causes of staphylococcal food poisoning (Argudin et al. 2010). Staphylococcal enterotoxin A, B, C and enterotoxin D genes, were confirmed in *S. aureus* isolates from cattle, workers and environmental samples in the dairy industry in Greece (Papadopoulos et al. 2019b; Papadopoulos et al. 2019c) in studies that screened only MRSA isolates. This study shows that 32% *S. aureus* isolates carried at least one SE gene. Several studies worldwide have demonstrated comparable results with ours identifying SEs genes from foods and animals which underscores the significance of these toxins in food poisoning (Vitale et al. 2015; Cheng et al. 2016; Bastos et al. 2017; Zhang et al. 2018).

To our knowledge this is the first study in Greece targeting risk factors of colonization exclusively among veterinary students. Considering the fact that in Greece there are only two Veterinary schools with approximately 200 students per year, we think that sampling 160 students from the one of the schools give our study enough strength to supports the findings. Nevertheless, a possible limitation of the study is that all the students by the time of sampling had already contacts with animals (companion or large animals) during their clinical years. In this aspect it was not possible to gather information and perform comparisons regarding differences between students in preclinical and clinical semesters. Moreover, it was not possible to collect information regarding the contact with different animal species which is of particular interest. The results have not been compared with a control population of non-veterinary students in Greece (suitable age- and gender-matched control group) in order to determine if there is anything unusual about staphylococcal nasal carriage among veterinary students. However, to our knowledge this is the first study in Greece targeting exclusively veterinary students as potential carriers of *Staphylococcus* spp. and *S. aureus.*

CONCLUSIONS

Albeit, studies worldwide have demonstrated veterinarians' occupational risk of *S. aureus* and MRSA colonization, this study showed that possibly colonization did not take place during veterinary studies. Although MRSA was not detected, the high rate of *Staphylococcus* spp. colonization suggests the need of sustained implementation of strict hygiene practices among students and the staff involved in veterinary training.

CONFLICT OF INTEREST

None of the authors declare a conflict of interest.

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Research article
Ερευνητικό άρθρο

Sand colic: A retrospective study of 6 cases

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ABSTRACT: Colic- loosely defined as abdominal pain- is very common in horses, with clinical signs ranging from mild to severe. More than ninety causes of colic have been described. When horses consume significant amounts of sand while eating, they may develop symptoms of sand colic. Sand accumulation in the gastrointestinal tract of horses can irritate the colonic mucosa (less likely gastric mucosa), leading, usually, to sand impaction. Six horses with sand colic were referred to the School of Veterinary Medicine of Aristotle University of Thessaloniki from 2014 to 2018. Age ranged from 7 years to 19 years. The horses were presented with a history of: mild to severe colic (6/6), anorexia (4/6), weight loss (3/6) and diarrhea (6/6). Gastrointestinal auscultation revealed a characteristic sound of "pouring sand" (4/6) while sand was found in the faeces in large quantities (6/6). In order to identify sand in the faeces, sand sedimentation test can be performed easily in the field. Rectal examination findings were: gassy dilation of the colon (2/6) and impacted segment of colon (4/6) with (2/6) or without (2/6) cecum tympany. These cases, depending on the severity of the symptoms, were treated either pharmaceutically (4/6) or surgically (2/6). Pharmaceutical treatment consisted of administration of fluids, NSAIDs, antibiotics and laxatives, with the later given via nasogastric tube. Psyllium and paraffin oil were selected as laxatives of choice in this study. Surgical treatment was elected in 2 horses due to failure of conservative treatment. One of the two horses, that underwent surgery, survived while the other developed postoperative peritonitis and died. Horses that were treated pharmaceutically, either showed improvement and survived to discharge (2/4) or did not survive due to poor response to treatment (2/4). Minimizing exposure to sand and dietary management were important in preventing recurrence of sand colic in all 3 cases. According to the authors' knowledge, this is the first report of sand colic cases in Greece.

Keywords: equine, colic, sand

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INTRODUCTION

Accumulation of sand in the colon of the horses is reported as a common cause of colic in places with loose sandy soil like California, Arizona, Michigan and coastal regions of USA (Kilcoyne et al. 2017, Rakestraw and Hardy 2012). It may also occur where horses selectively ingest dirt or gravel with food (Hart et al. 2013, Niinisto et al. 2014). The prevalence of sand colic in USA varies from 5% to 30% of all colic cases (Landes et al. 2008). Accumulation of sand in the colon of horses and subsequent colic related to it has been reported worldwide, treated either with conservative therapy or surgery (Kilcoyne et al. 2017). Even though surgical and pharmaceutical treatment of sand colonic accumulation has been described both in clinical cases and experimentally, retrospective studies of clinical cases are lacking (Rouhoniemi et al. 2001, Korolainen et al. 2002, Landes et al. 2008, Niinisto et al. 2014, Niinisto et al. 2018).

Sand accumulation in the colonic lumen causes significant irritation of the mucosa and disrupts normal motility patterns leading to diarrhea (Mair 2002a). The most common clinical signs at presentation included colic (chronic or acute), weight loss, diarrhea, abdominal distension, decreased appetite and poor exercise performance (Mair 2002a, Granot et al. 2008). Sand colic can be confirmed with gastrointestinal auscultation, rectal palpation, faecal evaluation for presence of sand and ultrasonography (Rouhoniemi et al. 2001, Korolainen et al. 2002, Rakestraw and Hardy 2012). However, radiography can be very helpful in determing both the amount of accumulated sand in the colon and the clearance of it following treatment (Rouhoniemi et al. 2001). Sand enteropathy or impaction can be treated with either conservative or surgical therapy. Pharmaceutical therapy includes intravenous or/and per os fluid administration, pain relief, antibiotic therapy and laxatives to promote the clearance of sand. Prognosis of sand colic cases is fair to good (Sanchez 2018).

According to the authors' knowledge, there have not been any reports of equine sand colic cases in Greece so far. The purpose of this study was to present cases of sand colic, presented to the School of Veterinary Medicine of the Aristotle University of Thessaloniki from 2014 to 2018. Aetiology, clinical signs, diagnosis and therapeutic protocol of the cases are described and discussed.

MATERIALS AND METHODS

Six horses, diagnosed with sand colic, were included in this study. These horses were presented to the School of Veterinary Medicine of Aristotle University of Thessaloniki between the years 2014 - 2018. Their age spans from 7 to 19 years (Table 1). All of them were living in the area of Northern Greece. According to their history, the majority of the horses were referred because of mild to severe colic with duration from 48 hours to 8 days and diarrhea (Table 1). Most importantly, these horses lived in fields with sandy soil or were fed in sandy paddocks. All the information about breed, gender, clinical findings and outcome are described in Table 1.

On clinical examination dehydration 5- 10%, and abnormal motility of the gut were mainly noted (Table 1). The findings upon abdominal auscultation are also thoroughly described in Table 1. Rectal palpation revealed gassy dilation or impacted segment in a part of the colon. Abdominocentesis was performed in only one case because of the severity of symptoms. The peritoneal fluid was modified transudate. Although haematology results were normal in two cases, in the majority of them (4/6) lymphopenia was noted (0.4-1.0 K/μl). Biochemical profiles were normal with no signs of hepatic or kidney failure. The diagnosis in all 6 horses was based on the sand identified in the faeces and the characteristic sound of "pouring sand" during the auscultation. In order to identify sand in the faeces, 2-3 faecal balls were diluted in water within a container or a rectal glove. The "solution" was left to rest for a few minutes up to 2 hours, leading sand to sediment at the bottom of the container.

Conservative therapy was elected in 3 horses, while surgery was recommended in the other 3. Despite our recommendation, the owner of one horse declined surgical treatment, therefore, conservative treatment was pursuit.

The medical therapeutic protocol (4/6) included the administration of a) fluids, lactated Ringer's (10 litres) and dextrose 5% (5litres) iv BID for 5 days, b) flunixin meglumine (1.1 mg/kg b.w. iv BID for 7 days and then SID for another 5 days) (Niglumine®, CALIER), c) antibiotics such as penicillin-streptomycin (20.000 IU im SID for 10 days) (Pen & Strep, Norbrook) with either metronidazole (15mg/kg b.w. p.o. BID for 5 days) (Flagyl®, Pfizer) (3/6) or trimethorpim-sulfonamide (30mg/kg b.w. im BID for 7 days) (Borgal®, Virbac) (1/6). In the treatment protocol vitamin B complex (10ml iv SID for 5 days) (Catosal®, Bayer), probiotics (Equigest Plus, Equiplanet) and also clanobutin sodium (10ml iv SID for 5 days) (Bykahepar®, MSD)

were included. Psyllium (250gr p.o. BID) (Flohsamen Psyllium, Waldhausen) along with 1litre of paraffin oil was considered the cornerstone of the medical management. These 2 laxatives were given to horses via nasogastric tube with 10 litres of water on a daily basis. Once appetite was restored, psyllium was offered to horses per os, with or without food.

Two horses were treated surgically. Abdominal laparotomy was performed under general anaesthesia in dorsal recumbency. A pelvic flexure enterotomy was performed, according to what has been previously described (Rakestraw and Hardy 2012) (Figure 1 and 2). Postoperative treatment was mostly similar to those patients treated pharmaceutically. Horses undergone surgery were given marbofloxacin (2mg/kg b.w. iv SID for 10 days) (Marbocyl 10%, Vetoquinol) instead of trimethoprim sulphonamide or metronidazole.

Table 1: Summary data for horses included in our study.

Case	Horse breed, age (years), gender	Symptoms	Clinical Findings	Findings upon Rectal Palpation	Treatment	Recovery
1	Fresian, 7, gelding	anorexia colic diarrhea weight loss	pyrexia dehydration 5-10% endotoxemia hypomotility of left, right colon and cecum	impaction in right dorsal colon with cecum tympany	conservative (even though surgical treatment was strongly recommended)	no
2	Greek, 11, gelding	colic diarrhea	tachycardia dehydration 5-10% sound of ‘’pouring sand’’ decreased frequency of borborygmi at cecum and hypermotility of the left colon	gassy dilation of colon	conservative	yes
3	Fresian, 16, stallion	anorexia colic diarrhea	tachycardia dehydration 5-10% sound of ‘’pouring sand’’ decreased frequency of borborygmi at cecum and hypermotility of the left colon	gassy dilation of colon	conservative	no
4	Warmblood, 9, mare	colic diarrhea weight loss	dehydration 5-10% hypomotility of left colon while in right colon and cecum the motility was normal	doughy impaction in left dorsal colon without cecum tympany	conservative	yes
5	Cob, 19, mare	anorexia colic diarrhea	tachycardia dehydration 5-10% endotoxemia sound of ‘’pouring sand’’ decreased frequency of borborygmi at cecum and hypermotility of the left colon	doughy impaction in cecum	surgical	yes
6	Warmblood, 13, mare	anorexia colic diarrhea weight loss	tachycardia dehydration 5-10% endotoxemia sound of ‘’pouring sand’’ decreased frequency of borborygmi at cecum and hypermotility of the left colon	impaction in right dorsal colon with cecum tympany	surgical	no

*** All six cases lived in sandy soils or were fed in sandy paddocks, according to their history.**

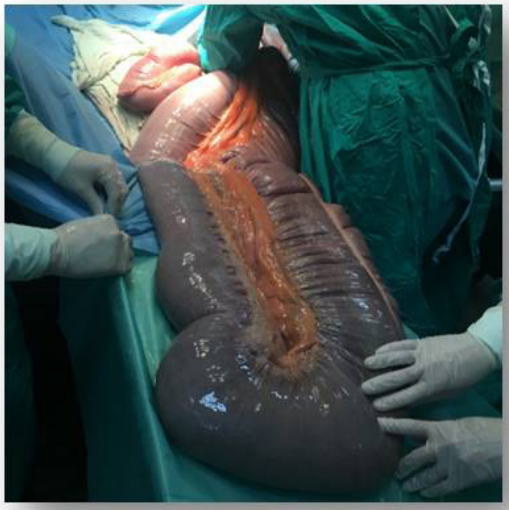

Figure 1: Sand colic surgery. Pelvic flexure enterotomy was performed.

Figure 2: Sand removal from the lumen of the colon.

RESULTS

Horses treated pharmaceutically were hospitalized in the School of Veterinary Medicine of the Aristotle University of Thessaloniki for 5 up to 20 days, depending on their clinical improvement. Two of 4 horses recovered fully, while the other 2 did not survive. Still, it is worth noting that one of these 2 horses followed the conservative protocol despite our recommendation for surgery, due to financial constraints (Table 1). One horse that underwent surgery survived while the other developed postoperative peritonitis and died.

DISCUSSION

Horses living in areas with sandy soil or selectively ingest gravel or dirt, are more prone to episodes of sand colic (Rakestraw and Hardy 2012, Hart et al. 2013, Niinisto et al. 2014). Acute ingestion of big amount of sand is an uncommon cause of sand colic (Mair 2002a). Poor pasture management, inadequate nutrition, dry weather, overstocking and feeding horses in paddocks can all result in horses consuming significant quantities of sand (Mair 2002a). It has been proven that ~80% of the consumed sand can be excreted within 5-11 days, while the other ~20% can remain in the lumen of the colon (Husted et al. 2005). This remaining amount of sand can eventually lead to an obstruction in a part of the gastrointestinal tract (Husted et al. 2005). This retrospective study is the first report of sand colic in Northern Greece. The small number of cases are maybe due to the fact that Northern Greece's soil is not sandy and drought or even because many veterinary practitioners are not familiar with this type of colic. However, poor management of the equine stables can lead to the appearance of sand colic, even in Greece. Interviewing horse owners of all 6 cases presented in our clinic revealed many management flaws, especially as far as feeding is concerned. In most cases, horses ate directly from a soil ground. In other words, although cases referred to our Hospital did not live in coastal regions where horses would graze on sand, problems arose because owners kept their horses in paddocks artificially covered with sand. Moreover, they would feed their horses on such grounds. According to literature, the majority of horses suffering from sand colic are more than 1 year old, like in this study (Rakestraw and Hardy 2012). However, many cases of sand colic in foals have been reported, due to pica (Rouhoniemi et al. 2001, Rakestraw and Hardy 2012). In our study no foal was presented with symptoms of sand colic, maybe due to the fact that the few equine reproductive farms of Northern Greece do not breed horses on sandy pastures. As mentioned before, all our cases were related to owners' mistakes.

Horses with sand colic are typically presented with

mild to severe abdominal pain, continuous or intermittent diarrhea, reduced faecal production (sand impaction), weight loss, decreased appetite, intermittent or continuous pyrexia, mild to severe dehydration, normal to gradually elevated heart rate and respiratory rate (Mair 2002a, Granot et al. 2008, Rakestraw and Hardy 2012). In cases with severe inflammation and irritation of the intestinal mucosa tachycardia, tachypnea, congested mucous membranes, prolonged capillary refill time and toxic rim at the gum margin may be seen (Mair 2002a). The horses of the study were admitted in our clinic with signs of mild to severe abdominal pain, diarrhea and dehydration. Three of them had signs of endotoxemia, such as congested mucous membranes and toxic rim at the incisor margin.

Intestinal mucosal inflammation and irritation exists in almost, every equine colic case (Mair 2002a, Sanchez 2018). In sand colic cases, the sand or the gravel accumulates in the gastrointestinal lumen. This accumulation has been described as "coffee sediment". This irritation influences the rate of the secretion and absorption, leading usually to intestinal hypersecretion and malabsorption (Mair 2002a, Sanchez 2018). Colonic absorptive processes are limited to surface epithelial cells, whereas secretory processes are a function of the crypt epithelium (Sanchez 2018). According to this, the sand can cause intestinal motility dysfunction leading to profuse, exudative diarrhea (Mair 2002a, Sanchez 2018). Severe mucosal inflammation and irritation may result in endotoxemia and secondary peritonitis that can be septic if bowel perforation occurs (Mair 2002a, Sanchez 2018). Chronic consumption of sand may lead to colonic impaction, although there are cases that successfully passed large amounts of the intestinal sand. The intestinal distention with gas before the impacted area is the main reason of pain during an episode of equine colic (Sanchez 2018). In our study 2 horses had gassy distention in a part of the colon, while another 2 had cecum tympany with an impacted segment of colon. In the rest of the cases (2/6) no gassy dilation was palpated. The sand may accumulate in any part of the gastrointestinal tract (Rouhoniemi et al. 2001). Usually, the majority of the sand is gathering in the right dorsal colon, transverse colon and cecum (Rouhoniemi et al. 2001, Mair 2002a). On the contrary, stomach, left dorsal colon, small intestine, descending colon and pelvic flexure have been rarely referred as sites of sand accumulation (Rouhoniemi et al. 2001). Coarse sand usually accumulates in the dorsal colon, whereas fine sand tends to accumulate in the ventral colon (Mair 2002a). In our study, impacted mass was found in cecum (1/6), in right dorsal colon (2/6) or in left dorsal colon (1/6). In 2 horses, doughy mass was palpated, while in the other 2 horses the impaction was hard.

Auscultation of the abdomen, sand sedimentation test, abdominal ultrasound, rectal palpation and abdominal radiography can help with the diagnosis of sand colic. The characteristic sound of "pourring sand" can be usually heard caudally to the xiphoid region (Mair 2002a, Husted et al. 2005, Rakestraw and Hardy 2012). This sound was present in 4 of our cases. Moreover, our auscultation findings revealed either hypomotility or hypermotility in different parts of the colon. In a study of 59 horses with sand colic, 33, 8% had normal intestinal gut sounds, 55,9% had hypermotility and 10,1% had hypomotility (Hart et al 2013). It is obvious that abdominal auscultation is not a reliable diagnostic test for this disease.

Peritoneal fluid can be a useful diagnostic tool, because it can show an elevation in total proteins (Mair 2002a, Rakestraw and Hardy 2012). According to many authors, abdominocentesis should be performed with extreme caution as there is a high risk of enterocentesis (Mair 2002a, Sullins 2017). Abdominocentesis was deemed necessary and performed in only one, critical case. Peritonitis was ruled out and the horse was treated medically. Abdominal radiology is the best way of sand colic diagnosis, since the amount of sand within the intestinal lumen can be approximately measured. Also, the effectiveness of the treatment can be monitored by measuring the quantity of the sand coming out from the gastrointestinal tract (Rouhoniemi et al. 2001, Rakestraw and Hardy 2012, Niinisto et al. 2014, Niinisto et al. 2018). Abdominal x rays can replace rectal palpation both in foals and ponies (Rouhoniemi et al. 2001, Mair 2002a). Abdominal ultrasonography, is useful in assessing the motility and peristalsis of the intestines but cannot assist in establishing a final diagnosis. Unfortunately, in this study, abdominal radiology was not performed as the size of the horses and the lack of large radiographic units was discouraging.

As in most colic cases, conservative treatment in sand colic consists of: non steroidal anti-inflammatories, intravenous or per os fluids and broad spectrum antibiotics. Having said that, the most important part of the treatment is the administration of laxatives such as paraffin oil, psyllium, magnesium sulphate and Dioctyl Sodium Sulfosuccinate (DSS) (Landes et

al. 2008, Kilcoyne et al. 2017). Psyllium hydrophilic mucilloid is a natural product, produced by *Plantago spp* seeds (Sullins 2017). Psyllium (as powder or flake) is a polysaccharide forming a sand-psyllium mucilloid mixture in the intestinal lumen (Mair 2002a). This bulk laxative hydrates intestinal contents and stimulates intestinal peristalsis, promoting intestinal evacuation (Mair 2002a, Baljit 2007). Psyllium can increase intestinal motility, by temporarily stimulating muscarinic and serotonergic receptors (Mair 2002a, Niinisto 2018). At the same time, intestinal calcium receptors get blocked causing suppression of the intestines' motility (Niinisto 2018). Arguments against the efficacy of psyllium usage are based on these 2 antagonistic ways of action (Niinisto 2018). The long-term use of psyllium can cause alterations in the normal gastrointestinal flora. For this reason, the concurrent use of probiotics has been advised in horses treated with psyllium (Rouhoniemi et al. 2001, Baljit 2007, Landes et al. 2008). Probiotic powder was given in all 6 cases of our study. Psyllium can be administered to horses suffering from enteropathy or impaction due to sand (Mair 2002a, Landes et al. 2008, Rakestraw and Hardy 2012). Many researchers argue about the optimal dose of psyllium that can be given per os or via nasogastric tube. The latest records indicate that the effective dose is 0,5- 1g/kg bw/24h (Rouhoniemi et al. 2001, Mair 2002a, Niinisto et al. 2014, Niinisto 2018). Taking into account all the above, we decided to add psyllium in the medical treatment protocol of the horses. Paraffin oil can promote sand excretion from the gastrointestinal tract (1-4 litres SID or BID, with 4-8 litres of water). Its effectiveness has been doubted, due to the fact that it may not go through the impacted mass (Rouhoniemi et al. 2001, Mair 2002a, Husted et al. 2005, Kilcoyne et al. 2017). According to previous reports, the combination of psyllium and paraffin oil can increase the excretion of the sand from the gastrointestinal tract, even if there is a large amount of sand (Rouhoniemi et al. 2001, Mair 2002b). Taking into consideration all the above, we decided to treat these horses with a combination of psyllium (250gr BID) and paraffin oil (2 litres BID with 8-10 litres of water).

Other types of laxatives that can be used in the treatment of sand colic are magnesium sulphate (Epsom salts) and DSS. Magnesium sulphate acts as an osmotic laxative, by moving water from the intestinal mucosa to the intestinal lumen (Rouhoniemi et al. 2001, Niinisto et al. 2018). Also, it can increase the motility of the intestines (Rouhoniemi et al. 2001, Niinisto et al. 2018). Magnesium sulphate is more effective in the small intestine rather than the colon, where sand usually accumulates (Murray 2004). It should be used continuously in order to be effective; in particular, it should be used daily for 3 days. If need be, this treatment can be repeated after 7 days (Rouhoniemi et al. 2001). Big and continuous amounts of magnesium sulfate may cause severe dehydration, colitis or even magnesium toxicosis (Murray 2004). The recommended dose is 0,5g/kg-1g/kg bw (Rouhoniemi et al. 2001, Niinisto et al. 2014, Niinisto et al. 2018). So, we decided not to add magnesium sulphate (Epsom salts) in the treatment protocol, considering the part of the gastrointestinal tract being affected by sand and the possible adverse effects of long-term use. DSS is a synthetic derivative that reduces surface tension and allows water to penetrate impacted material, increases intestinal secretion and alters mucosal permeability (Rouhoniemi et al. 2001, Murray 2004). The recommended dose is 10-25mg/kg bw diluted in 4-8 litres of water (Rouhoniemi et al. 2001, Murray 2004). High doses may cause mild to severe diarrhea and abdominal pain or even laminitis, endotoxemia and tachycardia (Rouhoniemi et al. 2001, Hotwagner and Iben 2008). Also, vitamin B complex and phosphorus were administered to horses as supportive treatment due to poor nutrition, weakness and exhaustion. Stimulation of hepatic function by promoting the bile secretion was attempted by the administration of clanobutin sodium in all 6 cases.

The decision for surgical treatment in a colic case has to be taken considering many clinical and laboratory factors. The patients' response to pain, heart rate, respiratory rate, temperature, intestinal motility and the gastric reflex should be taken into account as well as blood lactic acid (>3mmol/litre) and glucose (>180mg/dl) (Cook and Hassel 2014). The success rate in horses with sand impaction that underwent early exploratory abdominal laparotomy, was 75%-95% (Granot et al. 2008). On the contrary, the prognosis and the survival rate of horses receiving conservative treatment for some days and then treated surgically was poor (Hart et al. 2013). Meaning that, the decision for surgery has to be made when the equine patient is still stable before severe mucosal necrosis occurs (Granot et al. 2008, Hart et al. 2013). The decision to perform abdominal surgery is challenging, as in every colic case. It has to be made taking into consideration a number of clinical and laboratory parameters. Rectal palpation can be very simple and important diagnostic tool, but not so accurate, as impaction cannot always

be palpated (Husted et al. 2005, Cook and Hassel 2014, Kilcoyne et al. 2017). Distension in the ascending colon or cecum can be palpated in 51% of horses with sand impaction (Granot et al. 2008). Horses with obvious impaction in the colon diagnosed using abdominal radiology are far more likely to undergo exploratory laparotomy, when the appropriate equipment is available. In this study, surgical treatment was recommended in 3 of the cases. In 1 of these cases, surgery was strongly recommended due to the severe endotoxemia. In the other 2 horses, exploratory laparotomy was carried out due to failure of conservative treatment. In total, one horse survived and 2 died. We feel that the small survival rate in this study was a result of horses not being operated at all (1/3) or being operated in a critical stage (2/3). This is may be due to the fact that horses in Greece are not insured and horse owners are extremely reluctant to take up the financial burden of colic surgery.

Minimizing exposure to sand is important in preventing recurrence. Nutritional management of sand colic cases varies from highly digestible forage or concentrated feed for 2-3 weeks after sand has been excreted, to hay ab libitum, to no dietary change at all (House and Warren 2016). Administration of a moist bran mash containing 450gr psyllium, once a week, is recommended as good prophylactic measure to prevent the occurrence of sand impaction colic in horses exposed to sand (Mair 2002b). In order to prevent a recurrence, horses should not feed from the ground, but rather on a rubber mat, from a manger or raised buckets (Mair 2002b). Grazing in fields with not adequate grass seems to be a risk factor of sand ingestion (Mair 2002a). Also, a monthly faecal sample from horses with predisposition in sand colic should be examined in order to monitor this condition. In this study, horses discharged from our clinic (3/6) follow all the above preventing measures. At the same time, administration of psyllium (100gr p.o. BID) was advised, combined with moist bran mash (2-3kg/day) for 7 days and then psyllium (450gr p.o.) once a week as prophylactic measurement. Two years following discharge all horses are alive.

CONCLUSIONS

According to this study, sand colic should not be considered as an ‘exotic disease’ in Greece. For this reason, sand colic (impaction or enteropathy) should be included in the differential diagnosis of cases with chronic diarrhoea and/or abdominal pain.

CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Feeding with heat-killed *Gordonia bronchialis* affects growth performance, intestinal morphology and immunomodulation in Japanese quail (*Coturnix coturnix japonica*)

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ABSTRACT: Studies have shown that bacterial immunomodulators based on aerobic Actinomycetales such can switch off pre-existing Th2 preponderance and prompt Th1-mediated mechanisms, whatever the previous environmental immune priming of the individual. *Gordonia bronchialis* (*G. bronchialis*) is a Gram-positive, weakly acid-fast species of the genus Gordonia within the sub-order Corynebacteriaceae of the order Actinomycetales. In this study, the efficacy of heat-killed *Gordonia bronchialis* (*G. bronchialis*) on growth performance, immune system and intestinal structure in quail (*Corurnix coturnix japonica*) was evaluated. Quails (mean weight 7.8 g) were fed basal diet (control), or treatment diets containing 10^5 (low dose) or 10^6 (high dose) CFU per bird per day in food of heat-killed *G. bronchialis* continuously (for 42 days). Body weight gain (BWG) and feed consumption was recorded during grower period and finisher period. On days 7, 14, 28, 35 and 42 some of the quails were sampled for analysis of Newcastle antibody titer.Interleukin-4 (IL-4), Interferon- α (IFN-α), and interferon- γ (IFN-γ) concentrations were analyzed using ELISA kits. An indirect ELISA was performed to quantifying IgA. At the end of 14, 28 and 42 days old, three chicks from each group were selected for histopathological and histomorphometrical studies. Results showed that growth performance was significantly enhanced in both treatment groups compared with the control group. Serum anti Newcastle disease virus, IL-4 and IFN-α titers were higher in low dose treatment group compared with the control group. The length of the intestinal and pyloric caeca folds was increased in the high-dose group. Meanwhile, jejunum and ileum showed the most significant morphological changes in different days of sampling, particularly in high dose group. Among the evaluated factors, villous length and intestinal crypt depth demonstrated more significant differences. This study suggests that heat-killed *G. bronchialis* has the potential to enhance growth, immunological parameters and the intestinal structure in Japanese quail.

Keywords: Japanese quail; Growth; Newcastle disease; Interferon; *Gordonia bronchialis*

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INTRODUCTION

Newcastle Disease (ND) is one of the most devastating diseases of the domestic fowl, which can cause high level mortality of these animals. ND is caused by Newcastle Disease Virus (NDV), an avian Paramyxovirus type 1 (APMV-1) that belongs to the genus *Avulavirus*, family *Paramyxoviridae* (Silva *et al*., 2010). The NDV's are classified into four pathotypes; asymptomatic enteric, lentogenic, mesogenic, and velogenic (Rehman et al., 2018).

Despite the importance of the antibody-mediated response for protecting against NDV infection, the innate immune response induced by NDV challenge remains unclear. The host innate immune system provides the first line of defense against pathogens. The innate immune system can recognize components of pathogens called pathogen-associated molecular patterns (PAMPs) recognition receptor (Zhang et al., 2018).

The commercial production of Japanese quails (*Coturnix coturnix japonica*) is extensively distributed in several countries around the world and many studies showed that this species can easily adapt to commercial management conditions, with good performance in terms of meat and egg production (Lima et al., 2004). However, there is little information available on health control programs in this species. In addition, as today happens with broilers and turkey, quails will probably be intensively produced and the high bird concentration in some areas may cause the dissemination of infectious disease.

Currently, organic farms and foods are important to humans because the excessive use of antibiotics for treatment of diseases and animal husbandry has led to drug resistance in infectious agents, raising interest in products derived from nature to prompt human and animal health. Studies have shown that bacterial immunomodulators based on aerobic Actinomycetales such as *Mycobacterium vaccae* (*M. vaccae*) can switch off pre-existing Th2 preponderance and prompt Th1-mediated mechanisms, whatever the previous environmental immune priming of the individual (Tarreset al., 2012). Among the aerobic, near mycobacterial genera, within the Actinomycetales, are some species with adjuvant activities and antigens very similar to those of *M. vaccae*, but with subtle differences. *Gordonia bronchialis* (*G. bronchialis*) is a Gram-positive, weakly acid-fast species of the genus Gordonia within the sub-order Corynebacteriaceae of the order Actinomycetales. It is an environmental organism that rarely gives raise to human infections (Arenskotter et al., 2004). Killed preparations of *G. bronchialis* and some other genera within the order Actinomycetales are potent immune modulators useful in the prevention and treatment of many immune-related diseases in laboratory animal and veterinary medicine (Fontanella et al., 2007; Davila et al.,2011; Stanford and Stanford,2012). Rats treated with *G. bronchialis* and challenged with live *Trypanosomacruzi* show significantly reduced parasitaemias and less chronic myocarditis (Stanford and Stanford, 2012). *G. bronchialis* enhances growth and immunity in rainbow trout (Sheikhzadeh et al., 2016) and decreases the malondialdehyde (MDA), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) levels in serum of rainbow trout (Shabanzadeh et al., 2016).

Since there is no information about the effect of *G. bronchialis* on quail, in the present study the effect of dietary inclusion of heat killed *G. bronchialis* on the serum anti NDV titer, serum Immunoglobulin-A (IgA) titer, and serum cytokines [interleukin-4 (IL-4), interferon-α (IFN-α) and interferon-γ (IFN-γ)] of Japanese quail was investigated. Furthermore the growth performance and intestinal structure of Japanese quail fed with heat-killed *G. bronchialis* was also determined.

MATERIALS AND METHODS

Animal care and experimental design

Unsexed Japanese quails were obtained at one day of age acclimated to laboratory surroundings for one week before immunizations and measurements were begun. Quail were housed in cages measuring 61 × 56 × 81 cm. Temperature in the animal room was maintained at 23˚C. Food and water were available *ad libitum*. Quails were fed bird food containing approximately 20% crude protein that contained no coccidiostat or other medications (Supplementary file 1). Fluorescent lights provided a photoperiod of 12 h light and 12 h dark. The University of Tabriz Animal Care and Use Committee approved all protocols (FVM.REC. 1395.58).

Seventeen Japanese quails were assigned randomly to each of nine groups in a 3 × 3 factorial design with three bacterial treatments. The quails were randomly assigned to each treatment, because sexing could not be done until later development. The bacterial treatments consisted of (1) a control or no bacteria group, (2) a low dose group, and (3) a high dose group. The low- and high dose groups received 10^5 or 10^6 CFU per bird per day in food, respectively, from the first day of age.

Growth performance and sample collection

Body weight gain (BWG) and feed consumption was recorded during grower period and finisher period. On d 14, 28 and 42, two birds closet to the median weight from each pen (6 per treatment) were randomly selected, weighed, stunned and slaughtered by exsanguination. Bursa of Fabricius, and spleen were then precisely removed and weighed separately on a sensitive digital scale. Blood samples were taken from wing vein and centrifuged for 15 min at 1250 × *g* and 4 ˚C. Serum was collected and stored refrigerated at -20 ˚C pending analysis.

Immunological responses in serum

Interleukin-4 (IL-4), IFN-α, and IFN-γ concentrations were analyzed using ELISA kits (Cusabio, USA) following the manufacturer's instructions. An indirect ELISA was performed to quantifying IgA. The commercial chicken IgA ELISA quantification set (Cusabio, USA) was used according to manufacturer's instructions. Antibody titers against NDV was measured by haemagglutination-inhibition (HI) test according to Sun et al. (2018), and using ELISA kits (IDDEX, USA) following the manufacturer's instructions. Haemagglutination-inhibition results were expressed as $\log_2$ of the reciprocal of the last dilution.

Intestinal morphology development

At the end of 14, 28 and 42 days old, three chicks from each group were selected for histopathological and histomorphometrical studies. The samples were taken from different organs including liver, kidney, heart and brain (to evaluate the hepatotoxicity, nephrotoxicity, cardiac toxicity and neurotoxicity or other side effects of *G. bronchialis*, respectively). Besides, different parts of the small intestine (duodenum, jejunum, and ileum) were obtained for histomorphometrical study.The mentioned tissues were fixed at 10% buffered formalin, embedded in paraffin, sectioned at about 5 µm, stained with hematoxylin and eosin and studied microscopically with a light microscope (Olympus-CH30, Japan). According to previous studies (Sakamoto et al. 2000; Aptekmann et al. 2001), the measured morphometric variables included: villous height (measured from the villous-crypt junction), villous thickness (measured at mid-villous height), intestinal crypt depth (measured from the villous-crypt junction until the end of glands), intestinal crypt number, and goblet cell number.

Statistical analysis

The results were expressed as means ± standard error of mean (SEM) and all data were statistically analyzed by one-way ANOVA, using SPSS version 22.0 software for Windows (SPSS Inc., Chicago, IL). Differences between treatment groups were tested by LSD test, and differences were significant at $P< 0.05$.

RESULTS

The decrease in dietary *G. bronchialis* content from 10^6 to 10^5 Bacilli/Bird/Day caused a significant ($P< 0.05$) decline in weight gain (Table 1); feed conversion efficiency; however, was not affected by dietary *G. bronchialis* level.

The level of the IL-4 cytokine, which corresponded to Th_2 cytokines in birds, significantly increased following stimulation with *G. bronchialis* in low dose group compared to the control. IFN-α concentration was enhanced at both treatment groups (Figure 1).

The HI serum antibody results from day 1, 7, 14, 21, 28, 35, and 42 are given in Table 2. The findings by the ELISA method are given in Table 3. Although higher titers were obtained when *G. bronchialis* was used in high dose in day 42, the statistical analysis did not reveal differences between different treatments.

The effect of dietary *G. bronchialis* on serum IgA is presented in Figure 2. As the dietary *G. bronchialis* enhanced, IgA rose on day 7 (low dose group) and day 42 (high dose group), significantly.

Spleen and bursa of Fabricius were increased in relative weights as a consequence of increasing dietary *G. bronchialis* (Table 4). Both relative spleen and bursa weight increased.

Microscopically, there was not hepatotoxicity, nephrotoxicity, cardiac toxicity, and neurotoxicity or other side effects in the liver, kidney, heart, and brain, respectively. The most morphological changes were observed between low dose and high dose groups with the control group, which was seen more on day 42 of sampling in comparison. Interestingly, jejunum and ileum showed the most significant morphological changes in different days of sampling, particularly in the high dose group. Among the evaluated factors, villous length and intestinal crypt depth demonstrated more substantial differences (Figures 3 and 4). On the 42nd day of sampling, there were also significant differences in the goblet cell number between various groups. While, intestinal crypt numbers showed less morphological changes in both experimental groups.

Table 1. The effect of *Gordonia bronchialis* administration on performance parameters on different days in various experimental groups of Japanese quail

Days	Parameters	groups		
		Control	Low dose	High dose
1-14	BWG	53.7±0.89	58.7 ± 0.95	56.6 ± 1.50
	FI	126.41	121.19	120.51
	FCR	2.35	2.06	2.12
1-28	BWG	128.9±0.96[a]	126.7 ± 1.69[a]	140.1 ± 0.94[b]
	FI	318.92	294.09	294.11
	FCR	2.47	2.32	2.09
1-42	BWG	208.6±1.10[a]	209.4±0.92[a]	219.8 ± 1.16[b]
	FI	575.38	532.06	530.55
	FCR	2.75	2.54	2.41

BWG: body weight gain (mean ±SD, g/bird); FI: feed intake (g/bird); FCR: feed conversation ratio.
[a-b]Means within a row with no common superscripts differ significantly ($P \leq 0.05$)

Table 2. Effects of dietary heat-killed *Gordonia bronchialis* on antibody titers to Newcastle disease virus (log_2) in Japanese quail (Haemagglutination Inhibition test) [1]

Sampling day	Groups		
	Control	Low dose	High dose
d 1	3.77 ± 1.56	3.77 ± 1.56	3.77 ± 1.56
d 7	1.77 ± 0.44	1.55 ± 0.52	1.77 ± 0.66
d 14	2.55 ± 1.94	2.88 ± 2.26	2.66 ± 2.54
d 21	3.66 ± 2.59	4.88 ± 1.61	4.44 ± 2.74
d 28	4.55 ± 2.69	5.55 ± 0.72	5.77 ± 1.09
d 35	5.33 ± 2.54	5.77 ± 0.66	5.44 ± 2.65
d 42	6.33 ± 2.12	6.33 ± 0.5	7.33 ± 1.58

[1]Japanese quails were vaccinated with Newcastle disease virus vaccine at 10 and 20 days of age.

Table 3. Evaluation of immune response to Newcastle disease virus by ELISA method in different days in Japanese quail receiving 10^5 or 10^6 bacilli of heat-killed *Gordonia bronchialis* per day (mean ± SEM)

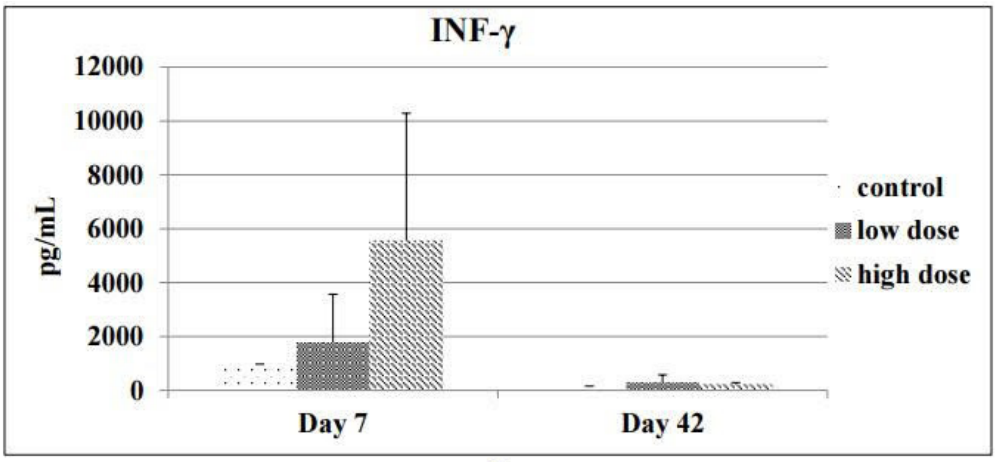
Group	Age (days)						
	D 1	D7	D 14	D 21	D 28	D 35	D 42
Control	2507.2 ± 1218.83	400 ± 0[a]	920 ± 83.66	1220 ± 228.03[a]	1736 ± 539.56	1779.2 ± 84.17	1916.6 ± 358.41
Low dose	2507.2 ± 1218.83	520 ± 130[a]	1300 ± 300	1620.2 ± 375.1[ab]	1966 ± 914.61	2164.8 ± 869.21	2187.4 ± 1413.28
High dose	2507.2 ± 1218.83	720 ± 178.88[b]	1620 ± 884.3	1903.6 ± 598.52[b]	2314 ± 1077	2458.6 ± 428.35	2953.8 ± 1902.85

[ab]: Different superscripts within columns indicate significant difference among doses of bacteria ($P < 0.05$).

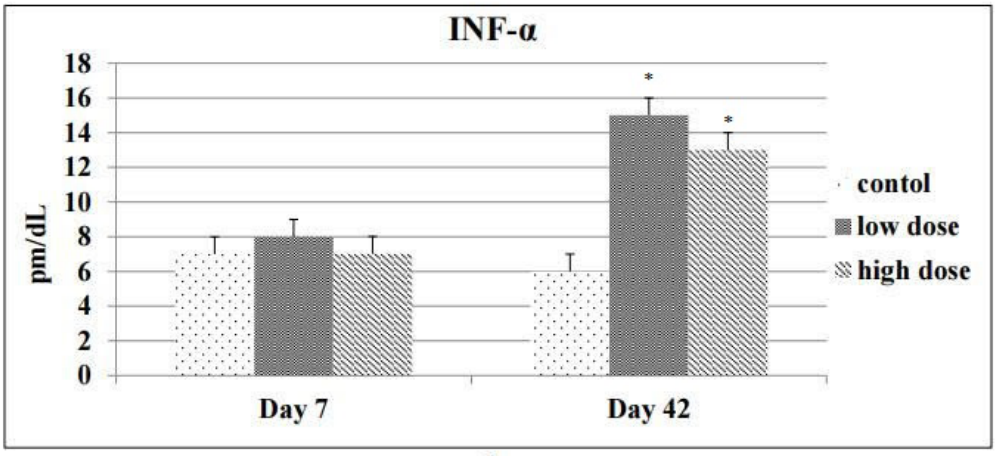
Table 4. Influence of varying dietary *Gordonia bronchialis* levels on relative lymphoid organ weights (% of live BW) at 14, 28 and 42 days of age (n = 4) (Mean ± SD)

Group	Day 14		Day 28		Day 42	
	Spleen	Bursa of Fabricius	Spleen	Bursa of Fabricius	Spleen	Bursa of Fabricius
Control	0.07 ± 0.00	0.07 ± 0.02[a]	0.08 ± 0.04	0.06 ± 0.01[a]	0.09 ± 0.00[a]	0.07 ± 0.02[a]
Low dose	0.07± 0.01	0.07 ± 0.01[a]	0.08 ± 0.00	0.06 ± 0.00[a]	0.09 ± 0.00[a]	0.08 ± 0.00[a]
High dose	0.07 ± 0.00	0.12 ± 0.00[b]	0.09 ± 0.00	0.13 ± 0.00[b]	0.12 ± 0.01[b]	0.14 ± 0.00[b]

Values within a column followed by different letters are significantly different (P < 0.05).



a



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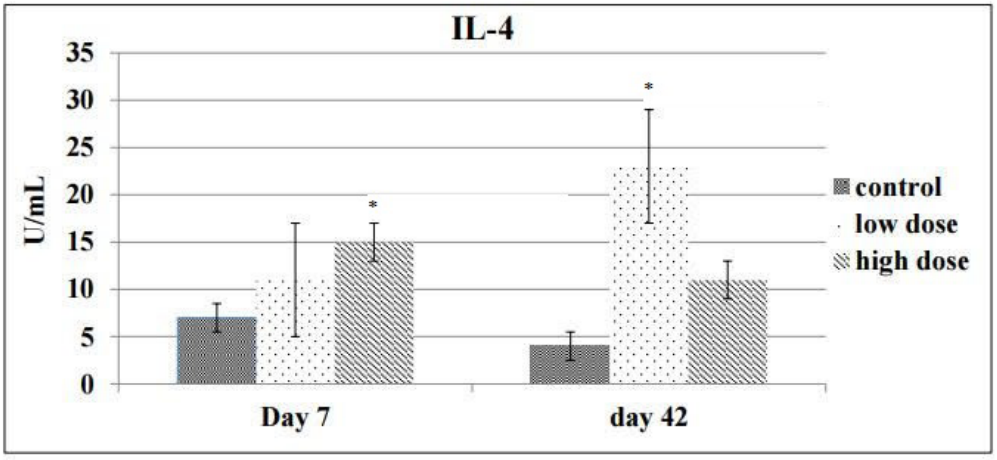
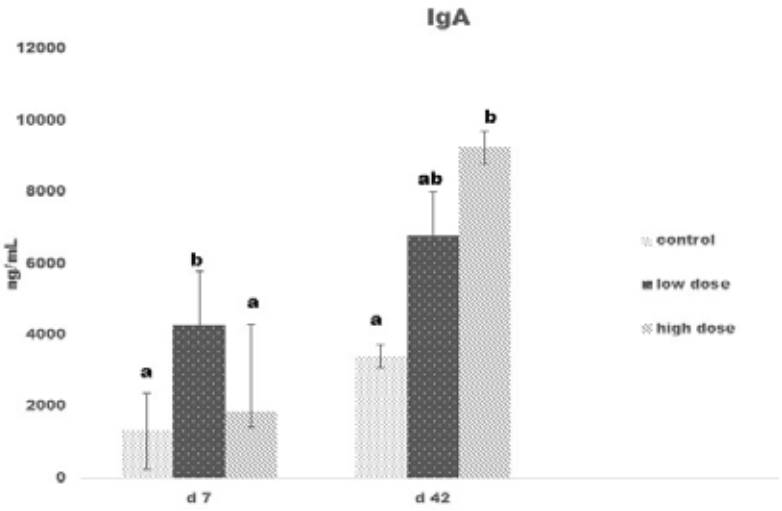


c

Figure 1. IFN-γ (a), IFN-α (b), and IL-4 (c) productions were measured in blood. The cytokine response to heat killed *Gordonia bronchialis* was compared with control group,*$p < 0.05$.



[ab]: Different superscripts within columns indicate significant difference among doses of bacteria ($P < 0.05$).

Figure 2. Ig A levels in different days from Japanese quail receiving low dose (10^5 cells) or high dose (10^6 cells) of heat-killed *Gordonia bronchialis* per day (ng/mL)

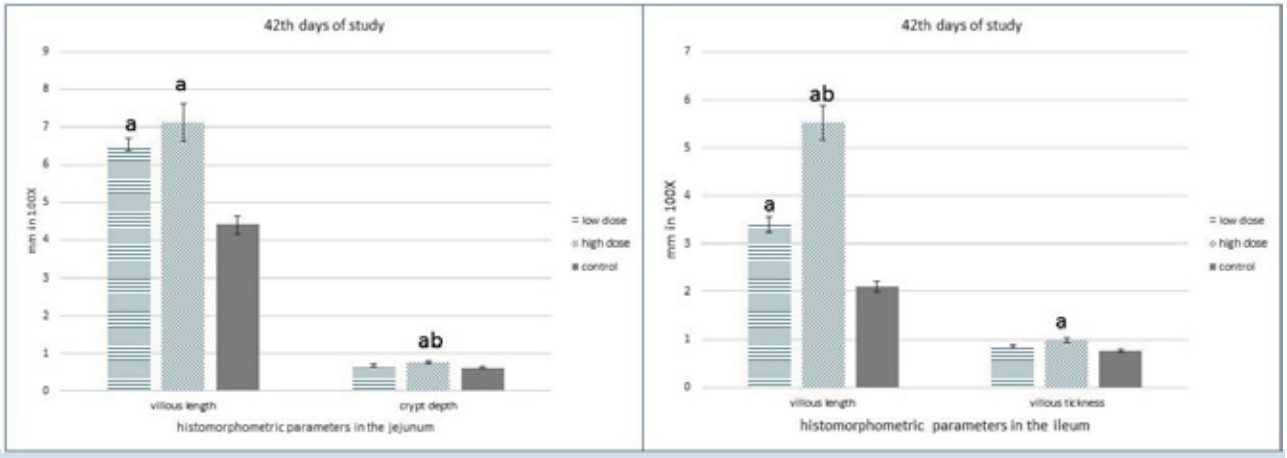


Figure 3. Significant differences ($P < 0.05$) in histomorphometric parameters between various groups on the 42nd day of sampling in the jejunum and ileum. a: significant statistical difference with the control group; b: significant statistical difference between low dose and high dose groups.

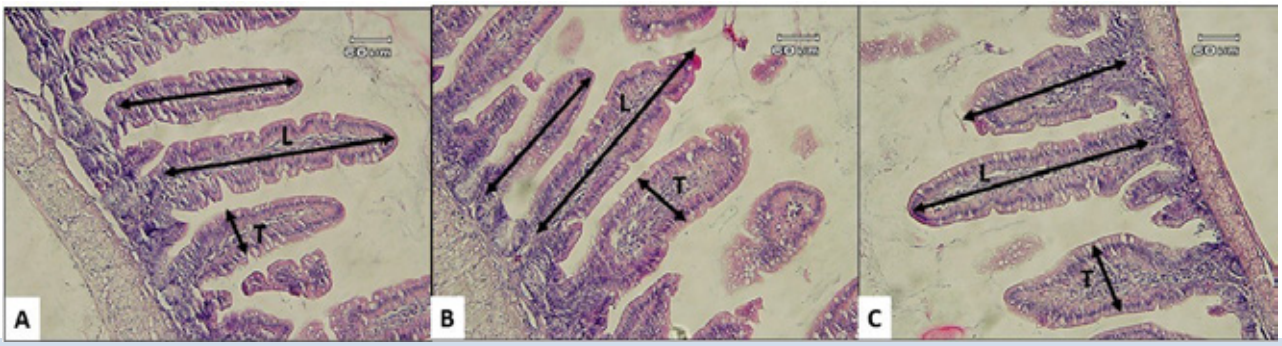


Figure 4. Small intestine (jejunum), Japanese quail. A: control group; B: low dose group; C: high dose group; a remarkable improvement was found in the small intestine of both treated groups. Indeed, there were the significant differences in the villous length (L) and thickness (T) of the small intestine in both *G. bronchialis*-recepient groups compared to the control group. H&E.

Supplementary file 1. Composition and nutrient contents of corn-soybean based diets

Feed ingredients	1-14 d	15-42 d
Ingredient (%)		
Corn	46.45	52.45
Soybean meal	48	42
Poultry fat	1.50	1.5
Dicalcium phosphate	1.2	1.2
Calcium carbonate	1.3	1.3
Salt	0.20	0.20
DL-Methionine	0.20	0.20
Vitamin premix	0.50	0.50
L-Lysine HCL	0.15	0.15
Mineral premix	0.5	0.5
Total	100	100
Calculated		
ME (kcal/kg)	2912	2910
CP (%)	23.1	20.2
Lys (%)	1.44	1.22
Met + Cys (%)	0.92	0.85
Calcium (%)	0.97	0.93
Nonphytate phosphorus (%)	0.49	0.47

DISCUSSION

With regards to immunomodulatory effects of Actinomycetes, there are some reports that Actinomycetes (*G. bronchialis*) enhances immune responses in rainbow trout (Sheikhzadeh et al., 2017). *G. bronchialis* also increases most parameters of blood profile including superoxide dismutase, GSH-Px, glutathione reductase in rainbow trout (Shabanzadeh et al., 2016). In current research, based on the absence of clinical signs, quails were considered healthy throughout the trials. This could be attributed to the low density of quails and high hygienic condition of cages used in the current study compared to commercial floor pens.

Dietary *G. bronchialis* supplementation improved weight gain. FCR value is less than control group without significant differences between treated and control group. In present study, it was observed that the use of *G. bronchialis* as an additive improved the fattening performance and there was dose response. In agreement with present observations, Shabanzadeh et al. (2016) indicated that heat-killed *G. bronchialis*inboth treatment groups enhanced the growth performance in rainbow trout similar to the resultsreported previously for Koi carp and shrimp (Stanfordand Stanford,2012).Shabanzadeh et al. (2016) observed that *G. bronchialis* increase in fish villi height and fold height in the intestine and pyloric caeca and therefore, may enhance the activity of digestive enzymes, resulting in higher nutrient absorption, better feed conversion ratio, and greater growth rate.

Interferons were so named due to their anti-viral properties. Our results confirmed the opinion of a number of researchers (Bailey et al., 2007; Karakolev et al., 2015), that bacterial endotoxins induce interferon synthesis after subcutaneous or intramuscular application.

The measurement of serum antibody titers to determine the potency of inactivated ND vaccines is a reliable alternative to the measurement of the protective dose 50% (PD_{50}) of these vaccines (Mass et al., 1998). Antibody titers increased following first vaccination (live) and reached the highest level on day 42 of age (three weeks post 2nd vaccination). In this study, which was performed with a design comparable with the *T. inchonensis* trials in quails, no differences between antibody titers of quails of treated groups and those of quails of the control group were shown, but in *T. inchonensis* trial, in low dose in day 42, there was significant difference (Nofouzi et al., 2019). There was also an obvious beneficial effect of Actinomycetes as immunomodulatory against chicken RBC in mouse, probably due to activation of

macrophages, induction of transcription of cytokine genes and release of inflammatory cytokines (Nofouzi et al., 2017). Immunoglobulin A, as the major class of antibody present in the mucosal secretions of most animals, represents a key first line of defense against invasion by inhaled and ingested pathogens at the vulnerable mucosal surfaces. IgA is also found at significant concentrations in the serum of many species, where it functions as a second line of defense mediating elimination of pathogens that have breached the mucosal surface. In this work, the numerically highest serum IgA was obtained by d 42, for high dose group. In fact, we showed long-lasting serum IgA response. Both treated groups showed higher IgA titers when compared to control at varying times throughout d 7 to 42. This implies that *G. bronchialis* may stimulate the humoral immune system to produce more antibodies. Because IgA is a non-inflammatory antibody that binds complement only weakly, it protects the tissues from excessive immune-mediated damage.

Our results suggested that *G. bronchialis* improved the immune response of quails at low *G. bronchialis* level, probably due to the up-regulation of IL-4 and IFN-α production. The changes of both Th1 and Th2 cytokines in our study could be attributed to the immunological balance and cross-regulatory effects between both inflammatory and anti-inflammatory cytokines, suggestion that *G. bronchialis* could maintain immune homeostasis and prevent further activation of immune system.

The immune system guards the body against foreign substances and protects from invasion by pathogenic organisms. The immune response against viral infection may affect the host defense against virus. The immune system is affected by not only infectious disease but also the sexual cycle, stress, and growth of animals. Therefore, a better understanding of the quail's immune system may also make quail a more useful experimental animal and improve their breeding in farms.

Immune tissue development can in some cases reflect immune response and functionality. Effects of control group and *G. bronchialis* supplementation on lymphoid organs are shown in Table 4. In the present study, quails which treated with high dose *G. bronchialis* had a significant increase in bursa of Fabricius and spleen weights, in day 42. The current observation indicates that the *G. bronchialis* needs for optimum cellular immune response may be higher than those for maximum growth rate.

The results of the current study demonstrated thatoral administrationof *G. bronchialis*improved the development of histomorphological structure of small intestine in Japanese quail, especially in high dose. Interestingly, the improvement was more effective on jejunum and ileum particularly in crypt length and thickness. Besides, the crypt number exhibited slight alteration. Recently, some researchers reported significant increase in the crypt depth due to using alphamune and biomin in broiler chickens (Erfani-Majd et al., 2013) which are in agreement with the results of the present study. In recent years, it is understood that greater villous height is an indicator that the function of intestinal villi is activated (Shamoto and Yamauchi, 2000). Moreover, it was stated that shortening of the villi and deeper crypts may lead to poor nutrient absorption, increased secretion in the gastrointestinal tract, and lower performance (Xu et al., 2003). Although, villous length did not show marked differences compare with other parameters in the present study. Also, the results of the present study showed a significant increase in goblet cell number in all parts of the intestine, especially in the 42th days of sampling. Similarly, a significant increase in the number of goblet cells and in mucin secretion on the surface of the jejunum villi had been observed when feeding broilers by a mixture of carvacrol, cinnamaldehyde, and capsicum oleoresin (Jamroz et al., 2006). It has been suggested that feeding wheat-based diet (containing enzyme Endofeed W (EEW) or growth promoters (thyme essential oil (TEO) or probiotic Primalac® (PP)) impact on intestinal histomorphology (jejunum and ileum) of broilers at 21 and 42 days of age (Khaksar et al., 2013), which is in agreement with the present results.

It seems that a period of adaptation is needed before the effects of *G. bronchialis* supplementation can be significant, because the changes in intestinal morphology and immune responses take time.

CONCLUSIONS

The results of the current study indicate that *G. bronchialis* improves growth performance and affects immune functions, cytokine level, and intestinal mucosal morphology of quails. Body weight gain was best for both *G. bronchialis* supplemented under the experimental conditions of this study. Immune function could be modified with dietary *G. bronchialis* supplementation. The present results suggest that oral administration of *G. bronchialis* (in low dose)can improve the histomorphological structure of the small

intestine without side effects in other vital organs in Japaneese quail environmental pollution, outburst of infectious disease and food safety concerns are there serious problems, which effected modern Iranian-farming industry. The current study gave us a cue of using *G. bronchialis* as an immunological stimulant of improve quail's resistance of disease. The multibeneficial effects of *G. bronchialis*, its easy access and the low cost all together made *G. bronchialis* a strong candidate in quail health feeding in Iran.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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Research article
Ερευνητικό άρθρο

Seasonal variation on bee venom collection. The impact on some biological aspects on *Apis mellifera*

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ABSTRACT: Bee venom was collected by electrical stimulation from *Apis mellifera* macedonica every six and twenty-four days respectively for two years.Collections were accomplished from April to October the first year, and from May to October the second year. The bee venom yield and the bees' behavior like the aggressiveness, the number of dead bees on the collecting device and the hoarding behavior were studied. A great variation was found among the colonies regarding the collected amount of bee venom. The production was high in spring, decreased in summer and increased again in autumn in both years. Two different tests were used to study the defensive response of honeybees. The rhythmic reflux of a leather ball in front of the hive and the test of rating assay. Both tests showed that bees' aggression did not significantly increase after collection. Furthermore, the aggressiveness of bees did not change during the period of collection. The average number of dead bees found on the wires of collecting device,was below 20 in each collection. Hoarding test indicates that no significant differences existed between before and after the stimulation of worker honey bee by electrical impulses.The collection of BV did not affect brood and adult population of bees.

***Keywords*:** *Apis mellifera* macedonica, bee venom collection, seasonal variation, bee behavior, aggressiveness, hoarding behavior

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INTRODUCTION

Bee venom (BV) is a valuable product for the pharmaceutical industry, and its production and placing on the market are expected to be of great concern to the beekeepers. It's composition is a complex mixture of active peptides (melittin, adolapin, apamin, MCD, secapin) enzymes (phospholipase A2 and B, hyaluronidase, phosphatase and α-glucosidase), biogenic amines (histamine, dopamine, noradrenalin), amino acids (aminobutyric acid, a-amino acids), phospholipids, sugars, volatiles substances, minerals and other components (Bogdanov, 2014) which have variety of pharmaceuticals properties, such as arthritis, chronic pain, multiple sclerosis, some types of cancer and others (Orsolic et al, 2003;Gajski and Garaj-Vrhovac, 2013; Liu et al, 2008;Dantas et al, 2014; Son et al, 2007; Mirshafiey, 2007).It is also widely used in cosmetology (Kurek-Gorecka et al, 2020; Lee et al, 2014; Kim and Kim, 2010).

The collection procedure involves the stimulation of bees with electrical impulses. However, the defensive behavior of bees is an obstacle to the collection of venom since bees become particularly aggressive. There is a view that no humans and animals should be near the area of the BV collection and honeybees must be moved to a distant location for the bee venom collection (Morse and Benton, 1964). Although, this aggressiveness during the collection is very well known, limited published information documented it.

In addition to the increased defensive behavior, the collection of BV results in decreasing of the sealed brood area from 11.3% to 18.1% (Sanad and Mohanny, 2013), in the number of dead bees (Simics, 1995) in decreasing honeybee population, and in diminishing productivity of honey (Mitev, 1971; Balzekas 1978) and of royal jelly (Zhou et al., 2003). On the other hand, these adverse effects were not justified by other researchers (Rybak et al, 1995; Skubida et al, 1995;Bahreini et al, 2000). In oppose, field studies,showed that the collection of BV increased the hygienic behavior and the hoarding behavior of bees (El-Saeady et al, 2016).

Besides those discrepancies, the amount of venom collection was also a topic of discussion with diverse results mainly because it depends on many parameters such as the frequency of collection, the time of the day, the season of collection, the colony strength, the race and the age of the bees, the device of collection and other factors (Lauter and Vria, 1939;Omar, 1994;Haggag et al, 2015).

The different findings on the effect of BV collection on both their defensive behavior and their biological responses, have encouraged this investigation. We collected bee venom by electrical stimulation at regular intervals for two years, and we studied characteristics of bee colony behavior, like bee aggressiveness, mortality, growth of population and hoarding behavior. In addition we determined the amount of bee venom that is produced per colony at different seasons to compare it with studies that had been conducted in other countries.

MATERIALS AND METHODS

The study was carried out from April to October in 2016 and from May to October in 2017, at the experimental apiary of the Laboratory of Apiculture-Sericulture which situated at the farm of Aristotle University Thessaloniki in Greece. Nine honey bee colonies of *Apis mellifera* macedonica, equal in both strength and population, with ten honey bee combs, housed in Langstroth hives classified into three experimental groups. In groupA bee venom was collected every six days, in group B every twenty-four days and the C group used as control. Control group was handled as the other two groups with the exception that no BV was collected from this group.

Collection of bee venom during different periods

The venom collector was in the form of a hive frame with dimensions 23.5x43.9 cm. It consisted of two glass plates with the electric wires powered bytwo batteries AA. The current was continuous but interrupted by a microprocessor withimpulse durationof two seconds. This electric shock device was placed inside the hive on a second empty floor, for twenty-five minutes (Fig. 1). Bees that came into contact with the wires received a mild electrical shock that forced them to sting onto two glass sheets of the device (Fig. 2). The BV collector device was removed from the five without the use of smoke and the attach bees were detached by shaking. The two glass surfaces were transferred into the laboratory and the BV collected as powder by using a full face mask (Drager X-plore 6300), gloves and all the necessary precautions. Bee venom weighed,packed in dark glass jars and stored in the freezer (-20^{o} C).

Figure 1. The bee venom collection device was placed inside the hive on a second empty floor.

Figure 2. Bees get in contact with the charged wire net received electrical stimulation that causes them to release of venom in the glass plate.

Defensive response of the honeybee

To measure the aggressiveness of worker bees, a black leather ball attached to white twine, bound to a wood (100 cm length) was swung rhythmically in front of the beehive for 1 minute as first described by Free (1961) and Stort (1974) as leather -patch assay. The rhythm of movement was about one turn per second. For each colony and each test, different ball was used to avoid the effects of remaining alarm pheromones. Measurements concerned the number of stings in ball and operator's gloves.Measurement was scheduled every six days on groups A and C and every twenty- four days on groups A B and C, just before venom collection. Bee colonies before the suspension of leather balls were not disturbed, so different components did not affect the defensive sequence and create perplexing effects as described by Collins and Kubasek(1982).

Rating assay which is the most reliable assay to test the defending behavior of honey bees,according to Guzman-Novoa et al (2003), was also used. This test was performed by two operators who evaluated the sound intensity of the bees, the tendency of workers to fly around the hive, the running on the combs, to hit the veil, and to sting the gloves of the operators during manipulations. The rating scale of the above measurements was 1-5 (Guzman-Novoa et al, 2003). The inspections were done every 7 to 10 days during the experimental period. The higher the score the higher the aggression of the bees.

The effect of bee venom collection on the number of dead workers

The effect on dead worker bees was recorded by counting the dead bees on the wire of the collecting devices after each collection as previously described by Sanad and Mohanny (2013)

The effect of bee venom collection on honeybee hoarding behavior

A number of thirty adult worker bees gathered from the brood area of each experimental bee colony and placed in cages (10x10.3x4.2 cm). Each cage supplied with a piece of a dark comb of 40 cm^2 surface area. The cages were placed in an incubator at 35°C and 50% relative humidity.

After 24 hours of starving, caged bees fed with sucrose solution (1:1) which was supplied in a gravity feeding vial. The decrease of syrup from the feeder vial was recorded and refreshed daily. Measurements continued for fifteen days. Four complete replications were carried out with bees from the same colonies. Hoarding results expressed as μl sucrose solution removed from the feeder per bee in one day.

The effect of bee venom collection on population and brood area

During the two-year research, we kept all the bee-colonies with the adult population covered 10 frames so that the production of BV did not depend on the size of the bee colony. To achieve this we removed the frames of the experimental hives with sealed brood in cases where the bee exceeded the 10 frames population and needed extra space. The effect of bee venom collection on the brood and the bee populations was estimated in comparison with the number of brood combs that were removed from the three groups so that the bees could be kept in a population of 10 frames.

Statistical analysis

Results are presented as the Mean±SD. Means and standard deviations were calculated using Microsoft Excel. Experimental results were statistically analyzed using Duncan's *t*-tests and one-way Analysis of Variance (ANOVA) (IBM SPSS Statistics ver. 25.0). For all analyses, the differences with p-values ≤ 0.05 were considered statistically significant.

RESULTS

The amount of bee venom collection

The mean values (± SD) of BV collected from each colony are shown in Table 1. The differences among the colonies were not significant (p-value group A = 0.230, p-value group B = 0.183), although there was a great variation among them as indicated by the high SD and the range. Some colonies yield very little or no BV and others produced as much as four times the mean.The total amount of BV collected from a single colony variedbetween 753.2 to 879.7 mg when it was collected every 6 days and between 131.7 to 240.6 mg when collected every 24 days. The higher the frequency of collection the more BV was collected. Climate factors may influence the amount of BV but not significantly as indicated by the results of 2016 and 2017.

Figure 3 shows the fluctuation of the amount of BV collected every 6 and 24 days respectively in 2016. The BV production was high in spring (average 48 and 38 mg respectively) decreased in summer (average 13 and 26 mg respectively) and increases again in fall (19 and 28 mg respectively). The tendency of higher production BV during spring was apparent in 2017 too (figure 4).

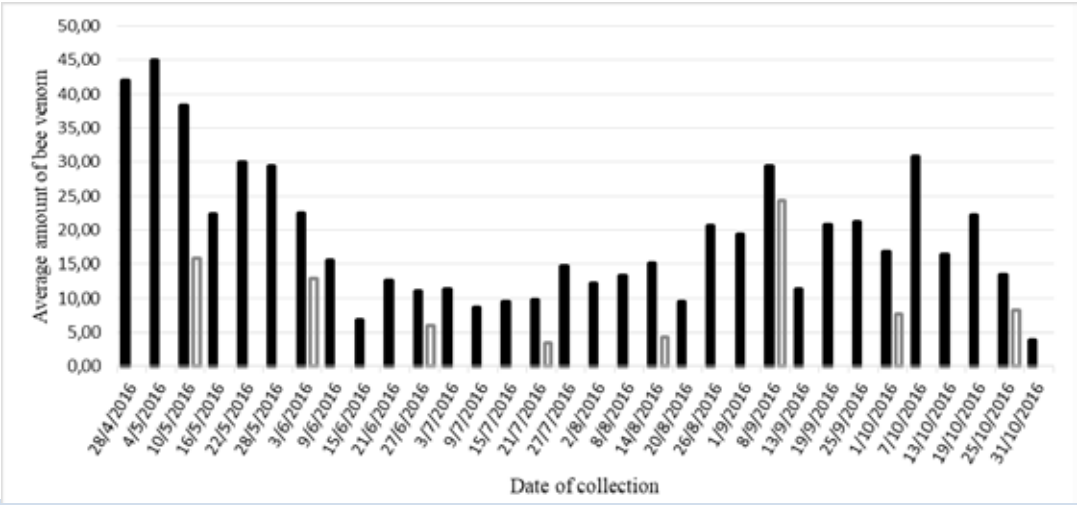

Figure 3. The average amount of bee venom (mg) collected during 2016. ▮ Collection every 6 days, ▯ collection every 24 days

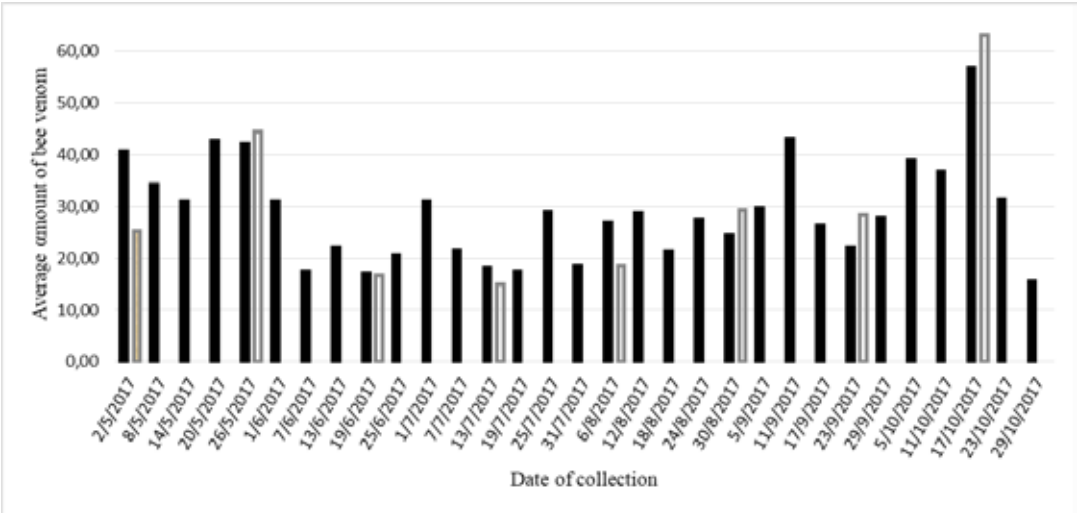

Figure 4. The average amount of bee venom (mg) collected during 2017. ▮ Collection every 6 days, ▯ collection every 24 days

Table 1. Bee venom production from bee colonies from April to October of two years (mg)

Frequency of collection	Mean ± SD	min & max	Total amount per colony
Every 6 days (A)			
2016	22.15[a] ± 16.2	0.3-100.7	753.2
2017	28.38[a] ± 11.52	4.7-115.0	879.7
Every 24 days (B)			
2016	14.63[a] ± 13.62	0.6-55.1	131.7
2017	30.08[a] ± 15.32	0.0- 82.7	240.6

*The statistical analysis was performed between the two experimental periods for each group

Table 2. The average number of stings (±SD) in leather ball measuring the defensive behavior of bees

	Collection		
Year	**Every 6 days (A)**	**Every 26 days (B)**	**Control (C)**
2016	3.4[a] ± 13.3	3.9 [a] ± 12.8	5.32 [a] ± 11.22
2017	1.0[a] ± 2.7	0.9[a] ± 3.26	5.37[b] ± 15.57

*The statistical analysis was performed between groups for each experimental period

Table 3. The tendency of defensive behavior of honey bees (scale 1-5)

		Collection		
Rating assay	**Year**	**Every 6 d (A)**	**Every 26 d (B)**	**Control (C)**
Sound intensity	2016	1.38[a]	0.78[b]	1.28[a]
	2017	3.05[a]	2.58[a]	2.71[a]
Flying around the hive	2016	1.80[a]	1.15[b]	1.49[c]
	2017	2.97[a]	2.65[a]	2.86[a]
Running on combs	2016	1.70[a]	1.22[b]	1.42[ab]
	2017	3.08[a]	2.73[a]	2.63[a]
Hitting operator's veil	2016	0.31[a]	0.17[a]	0.33[a]
	2017	2.68[a]	2.00[a]	2.40[a]
Stinging operator's hands	2016	0.45[a]	0.12[a]	0.33[a]
	2017	0.98[a]	0.62[a]	0.99[a]

*The statistical analysis was performed between groups for each experimental period and for each parameter

Defensive response of the honeybees

Applying the leather -patch assay we found that the collection of BV did not increase the defensive responseof the bees. No statistically significant differences were observed between colonies collected BV and controlsin 2016 (p-value = 0.481).In the second year the bees of the control hives appeared to be more aggressive than bees used to collect BV but this is misleading as the number of stings in the leather ball was small and cannot be considered as aggressive behavior (Table 2).

During the experiment, we found differences in the defensive response among the bee colonies. Most of the colonies did not react to the leather ball swinging in front of their hive. The increased aggression of some colonies was considered as random and unrelated to the existing weather conditions as bees from other hives on the same day showed calm behavior.In addition, their aggression decreased in the subsequent assays.

Table 3 indicates that the rating test yielded low values in all cases that never reached the upper levels of aggression. Statistically significant differences were found between the three experimental groups only in 2016 and were restricted in the sound intensity (p-value group B-group C = 0.001), the flying of bees (p-value group A-group C = 0.007, p-value group B-group C = 0.004) and the running on combs (p-value group A-group C = 0.008). The application of the assay during the full collection period showed that the bees did not change their behavior and did not become more aggressive as the project progresses.

The effect of collecting bee venom on the number of dead bees

As Table 4 indicates, the average number of dead bees found on the wire of collecting device was higher in colonies collected BV every 6 days to those collected every 24 days in both years. Differences between the two protocols were significant in 2016 (p-value=

0.001). The maximum numbers of 70 and 86 dead bees found in one collection were considered random and outliers, thatthey did not participate in the estimation of the mean value. The number of dead bees in group collecting BV every 6 days that were below 25 constituted 91.2% in 2016 and 96.7% in 2017. In group collecting BV every 24 days, the number of dead bees in most cases was below 10.

The effect of collecting bee venom on honeybee hoarding behavior

Table 5 presents the daily consumption of syrup by caged bees. There were nosignificant differences (p-value group A 2016= 0.130, p-value group B 2016= 0.895, p-value group A 2017= 0.423, p-value group B 2017= 0.472) in syrup consumption before and after BV collection in both years. Substantial variation exists between cages indicating that other factors such as the age of the bees, the number of remaining bees, and time of the year may be involved.

Population and brood area during the collection of bee venom

During the experiment, the bee colonies became overcrowded and we withdrew frames with sealed brood, indicating that the collection of BV did not affect their development. We removed 3, 2 and 10 frames of sealed brood respectively from groups A, B and C in 2016 and 12, 9 and 5 frames during 2017. The removal of brood frames seems to stop the swarming impulse.

Table 4. Number of dead bees found in the wire of collecting device

	Collection every 6 days (A)		Collection every 24 days (B)	
	mean	**Min-max**	**mean**	**Min-max**
2016	$13.45^{a} \pm 16.2$	0-70	$3.8^{b} \pm 5.2$	0-17
2017	$19.29^{a} \pm 19.47$	0-86	$9.27^{a} \pm 8.2$	0-25

*The statistical analysis was performed between groups for each experimental period

Table 5. Consuming syrup in vivo μl/bee

		Collection every 6 days (A)		Collection every 24 days (B)		Controls (C)	
Hive		Before	After	Before	After	Before	After
1	**2016**	80±30	40±60	50±20	40±50	60±30	40±40
	2017	60±70	60±30	50±30	80±60	60±40	60±30
2	**2016**	60±30	60±90	50±20	40±10	100±50	70±80
	2017	50±20	60±30	80±60	80±40	70±40	60±30
3	**2016**	50±20	40±40	20±10	40±70	40±20	20±20
	2017	60±40	70±40	70±30	70±30	40±50	80±30
Mean	**2016**	60^{a}**±30**	50^{a}±70	40^{a}±20	40^{a}±50	70^{a}±40	40^{b}±60
	2017	60^{a}±50	60^{a}±40	70^{a}±40	80^{a}±40	60^{a}±50	70^{a}±30

*The statistical analysis was performed on the average consumption of each group before and after the experiment for each experimental period

Table 6. Comparison of the amount of venom collection from different bee races

a/a	Race of bees	Period of collection	Amount of BV (mg/colony/ collection)		References
			Average	Range	
1	*Carniolan* and *Caucasian* breeds	June-July	37.3	20.8-53.0	Rybak, 2008
2	Not mentioned	Mar-Nov.	115.7	24.0-383.0	Sanad and Mohanny, 2013
3	*Carniolan*	Jan-Dec.	39.0	32.0-45.0	Omar et al, 2014
	Italian		33.0	26.0-40.0	
4	*Apis ligustica*	July-Oct.	31.9 (2014)	20-42	Nowar, 2016
		2014-2015	35.6 (2015)	20-50	
5	*Carnional*	Feb.-Oct.	123.3	96,6-150,0	Omar, 2017
	Italian	Feb.-Oct.	130.0	96,6-150,0	
6	*Apis macedonica*	April - Oct.	22.15	0.3-100.7	Current study
			28.38	4.7-115.0	

DISCUSSION

It is difficult to compare the results of BV collection with those of other studies because different collecting devices in a different climate and vegetation, with variable bee races and seasons of collection were used. In most studies, BV was collected in a different frequency, using a different number of colonies and duration of collection and finally, they presented the total amount of BV. In order to have compatible results with other studies, we calculated the amount of BV per colony that was collected after a single treatment by different authors. As Table 6 shows, the average amount of BV that was collected by a colony in one treatment in our research is close to those found by Rybak (2008), Omar et al (2014) and Nowar (2016) and lower than those of Sanad and Mohanny (2013) and Omar (2017).

One dissimilarity between our results and the published information is the differences between the lowest and the highest values (range). In our work, we found values with a wide range from near zero to values that were higher than 100 mg, while most of the other researchers, with the exception of Sanad and Mohanny (2013), gave a narrow range. These different results may be due to the number of observations, the number of hives, the frequency and the duration of collection. The low yields of venom production found in this work probably were due to the extremely low yields of specific bee colonies that were not substantially stimulated to sting in order to leave their venom on the glass plate.

The widely accepted notion that the procedure of collecting BV significantly increases their aggression was not confirmed in this study by either of the two tests used. Furthermore, the aggressiveness of bees remained stable and did not increase during the seven months weekly collection manipulations in both years of experiment. The concept that collection devices when placed inside of the hive triggered alarm pheromones that cause great stress on the bees and that they are very damaging to the health of the bees was not also verified. The gentle behavior of the Macedonian bee (*Apis mellifera* macedonica) may have played an important role. The significant variation among colonies in assays measuring the defensive behavior of honey bees was also noted by Guzman-Novoa et al (1999).

An average number of dead bees found on the wires of collecting device as a side effect of the collection were below 20 in each collection but it is likely to reach in some cases extremely higher numbers. In one case we counted 273 dead bees in only one hive, but it was a single event that did not repeat in the same or another bee colony. The bee losses during BV collection did not have any consistency during the year. In opposition to our results, other authors (Sanad and Mohanny, 2013) found significant differences among the death of workers within the months of BV collection. They recorded a higher number of dead bees in summer (50.3 workers/day) and lower in autumn (31.7 workers/day).The death of the bees probably resulted from the stress imposed on the bees from the collecting device, the electric currents, and fights between bees that occasionally occur in colonies during collection.

Hoarding test indicates that no significant differences existed between before and after the alarming or stimulation of worker honey bee by electrical impulses. Similarly, in field experiments, the mean yield of honey, obtained from colonies in which venom was collected, was not significantly different than controls (Rybak, 2008). El-Saeady et al (2016) indicated that bee colonies used for the collection of BV in field studies (fed ad libitum) consumed more syrup after the collection than before, although the differences were not significant. They attributed the increase in feed conception to the stimulation of worker honey bees to collect more food to compensate for the loss of secreted protein (venom).

Similar to our results Skubida et al. (1995) found that the use of stimulation of honeybees with electrical impulses for honeybee venom collection, had no adverse effects on colony strength, brood rearing and productivity. Also Rybak (2008) found that collecting bee venom three times during the season did not reduce significant the yield of honey, while the mean mass of venom collected from one honeybee colony did not differ significantly between the years, but was differ the amount of collected venom between the examined colonies.

In our work, it has been shown that collecting venom from bees does not cause aggression. Since this result is different from others studies in other countries, it would be interesting to look at the effect of the BV collection on bee behavior for the same period, the same flora and the same methodology (device type, time, ambient temperature, etc) with different races. If we were able to conduct experiments under the same conditions of collection and environment in different countries at the same time, we may be able to

explainthe great variability observed in the research results of various studies.

CONCLUSIONS

In this study, we concluded that bee venom collection can take place for a long period with the highest yields being achieved in spring and autumn.No negative effects found on sealed brood, the number of dead bees, the honeybee population, and the productivity of honey. Furthermore, the aggressiveness of bees remained stable and did not increase during the collection period.

CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Genetic polymorphisms in *FSHR*/*ALUI* and *ESRα* /*BG1I* loci and their association with repeat breeder incidence in buffalo

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ABSTRACT: The objectives of this study were to explore the association between *FSHR* and *ESR*α genes polymorphism and repeat breeder incidence in Buffalo. DNA was extracted from 243 (96 normal fertile and 147 repeat breeder) Egyptian buffaloes. PCR-*Alu*Iof 306-bp of *FSHR* gene yielded two digested (243 and 63 bp) fragments for the genotype CC, four fragments (243, 193, 63, and 50 bp) for the genotype CG and three fragments (193, 63, and 50 bp) for the genotype GG. Logistic regression analysis presented a significant association of C and G alleles with the incidence of repeat breeder; where the G allele showed a significantly higher incidence compared to C allele in repeat breeder heifers. DNA sequencing of 306 bp of the*FSHR* gene confirmed the polymorphic patterns attained by RFLP analysis; where C/G SNP was detected and changed threonine into serine amino acid. PCR-RFLP/*Bg1I* of 248-bpfromthe*ESR*α gene revealed one monomorphic GG genotype (171, 77 bp) confirmed by DNA sequencing. There were no detected SNPs in all enrolled animals. The results herein suggest the effectiveness of*FSHR*/*Alu*Ilocus polymorphism as a candidate for the incidence of repeat breeder in buffalo than*ESR*α/*Bg1I* one results in marker-assisted selection (MAS) against infertile animals

Keywords: *FSHR, ESRα*genes, repeat breeder, buffaloes, PCR-RFLP, DNA sequencing.

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INTRODUCTION

Water buffaloes were firstly domesticated in India 5000 years ago to provide a source of milk powder. Two groups of buffaloes were identified: swamp buffaloes (2n=48) that were developed chiefly for draught purpose and river buffaloes(2n=50) served as milking animals for dairy industries (Kierstein et al., 2004).They have essential effects on the economy of several countries including livestock and agricultural industries (Yindee et al., 2010).According to the last estimates of FAO (2013), there are about 195 million buffaloes in the world, 97% of them in Asia, 2% in Africa mainly in Egypt, and 0.2% in Europe mainly in Italy.The reproductive performance of any animal is affected by the genotype of both sexes including sire and dam (Mamta & Menaka, 2015).

Repeat breeder syndrome remainsthe most important type of infertility in domestic animals particularly in buffalo as it has a great economic impact for animal breeders. It contributes also to long calving intervals, increasing culling rates, and replacement costs (Garcia-Ispierto et al., 2007). Amiridis et al., (2009)classified the causes of repeat breeders into two major categories: fertilization failure and early embryonic death. The development of DNA markers is an essential tool for applications in animal breeding (Stamoulis et al., 2010;Arslan et al., 2019).Nguyen (2010) cited that buffalo suffer from many reproductive problems such as attaining puberty later than cattle, little numbers of follicles on their ovaries, unnoticed heat, ovulation at various times, seasonal breeding, and long postpartum anestrus. In buffaloes, fertility problems like repeat breeder are not easily recognized (Azawi et al., 2008). This syndrome are responsible for long service period and inter-calving interval that leads to low milk and low calf crop resulting in greater economic losses in the dairy industry. To minimize these losses, early and accurate diagnosis of the causes of these syndromes followed by appropriate timely interventions are required (Singh et al., 2008).

It was established that, chromosomal aberrations and autosomal recessive genes may be accounted for 20% of total early embryonic death that causes the repeat breeder condition (King, 1990). Additionally the Robertsonian translocation (t1/29) causes lower conception rate and early abortion which also caused females to be repeat breeder (Popescu & Pech, 1991). Genetic evaluation of animal reproductive performance depends chiefly on molecular markers technologies that identify genes related to reproductive efficiency (Beuzen et al., 2000). On the other hand, genetic polymorphisms have an important role in many fields of animal breeding (Stoneking, 2001). The development of breeding program strategies requires the description of the genetic structure of populations, breeds and species because it provides information that is necessary for genetic conservation programs. These characterizations offer support and intensify the traditional selection methods (Vasconcellos et al., 2003).

Follicle-stimulating hormone receptor (*FSHR*) gene ispositioned on chromosome number 11 and consists of 10 exons and 11 introns, the first 9 exons encompass the extracellular domain while exon 10 encloses the transmembrane domain (Houde et al., 1994). It is expressed in the ovaries of females and performs its actions by joining with follicle-stimulating hormone (Themmen & Huhtaniemi, 2000), to stimulate the gametogenesis process (Simoni et al., 1997). It has a major role in follicular development in the ovary (George et al., 2011). The FSHR gene is the main determinant of ovarian responsiveness to FSH for the induction of ovulation in females (Yang et al., 2012).

Estrogen receptors (ESR) are composed of two isoforms: ESR1 and ESR2 and each one of them is yielded from a separate gene and is situated on different chromosomes. They are recognized in multiple tissues but uterus, vagina, and ovaries are the chief positions of their expression in females (Enmark and Gustafsson, 1998). Estrogen receptor alpha (*ESRα*) gene is localized on chromosome 9 and contains 8 coding exons (Szreder et al., 2011). It plays a role in the regulation of reproduction, development of the mammary gland (Rani et al., 2016). The *ESR* gene revealed a strong affinity to impact the activity of animals during a period of estrus due to its presence in the ovary (Schams and Berisha, 2002). Estrogen receptors are nuclear receptors (Bjornstrom and Sjoberg, 2005). Previous studies reported the association between *FSHR* and ESR genes polymorphisms and reproductive problems in buffalo; however,controversial resultswerealso obtained(Yang et al., 2010; Othman & Abdel-Samad, 2013; Sosa et al., 2015; Rani et al., 2016; Shafik et al., 2017).

Consequently, the main objectives of this study were to detect polymorphisms of *FSHR* and *ESR*α-genes and their association with the incidence of repeat breeder in Egyptian buffalo heifers using PCR-RFLP and DNA sequencing techniques.

MATERIALS AND METHODS

Animals and Experimental samples

The present study was conducted on a total of 243 (96 normal fertile and 147 repeat breeder) Egyptian buffaloes (*Bubalus bubalis*) aged from 2 to 3 years old. Animals were selected from three localities: A buffalo nucleus herd kept in Nataff-Gedeed Station, Mahalet-Mousa Farm, Agricultural Research Centre, Ganat El-Reida, and El-Noor farms, Ismailia governorate. Based on farm history, animals conceived from one or two successive inseminations and became pregnant were represented as a normal fertile group. While animals that had not conceived after three or-more services and associated with true estrus (heat) every 21-25 days was considered as a repeat breeder. Blood samples were collected from the jugular vein into sterilized vacutainer tubes containing EDTA as an anticoagulant and then stored at −20°C for genomic DNA extraction. Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University approved the protocol of the study..

Genomic DNA extraction and PCR

Genomic DNA was extracted from the leucocytes using the Gene JET Genomic DNA purification kit following the manufacturer protocol (Thermo Scientific,Lithuania). The quality of the extracted DNA was assessed by 1% agarose gel electrophoresis. A 306-bp fragment from exon 10 of the*FSHR* gene and a 248 bp from the putative promoter of *ESRα* gene were amplified by PCR using primers shown in Table 1. PCR was carried out in a volume of 50 µl containing 19 µl H_2O, 1.5 µl forward primer, 1.5 µl reverse primer, 3 µl DNA and 25 µl PCR master mix (Bioline, England). The conditions of PCR program were shown in Table 2.Then PCR products were resolved by electrophoresis stained with ethidium bromide, and visualized using UV light of gel documentation system.

Table 1. Forward and reverse primers sequence for *FSHR* and *ESRα* genes, annealing temperatures and, size of PCR amplicon.

Gene	Primers		Annealing temperature (°C)	Size of PCR product (bp)	Reference
	Forward (5′-3′)	Reverse (5′-3′)			
FSHR (part of exon 10)	5′-CTGCCTCCCTCA AGGTGCCCCTC-3′	5′-AGTTCTTGG CTA AATGTCTTAGGGGG-3′	60	306	Marson et al., (2008)
ESRα (part of putative promoter)	5′-TTTGGTTAACG AGGTGGAG-3′	5′-TGTGACACAG GTGGTTTTTC-3′	56	248	Szreder & Zwierzchowski, (2004)

Table 2. Polymerase chain reaction (PCR) condition for *FSHR* and *ESRα* genes.

Gene	Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
FSHR (part of exon 10)	95/5 min	40	95/30 s	60/30 s	72/30 s	72/8 min
ESRα (part of putative promoter)	95/5 min	40	95/30 s	55/30 s		

PCR-RFLP genotyping

The PCR products were digested with the following restriction enzymes; *AluI* (New England Biolabs Inc) for the*FSHR* gene and *Bg1I* (Thermo Scientific,Lithuania) for *ESRα* gene with incubation at 37 °C for 15 min. The cleaved fragments were detected by 2% agarose gel electrophoresis and visualized under UV using a gel documentation system. The RFLP reaction mixture was carried out in 30 µl consisted of 10 µl PCR product, 1 µl restriction enzyme, 10 µl 10× buffer and 9 µl H_2O (dd water).

DNA sequencing

PCR products of normal and repeat breeder animals with different patterns attained by RFLP analysis were sequenced. The PCR bands with expected size were purified using the PCR purification kit following the manufacturer protocol (Jena Bioscience # pp-201×s/Germany) using the method described by Vogelstein and Gillespie (1979). The purified PCR products were sent to be sequenced in one forward direction using ABI 3730XL DNA automated sequencer (Applied Biosystem, USA). The obtained se-

quences were inspected using Chromas software. Sequence analysis and alignment were carried out using NCBI/BLAST and CLC Main Workbench7 software. Ambiguous sequences at the beginning and extreme end of each sequence were trimmed to avoid possible errors in base calling. The Sequences were analyzed using the Chromas Lite 2.1 program (http://technelysium.com.au/?page_id=13) (Altschul et al., 1990). The identity of the sequenced PCR products was examined using BLAST search against the GenBank database of buffalo (*Bubalus bubalis)* and cattle *(Bos taurus)*,(http://BLAST.ncbi.nlm.nih.gov/BLAST.cgi). The alignments and assembly of the sequences were performed using NCBI.

Data analysis

Gene and genotype frequencies of *FSHR* and *ESRα* genes were calculated according to equations described by Falconer and Macky (1997). Chi-square ($\chi 2$) used to check the Hardy-Weinberg equilibrium. The association between repeat breeder and the identified SNPs was assessed by unconditional multiple logistic regression modelsto obtain the odds ratio (OR) and the corresponding 95% confidence interval (CI). Codominant, dominant, recessive, overdominant and log-additive models were used to avoid the assumption of genetic models. The age and weight of fertile and repeat breeder animals were also adjusted and the statistical analyses were performed using statistical packagefor thesocial sciences (SPSS) software (version 21) **(**SPSS,2004).

RESULTS

The genomic DNA from 243 (96 normal fertile and 147 repeat breeder) buffaloes was extracted to amplify 306 bp (part of exon 10) of *FSHR* gene. The restriction digestion analysis of PCR products conducted with *AluI* endonuclease revealed three genotypes: the CC genotype with two digested fragments: 243 and 63bp, four digested fragments: 243, 193, 63 and 50 bp for the genotype CG and the GG genotype with three digested fragments: 193, 63 and 50 bp (Figure 1).The genotypic and allelic frequencies of the*FSHR* gene were calculated and presented in Table 3. For 96 normal fertile buffaloes, the genotypic frequencies of CC, CG, and GG genotypes were 34.4%, 39.6%, and 26% respectively. While in 147 repeat breeder heifers the genotypic frequencies were 21.1%, 36.7% and 42.2% respectively. Allelic frequencies of C and G alleles were 54% and 46% in normal animals and 39% and 61% in repeat breeders. The $\chi 2$-test presented the obtained *FSHR* gene genotypic distribution among normal fertile buffaloes that was deviated from Hardy-Weinberg equilibrium ($p<0.05$), while in repeat breeder heifers the genotypic distribution followed Hardy-Weinberg equilibrium ($p>0.05$). The different band patterns obtained by the RFLP marker were DNA sequenced. Nucleotide sequences for the forward primer direction of *FSHR* gene (306 bp) alignment between normal fertile and repeat breeder buffalo heifers were carried out using CLC Main Workbench7 program. The results confirmed the digested polymorphic patterns attained by RFLP analysis; where it was revealed the presence of C/G non-synonymous transversion SNP replaced threonine into serine (Figure 3).

As revealed in Table 4, logistic regression analysis presented a significant association of C and G alleles of the *FSHR* gene with incidence of repeat breeder (P=0.002). In repeat breeder heifers, the G allele showed a significantly higher incidence compared to C allele: 61% versus 39%. Indicating that heifers carrying the risk allele G have a higher susceptibility to repeat breeder in comparison with C allele carriers and increased the OR value of the risk for infertility to 1.81 with 95% CI= 1.26-2.62. Meanwhile, in normal fertile heifers, G allele appeared with lower frequency when compared to C allele: 46% versus 54%. The C>G transversion detected SNP showed significant association when tested under different genetic models. With the codominant model (P=0.016), animals with a homozygous GG genotype at this locus had an OR of 1.00 with 95% CI for being repeat breeder compared to CG genotype that had OR = 0.57 with 95% CI= 0.31-1.07, while CC genotype had OR of 0.38 and 95% CI= 0.19-0.74. Under the dominant model of the C>G SNP (G/G versus C/G+C/C), it showed a highly significant association (P=0.0095) with repeat breeder with OR of 0.48 and 95% CI= 0.28-0.85. Meanwhile, the recessive model of the*FSHR* gene (G/G+C/G versus C/C) SNP showed a significant association (P=0.022) with repeat breeder with OR of 0.51, 95% CI: 0.29-0.91. The overdominant model (G/G+C/C versus C/G) revealed a non-significant association (P=0.65) with repeat breeder with OR of 0.89 and 95% CI= 0.52-1.50. However, logistic regression models revealed significant associations ($P<0.05$) between codominance, dominance, recessive, over-dominant as well as log-additive effects of C>G SNP and the occurrence of repeat breeder in buffaloes. For this SNP, the log-additive effect (P=0.0041) can be interpreted as every additional copy of the risk

allele G at this locus resulted in an increased risk of repeat breeder by 0.61, 95% CI= 0.44-0.86 in buffalo heifers. Consequently, buffaloes carrying the heterozygous CG genotype are 0.61 more likely to develop repeat breeders compared to reference CC genotype. Furthermore, the mutant G allele is a highly risk allele that increased the susceptibility to repeat breeder by 0.61, CI= 0.44-0.86 than C allele which is a low risk allele.

Concerning the*ESRα* gene, the genomic DNA was extracted to amplify 248bp (a part of the putative promoter). Restriction analysis of 248 bp PCR products was digested with *Bg1I*. The results showed monomorphic GG pattern with two digested fragments at 171 and 77 bp in all the studied animals (Figure 2). The monomorphic band pattern was confirmed by DNA sequencing; where nucleotide sequence alignment for the forward primer direction of *ESRα* gene (248bp) from normal fertile and repeat breeder buffaloes revealed no variation exists between the sequences of the studied animals (Figure 4).

Table 3. Genotypic and allelic frequencies of the *FSHR* gene in normal fertile and repeat breeder animals.

Animals	No. of animals	Number/frequency of genotypes%			Allele frequency %		Risk allele	χ2 (HWE)	P-value
		CC	CG	GG	C	G			
Fertile	96	33/34.4	38/39.6	25/26	54	46	G	3.948	0.04692
Repeat breeder	147	31/21.1	54/36.7	62/42.2	39	61		7.852	0.04259

HWE-Hardy Weinberg Equilibrium. Hardy Weinberg test was done using the Pearson's goodness of fit test. P value<0.05 was considered to show significant deviation of the observed genotypes from Hardy-Weinberg proportions.

Table 4. Genotypic and allelic association of C>G SNP of *FSHR* gene polymorphism with repeat breeder incidence under different genetic models.

Comparative models	Genotypes	Fertile (n=96)	Infertile (n=147)	OR (95% CI)	P-value
Codominant	G/G	25 (26%)	62 (42.2%)	1.00 (reference)	0.016
	C/G	38 (39.6%)	54 (36.7%)	0.57 (0.31-1.07)	
	C/C	33 (34.4%)	31 (21.1%)	0.38 (0.19-0.74)	
Dominant	G/G	25 (26%)	62 (42.2%)	1.00 (reference)	0.0095
	C/G-C/C	71 (74%)	85 (57.8%)	0.48 (0.28-0.91)	
Recessive	G/G-C/G	63 (65.6%)	116 (78.9%)	1.00 (reference)	0.022
	C/C	33 (34.4%)	31 (21.1%)	0.51 (0.29-0.91)	
Overdominant	G/G-C/C	58 (60.4%)	93 (36.3%)	1.00 (reference)	0.65
	C/G	38 (39.6%)	54 (63.7%)	0.89 (0.52-1.50)	
Log-additive	---	---	---	0.61 (0.44-0.86)	0.0041
	Allele	Fertile (n=96)	Infertile (n=147)	OR (95% CI)	P-value
FSHR C>G	C	No. 104 % 54%	No. 116 % 39%	1.00 (reference)	0.002
	G	88 46%	178 61%	1.81 (1.26-2.62)	

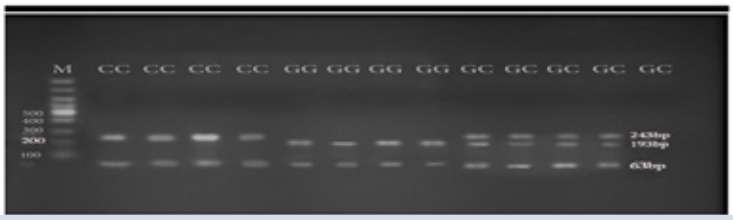

Fig 1. Ethidium bromide stained 2% agarose gel electrophoresis of representative samples of RFLP banding pattern of *FSHR* gene (306-bp) from normal fertile and repeat breeder buffaloes after digestion with *AluI*. M: 100 bp ladder

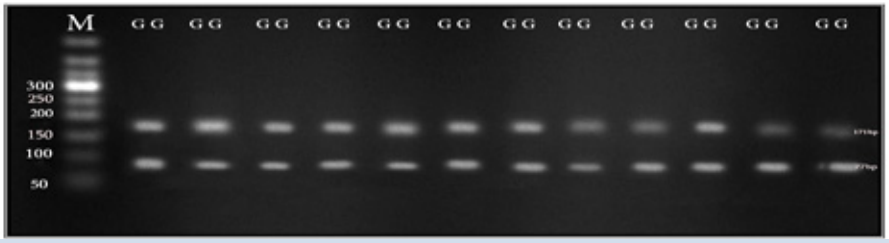

Fig 2. Ethidium bromide stained 2% agarose gel electrophoresis of representative samples of RFLP banding pattern of *ESRα* gene (248 bp) from normal and repeat breeder buffaloes after digestion with *Bg1I*. M: 50 bp ladder

Fig 3. (a) DNA sequence alignment of *FSHR* gene (306 bp) between normal fertile and repeat breeder buffalo heifers using CLC Main Workbench7 program, (b) Alignment of amino acids of *FSHR* gene (part of exon 10-306 bp) between normal fertile and repeat breeder buffalo heifers using BLAST, (c) DNA sequence alignment of ESRα gene (248 bp) between normal fertile and repeat breeder buffaloes using CLC Main Workbench7 program.

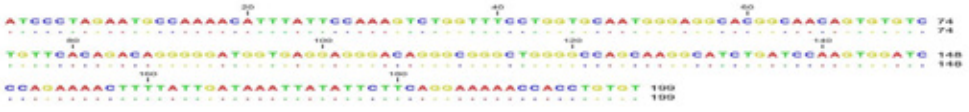

Fig 4. DNA sequence alignment of *ESRα* gene between normal fertile and repeat breeder buffaloes using CLC Main Workbench7 program.

DISCUSSION

In a breeding system, reproductive efficiency has attained remarkable interest, especially in seasonal breeder animals. Buffalo are able to breed throughout the year; however in developing countries, like Egypt, the chance of pregnancy attained by the seasonal trend of ovarian activity is time-limited (Barile, 2005). Buffalo have smaller ovaries, fewer primordial follicles, less over estrous signs than cattle(El-Wishy, 2007; Perera, 2011). This may be attributed to the peak concentrations of progesterone as well as oestradiol-17â is less detected. Field surveys on reproductive disorders revealed that anestrum and repeat breeder were the most common cause of infertility in buffaloes particularly in Egypt (Singh & Sahni, 1995; Ahmed et al., 2012).

In this study, the RFLP analysis revealed the presence of C/G non-synonymous SNP transversion which causes to replace threonine into serine. Interestingly, the logistic regression analysis presented a significant association of C and G alleles of the *FSHR* gene with the incidence of the repeat breeder; where G allele showed a significantly higher incidence compared to C allele. Previous studies reported the association between *FSHR/AluI* locus polymorphisms and the incidence of infertility traits in both cattle and buffaloes populations; however controversial results were also obtained. Moreover, different distribution and frequencies for the attained genotypes; that is may be attributed to the genetic background differences between the studied animals. Regarding the polymorphism in buffaloes, Othman & Abdel-Samad (2013) determined genetic polymorphism of the *FSHR-AluI* gene among healthy buffaloes. They obtained only one CC genotype (243 and 63 bp). According to the results, sequence alignment of the *FSHR* gene showed that the *FSHR* gene in the Egyptian buffaloes possessed identities at 99% with only G/A SNP at position 59 of this gene. Also, Sosa et al., (2015) worked to indicate the effect of *FSHR* gene polymorphism on anestrus and repeat breeder, no significant differences between animals were detected. All animals studied in this study were genotyped as CC. Shafik et al., (2017) determined the association between polymorphisms of the *FSHR* gene and infertility using DNA sequencing and they did not found any SNP in 306 bp in all examined buffaloes.

A number of related studies were carried out in different cattle populations. For instance, Marson et al., (2008) studied the effect of *FSHR* gene polymorphism on sexual brightness among 370 cattle beef heifers. PCR-RFLP/*AluI* yielded three genotypes: GG with three fragments (193, 63, and 50 bp), CG with four fragments (243, 193, 63, and 50 bp) and CC with two fragments (243 and 63 bp). It was found that the heifers with CG genotype presented a 66% pregnancy rate, Meanwhile, GG genotype exhibited 58% and CC genotypes revealed 64%. However, the effect of the *FSHR* gene polymorphism on pregnancy rates between the diverse breeds was not established.Hernandez et al., (2009) tried also to find a variation of the *FSHR* gene in different cross breeds of cattle. The authors used the same primer and restriction enzyme and found different gene and genotypic frequencies in the studied cattle population. They added that, Hardy-Weinberg equilibrium was detected in the Bos indicus and Bos taurus x Bos indicus cattle but not in the Bos taurus group. Moreover, the association between *FSHR* gene polymorphism and superovulation in Chinese Holstein cows was investigated by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing (Yang et al., 2010). The authors reported the presence of G278A and A320T SNPs. They showed also that *FSHR* gene is a possible predictor for superovulation in Chinese Holstein cows.In the same line, Sang et al., (2011) investigated the association between *FSHR* gene polymorphism and sperm quality traits in mature Holstein bulls. The authors added that bulls with AA genotype might have significantly higher sperm quality traits than AT genotype.Andreas et al., (2014) declared also a significant effect at ovulation rate, while individual animals with the CC genotype of *FSHR-AluI* had a higher ovulation rate than CG and GG genotypes.In the same respect, Arslan et al., (2017) reported that a digested fragment of 306 bp of the *FSHR* gene with *AluI* obtained three genotypes with a significant deviation from HWE between low insemination and high insemination cows. On divergence to our results, Omer et al., (2016) characterized Sudanese cattle and obtained only one CC genotype.

Mutations in the promoter region greatly affect the post-transcriptional regulation of gene expression and impact the accuracy of the translation process (Fürbass et al., 1997) Although some mutations are positioned in promoter regions of the genes and they would not change the amino acid sequence of protein products but they play a role in the functions of estrogen as they are found in the transcription factors' binding sites and so leading to an alternation of the expression levels of the genes. According to our re-

sults, there was no association between the*ESRα* gene polymorphism and the incidence of repeat breeder. For the *ESRα* gene, the results herein showed monomorphic GG pattern with two digested fragments at 171, and 77 bp in all the studied animals confirmed by DNA sequencing. Previous studies reported opposing results for the latter polymorphism. For instance Othman & Abdel-Samad (2013)used the same primer, endonuclease enzyme, and amplified the same fragment of *ESR* gene.However,PCR- *Bg1I* 248 bp of the *ESRα* gene elicited two genotypes; GG genotype with two digested fragments: 171 and 77 bp and three fragments 248, 171, and 77 bp for the AG genotype. Additionally,they found that 18% of animals are the AGgenotype and 82% are the GG genotype.On the contrary, the results ofRani et al., (2016) agreed with the obtained results. They used two endonucleases and obtained a monomorphic pattern in all the studied animals.

Exploring the polymorphism was carried out on different regions ofthe *ESR* gene and its association with reproductive traits.Zahmatkesh et al., (2011) digested 245 bp of *ESRα* gene with *BglI* endonuclease, they found three genotypes: AA (245 bp), AG with three digested fragments (168, 77, and 243 bp) and GG had two fragments (168 and, 77 bp) with genotypic frequencies: 0.010, 0.129, and 0.861. Allelic frequencies of A and G were 0.0742 and 0.9257. It was presented that, A/G transition had no significant effect on reproduction traits. In a cohort of cattle population, Szreder &Zwierzchowski (2004)used PCR-RFLP and DNA sequencing to determine the genetic polymorphism of *ESRα* gene among different breeds and obtained AG and GG genotypes, the results of DNA sequencing revealed A/G transition in the promoter region leading to silent mutation. Sangdehi et al., (2015)used also *SnaBI* endonuclease to digest a 340 bp fragment from the promoter region of *ESRα* gene and obtained three genotypes: AA, AG, and GG between four breeds of cattle: Mazandarani, Taleshi, Sistani, and Simmental. The genotypic frequencies were: 0.65, 0.75, 0.35, and 0.91 respectively for AA genotype, 0.30, 0.25, 0.40, and 0.09 for AG genotype and 0.05, 0.0, 0.25, and 0.0 for GG genotype. Kathiravan et al.,2017detected genetic polymorphism of *ESRα* gene among different Indian murrah buffalo breeds. Genomic DNA was extracted to amplify 870 bp from exon 13 that was digested by *MboI* restriction enzyme. All animals enrolled in this study were of monomorphic pattern and genotyped as AA genotype with 614 and 256 bp fragments. This result shows the conservation of *ESRα*/AA genotype in murrah buffaloes.

The limitations of this study should be acknowledged. First, a small sample size may not allow obtaining a concrete conclusion for elucidating the *ESRα* gene polymorphisms. Second, a limited number of candidate gene markers for the incidence of repeat breeder may also influence the conclusion. Third, other buffalo breeds should also be considered. Accordingly, such shortcoming should be considered in further investigations.

In conclusion, a remarkable significant association was detected between *FSHR*/*AluI* locus polymorphism and incidence of repeat breeder in Egyptian buffaloes. The association of observable phenotypic variationin the fertile and repeat breeder buffaloes with identified polymorphisms can potentially be explained by allele-specific differencesin *FSHR* gene expression. These findings suggest that *FSHR/AluI* gene could be used as a marker for early culling of repeat breeder heifers resulting in preventing economic losses afforded by the breeder resulting in efficient marker-assisted selection (MAS).

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CON FLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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Research article
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Effect of light colour temperature on expression and serum profile of selected immune markers in layers

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ABSTRACT: This study was carried out to quantify the effects of different temperatures of light colour on expression and serum profile of selected immune markers in Fayoumi layers. A total of 165 Fayoumi healthy pullets, 17 weeks of age were used. At laying, birds were separated in well ventilated environmentally-controlled rooms and allocated into three groups of 55 birds each (5 males and 50 females) for 3 months and these birds represented the base generation (F_0). Fertile eggs were collected and the newly hatched chicks were also divided into three groups from first day of life till 3 months after laying and these represented the first generation (F_1). In the two generations, the first group (control) was exposed to cool white LED light (day light) (6500 kelvin), the second group was exposed to very cool white LED light (sky blue light) (10000 kelvin) and the third group was exposed to warm white LED light (yellow light) (2700 kelvin). Birds of each group of the two generations were evaluated for expression profile of *TLR4 and IL10* genes and serum level of IL10. Results showed that blue light-exposed groups, in the two generations, exhibited a higher up-regulation of *TLR4 and IL10* genes and increased serum level of IL10 compared to groups experienced either white or yellow light colour. Comparison between F_0 and F_1 individuals revealed improved genetic profiles for F_1 birds. The results therefore elucidate the benefits of using blue light in improving the immune status of layers in order to predict the most susceptible risk time for disease incidence and to build up an effective management regimen.

Keywords: Layers; gene expression; immune markers; light; Fayoumi chickens

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INTRODUCTION

Poultry production is an important component of agriculture all over the world. Chickens are considered one of the most popular types of poultry all over the world regardless culture and religion. The reason for popularity could be the high nutritive values poultry products have (Bell et al., 2002; da Silva et al., 2017; Kralik et al., 2018). Fayoumi is one of the native old breeds of chicken in Egypt. It is named for the Faiyum Governorate, southwest of Cairo and west of the Nile (Meyer, 1997; Zhou and Lamont, 2003). They are a lightweight fowl, with roosters weighing in around 2 kilograms (4.4 lb) and hens 1.6 kg (3.5 lb). In roosters, the plumage is silver-white on the head, neck, back and saddle, with the rest in a black and white barring. Hens have heads and necks in the silver-white hue, with the rest barred. Fayoumi has a single comb, red moderately large earlobes and wattles, with a white spot in the earlobes. Fayoumi also has dark horn coloured beaks, and slate blue skin (Zhou and Lamont, 2003).

Artificial light source as an external environment factor is an important aspect affecting growth and immunity in layers. It is well known that lighting factors, such as intensity, exposure time and colour affect physiology and immune competence of chickens (Foss et al., 1972; Rozenboim et al., 1999; Olanrewaju et al., 2006; Xie et al., 2008; Blatchford et al., 2009; James et al., 2018). Light colour is described by chromaticity. Chromaticity is the measure of warmth of the light source (warm light) or coolness (cool light) expressed in degrees Kelvin. The scale ranges from 2000 to 7000K. Chromaticity values of 4000 K and above are considered cool (mostly blue light), while those around 3500 K or 3600 K are called neutral, and those of about 3000 K or below are considered warm (more red light) (Knisley, 1990). Light-emitting diodes (LED) saves energy efficiently and provide sufficient brightness (Hassan et al., 2014). Natural daylight can be also effectively simulated by the application light-emitting diode (LED) than the spectral gaps of other lighting sources (El-Sabrout and Khalil, 2017). Additionally, LEDs are potentially beneficial to the poultry industry due to long life span, moisture resistance, and narrow spectrum (Olanrewaju et al., 2015; Sharideh and Zaghari, 2017). Thus, most of the poultry producers have replaced ICD (inductively coupled discharges) lamps with LEDs. It has been established that the colour of light is a remarkable physical component of light that has a great impact on different productive, reproductive and immune parameters of chickens (Olanrewaju et al., 2015). Moreover, as long as the longer wave lengths are possessed, the higher penetration power of light is attained (Yang et al., 2016).

Light is also a key microclimatic factor that hits chicken skull at the retinal receptors and travels through neurons to the pineal gland and hypothalamus regulating centers (Egbuniwe and Ayo, 2016). Poultry detect light through the photoreceptors of retina and the extra-retinal photoreceptors in the brain. The brains of birds are equipped with active extra-retinal photoreceptors that receive light energy and transmit it through the skull and tissues. The chicken eye is capable of discriminating light colour due to 7 photoreceptors (1 rod and 6 cones) in the eye (Hartl and Hayer-Hartl, 2002). The chicken retina consists of four types of single cones and a double cone, which are highly responsive to violet, blue, green, and red light (Bowmaker and Knowles, 1977). Photoreceptive pigments located at cones are characterized by a high sensitivity to violet (415 nm), blue (455 nm), green (508 nm), and red (571nm) (Parry et al., 2004). Therefore, light colour has been studied in poultry over the last three decades and its use has increased recently. The use of coloured lighting systems is an option to enhance production of layers in the modern layer industry. Many kinds of lights have been introduced commercially however, light emitting diode (LED) can dramatically save energy and provide adequate illumination (Rozenboim et al., 1998).

Chicken Toll-like receptor (TLR) repertoire consists of ten genes similar to that found in human and is two fewer than mouse (Higgs et al., 2006). The identified TLRs include TLR1 type 1, 2, TLR2 type 1, 2, TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21. Chicken TLRs are present in different organs as thymus, liver, kidney, brain, muscle, spleen, bursa, and testis (Bekeredjian-Ding and Jego, 2009). Toll-like receptors (TLRs) are a family of transmembrane-spanning proteins, which recognize molecules unique to microbes, discriminate self from non-self-antigens, trigger appropriate immune responses, act as sentinels of tissue damage, and mediate inflammatory responses to aseptic tissue injury (Marsh et al., 2009).

Interleukin-10 encodes a 178-aa polypeptide, with a predicted 162-aa mature peptide. It has 45 and 42% aa identity with human and murine IL-10, respectively. The chIL-10 gene structure is similar to (five exons, four introns), but more compact than, that of its mammalian. Chicken IL-10 mRNA expression

was identified mainly in the bursa of Fabricius and cecal tonsils, with low levels of expression also seen in thymus, liver, and lung (Rothwell et al., 2004). IL-10 is one type of the anti-inflammatory cytokines that could control the nature and degree of inflammation responses during infection, and also share an important role in immunity of intestine, and hemostasis (Manzanillo et al., 2015). IL-10 expression is extensively regulated at the post-transcriptional level, which may involve control of mRNA stability via AU-rich elements and by microRNAs such as let-7 or miR-106 (Sharma et al., 2009).

Research has elaborated the effects of light colour temperatures on different parameters of broiler including performance (Hassan et al., 2014; Sultana et al., 2014; Archer, 2016; Shariadeh and Zaghari, 2017; Abdel-Azeem and Borham, 2018), behaviour (Prayitno et al., 1997; Blatchford et al., 2009), welfare (Mohamed et al., 2014), health and productivity (Blatchford et al., 2009; Deep et al., 2010), heat stress (Abdo et al., 2017; Mousa-Balabel et al., 2017), carcass characteristics (Onbaşılar et al., 2007; Olanrewaju et al., 2015), immune parameters (Hassan et al., 2014; Firouzi et al., 2014), blood properties (Firouzi et al., 2014; Seo et al., 2016), and physical traits (Alattar et al., 2019). In layers, research has focused also on many aspects including performance (Kamanli et al., 2015), egg production (Han et al., 2017; EL-Emam et al., 2019), egg quality (Er et al., 2007; Borille et al., 2013; Kamanli et al., 2015; El-Sabrout and Khalil, 2017), behaviour (Mohamed et al., 2010; Sultana et al., 2013; Shi et al., 2019), hatching performance (Yu et al., 2018) and stress response (Liu et al., 2018; Archer, 2019).

Research carried out on the effect of light colour in broiler showed conflicting findings. Blue light colour has been suggested to improve the immune status of birds (Hassan et al., 2014; Mohamed et al., 2014; Seo et al., 2016; Guo et al., 2018; Soliman and Hassan 2019). However, warm light colour has been reported to enhance immunity (Sharideh and Zaghari, 2017). There is a little information regarding the effect of light colour temperature on immune status of layers. Additionally, no previous studies have considered how environmental factors such as the temperature of light colour may affect immunity of layers through successive generations, as well as how these effects can be explored through the approach of gene expression profile of immune markers.

Therefore, the objectives of this study were to evaluate the effect of different light colour temperatures on the immune status of Fayoumi layers by investigating the expression pattern of *TLR4* and *IL10* genes and the serum profile of IL10.

MATERIALS AND METHODS

Experimental birds and design

Base generation (F_0)

A total of 165 Fayoumi healthy pullets (17 weeks) with a similar body weight (900 ± 30 gram) were used in this experiment. Pullets were purchased from a governmental farm for poultry breeding in Fayoum Governorate, Egypt. All birds were housed in the same room till the time of laying at a density of 8 birds/m^2. The photoperiod was 12L: 12D, the relative humidity ranged from 67 to 77 % (Cao et al., 2008), and the house average temperature was 28 °C. Ventilation and temperature were checked daily and kept adjusted throughout the experiment (Rosa et al., 2019). From the 19th week the lighting schedule was gradually increased half an hour every week till it reached 16L: 8D lighting schedule at laying time (Han et al., 2017). As soon as laying started, at 24 weeks, the birds were allocated into three groups in three separate, well-ventilated, environmentally-controlled rooms according to the light colour temperature. Each room had a floor area of 9 m^2 (3m width x 3m depth) and was used for housing of 55 birds (5 males and 50 females). The first group (control) was exposed to cool LED white light (day light) (6500 kelvin). The second group (sky blue light) was exposed to very cool LED white light (10000 kelvin) and the third group (yellow light) was exposed to warm LED white light (2700 kelvin) till the end of the experiment. Light intensity was 25 lx (1.4-ft candle) during the light phase and 0 lx during the dark phase of the photoperiod (Mohammed et al., 2010). The intensity of light was recorded near the floor, nearly at the level of bird height. Artificial light systems were placed 10 cm above the birds using plastic crosses attached to the ceilings of the rooms. Feed intake was calculated daily according to standard farm husbandry practices to meet the nutrient recommendations for poultry of National Research Council (NRC, 1994) and drinking water were allowed ad-libitum throughout the experimental period.

First generation (F_1)

Eggs were collected daily, and egg number and egg weight were recorded daily for each group. All eggs

for incubation were sorted in order to remove cracks, morphological deformities and dirt. At 28 weeks of age, fertile eggs were collected for 5 days from each group. They were incubated in a humidified egg incubator at 37 °C and 70% RH. The newly hatched chicks (F_1) were wing banded, weighed at hatch and then every two weeks, and were inoculated based on the program of vaccination of the Local Veterinary Organization. Chicks were divided into three groups from first day of life as the base generation control cool white, sky blue light and yellow light but were subjected to a continuous artificial lighting during the first 8 weeks of age. This artificial light was decreased to 12 hours light and 12 dark at 17th week of age, then was gradually increased by one hour/month till reached 16 hours light at the 21st week of age (Han et al., 2017). Chicks were offered a ration for starters (19 % CP and 2800 Kcal/Kg) from the time of hatch to the age of 8 weeks, a ration for growers (15 % CP and 2700 Kcal/kg) from the age of 9 to 20 weeks, and then were fed a balanced ration for layers covering their nutritional requirements (16 % CP and 2700 Kcal/kg) till the end of the experiment (Baghban-Kanani et al., 2020). Table 1 shows the ingredients and chemical composition of the diet.

Sample collection

In both base and first generations, tissue and blood samples were taken in each group from 50 females and 5 males. Tissue samples were taken from spleen for RNA extraction. The samples were put in Eppendorf containing RNA later (Qiagen, Germany), to minimize the action of endogenous RNase. The blood samples were collected without anticoagulant from wing veins into clean and dry centrifuge tubes, were allowed to clot at room temperature, and were then centrifuged at 3000 rpm for 5 min. Serum was stored at -20 °C until biochemical analysis. Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University approved the protocol of the study.

Table 1. Ingredients and chemical composition of the diet used in the experiment.

Ingredient	Starter (0-8 wk.)	Growing (9-20 wk.)	Laying (21wk.-till end of experiment)
Yellow corn (kg)	63	63	65
Soybean meal (44% cp) (kg)	30	16.50	23.3
Wheat bran (kg)	3	16.70	1.90
Di- calcium phosphate (kg)	1.80	1.30	1.50
Limestone (kg)	1.50	1.80	7.6
Nacl (kg)	0.30	0.30	0.30
Premix (vitamins minerals mixture) (kg)	0.30	0.30	0.30
Methionine (kg)	0.10	0.10	0.10
Total (kg)	100	100	100
Calculated analysis:			
Metabolizable energy k Cal /kg	2800	2700	2700
Crude protein %	19	15	17
C/P ratio	147	193	168
Calcium %	1	.90	3.30
Available phosphate %	0.45	0.40	0.40
Lysine %	0.95	0.70	0.73
Methionine %	0.38	0.30	0.32
Methionine and cystine %	0.70	0.54	0.62

Table 2. Oligonucleotide primers sequence, accession number, annealing temperature and PCR product size of *TLR4, IL10 and β-Actin* genes.

Gene	Primer (forward)	Product length (bp)	Accession number	Reference
TLR4	F:5-GAGAACCTCAATGCGATGC-3 R:5-ATAGGAACCTCTGACAACG-3	272	NM_001030693	(Lu et al., 2013)
IL10	GGAGCTGAGGGTGAAGTTTG-3 -5: F TAGAAGCGCAGCATCTCTGA-3 -5: R	416	AJ621254	(Lu et al., 2013)
β-Actin	F:5-GAGAAATTGTGCGTGACATCA-3 R:5-CCTGAACCTCTCATTGCCA-3	152	NM_205518.1	(Yuan et al., 2007)

Table 3. Reverse transcription and real time PCR program for *TLR4, IL10 and β-Actin* genes.

Gene	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		
			Secondary denaturation	Annealing	Extension	Secondary denaturation	Annealing	**Final extension**
TLR4	50˚C 30 min	94˚C 15 min.	94˚C 15 sec	56˚C 30 sec.	72˚C 30 sec	94˚C 1 min.	56˚C 1 min.	72˚C 1 min.
IL10	50˚C 30 min.	94˚C 15 min.	94˚C 15 secs	59˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	59˚C 1 min.	72˚C 1 min.
β. actin	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	51˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	51˚C 1 min.	72˚C 1 min.

RNA extraction and real time PCR

The RNA extraction was done using RNeasy Mini Kit (Qiagen, Germany), according to the protocol of the manufacturer. To remove any contaminating genomic DNA, RNA was treated with RNAse free-DNAse I (Qiagen, Germany). The expression profile of *TLR4* and *IL10* genes was carried out in spleen. The relative expression was quantified using SYBR Green PCR Master Mix (2x SensiFast™ SYBR, Bioline). Primer sequences and annealing temperatures are shown in Table 2. The housekeeping β-actin gene was used as an internal control. The reverse transcription of the extracted mRNA and the real time PCR program schedule for each gene is illustrated in Table 3. The real time PCR procedures for selected immune genes were carried out according to procedures described by Ateya et al., (2019). Stratagene MX3005P software was used to determine CT values. In order to detect variation of gene expression on the RNA of different samples, CT of each sample was compared with that of the control group according to "ΔΔCt" method stated by Yuan et al., (2006).

Biochemical analysis

IL-10 was determined using ready-made interleukin-10 (IL-10) ELISA Kits provided by Quantikine Company according to the method described by Zdanov et al. (1996).

Data analysis

Results were expressed as means ± standard error of the mean. Analysis was done using one-way analysis of variance (ANOVA) to test all groups' unpaired values. Duncan Multiple Range Test was used to separate the means among the treatment groups. Differences were considered to be significant at the level of ($P \leq 0.05$).

RESULTS

The impact of light colour temperature on the pattern of expression of immunity genes (*TLR4 and IL10*) was explored in males and females of F_0 and F_1 generations (Figure 1). Blue colour light-exposed groups exhibited a significant up-regulation of the *TLR4 and IL10* in both males and females compared to both white (control) and yellow colour light-exposed groups. Comparison of F_0 and F_1 generations revealed that F_1 generation had a higher up-regulation of *TLR4 and IL10* genes than F_0 in both males and females.

The impact of light colour temperature on the serum profile of IL10 was explored in both males and

females of F_0 and F_1 generations (Figure 2). There was a significant effect to the light colour temperature on serum levels of IL10. Blue colour light-exposed groups exhibited a significant increase in serum IL10 values in both males and females compared to both white (control) and yellow colour light-exposed groups. Comparison of F_0 and F_1 generations elucidated that F_1 generation had higher values of IL10 than F_0 in both males and females.

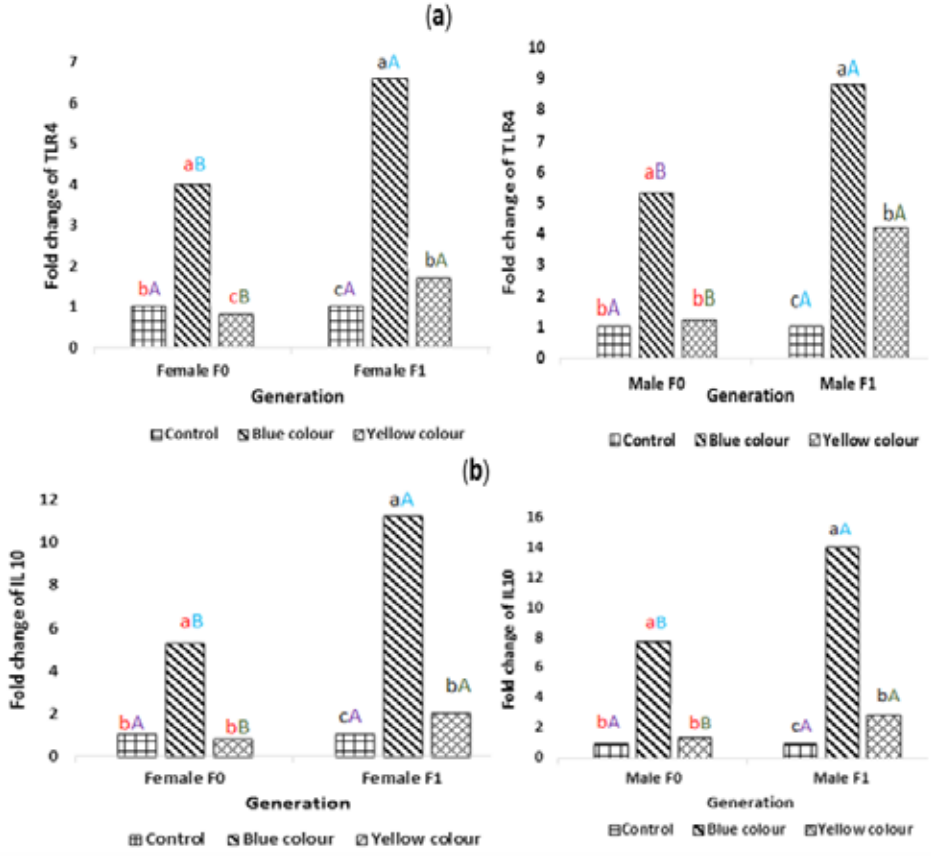

Figure 1. Relative expression of *TLR4* (a) and *IL10* (b) in males and females of base (F_0) and first (F_1) generation. Small letter indicates a significant difference between groups at the same generation. Capital letter indicates a significant difference between the two generations.

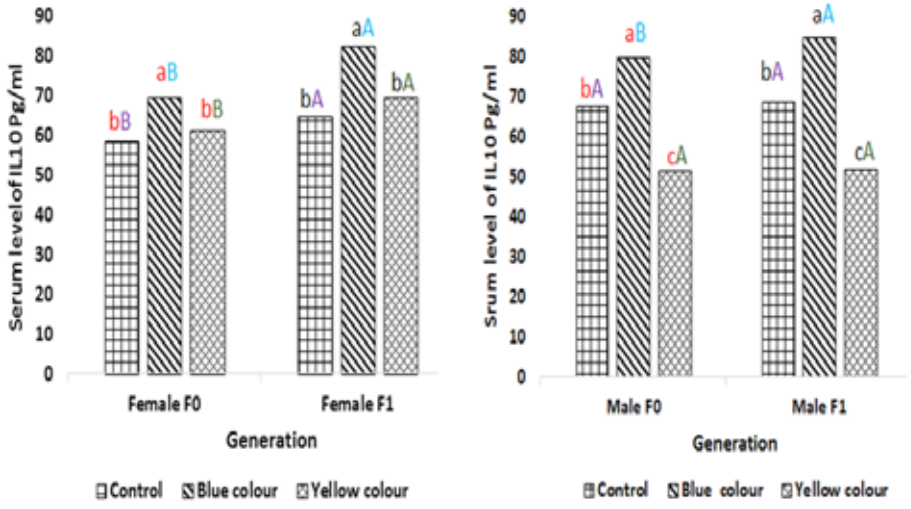

Figure 2. Serum level of IL10 in males and females of base (F_0) and first (F_1) generation. Small letter indicates a significant difference between groups at the same generation. Capital letter indicates a significant difference between the two generations.

DISCUSSION

Light is as an important management tool to manipulate layer immunity. Light colour in particular is considered an important aspect of light that has been considered at one time as a management tool in poultry production (Prayitno et al., 1997). Photoperiod, wavelength, light intensity and more importantly light colour are characteristics that have to be taken into consideration in the selection of artificial light sources and the design of lighting programs for chickens (Thiele, 2010).

Maintenance of layers immune function has therefore become a necessity to avoid reduction in disease resistance and productivity. It has been shown that chicken serum composition could be modulated by lighting program (Onbaşılar et al., 2007). Toll-like receptors (TLRs) are highly conserved proteins secreted from macrophage and dendritic cells, participate in pathogen detection, enhance the production of inflammatory cytokines and up-regulate co-stimulatory molecules (Krishnan et al., 2007; Underhill and Ozinsky. 2002). In avian species, ten TLRs have been identified. Chicken *TLR4* is expressed in different locations including blood and spleen (Kogut et al., 2005). IL-10 is known as the anti-inflammatory cytokine secreted by macrophages, monocytes, and B cells (Eskdale et al., 1997). It also possesses pleiotropic effects in inflammation and immunoregulation (Pestka et al., 2004; Saraiva and O'Garra, 2010). Moreover, gene expression regulation can be exerted at a posttranscriptional level (Said et al., 2010; Haritova and Stanilova, 2012).

Findings of the current study revealed that blue colour light-exposed group had the highest up-regulation of *IL-10 and TLR4* gene expression in both males and females of F_0 and F_1 generations. As far as we are concerned there is a lack of studies exploring the effect of light colour temperature on immune status in layers particularly those considered gene expression of immune markers. Light has been shown to have a remarkable effect on immune response (Moore and Siopes, 2000; Onbaşılar et al., 2007; Blatchford et al., 2009) however, this effect may be poorly understood (Xie et al., 2008). The improved immune profile of the birds experienced blue colour light in the current study could be attributed to specific action of the colour blue of light on immune system of birds. Blue light colour has been shown to have a remarkable positive effect on splenocyte and mononuclear cells proliferations and to increase levels of nitric oxide that activates macrophage for phagocytosis and production of antimicrobial compounds (Seo et al., 2016). The improved immune profile emerged by blue light colour could also be due to a higher peripheral blood T-lymphocyte proliferation (Xie et al., 2008; Zhang et al., 2014; Chen et al., 2016; Guo et al., 2018), a higher H/L ratio (Mohamed et al., 2014; Mousa-Balabel et al., 2017) and its role in modifying heat shock biomarker activities toward enhancing immunity levels and reducing negative impacts of heat stress (Abdo et al., 2017). Other causes for the beneficial effect of blue light colour could be the ability of blue light to improve blood antioxidant (total antioxidant capacity, superoxide dismutase, and glutathione peroxidase), and increase B-lymphocyte proliferation in broilers (Li et al., 2015). Nevertheless, improved immune profile in Japanese quail exposed to warm white colour has been also reported (Moore and Siopes, 2003), and was referred to the release of melatonin that stimulates cellular and humoral immune response. Warm light colour was also reported to increase number of WBC (Abu Tabeekh, 2016).

In the current study we found that blue colour light-exposed group showed the highest level of IL-10 in both males and females of F_0 and F_1 generations. The results of serum profile also coincided with those of the gene expression pattern. There is also little information on serum profile of immune markers in layers exposed to different light colour temperatures. There is also controversy between the results reported in the current experiment and those of previous work. The reason for such controversy could be differences in the light source, light colour temperatures, light intensity and species/strain of the bird. It could also be that previous experiments were conducted on only one generation of birds. For instance, Abu Tabeekh, (2016) investigated how light colour affected some blood parameters of layers and reported that birds experienced warm light colour exhibited higher white blood cell counts than those received red light (RL), blue light (BL), green light (GL), and blue-green mix light (BGL).

The effect of light colour temperature was previously investigated in broiler chickens, and there were also controversies between the results. An enhanced IgG and IgA was reported in broiler receiving mixed green-blue light compared to those receiving either monochromatic green or blue light (Hassan et al., 2014). A significant enhanced proliferation of splenocyte and blood mononuclear cells was observed in chickens reared in blue compared to those reared in

green light-emitting diode (LED) (Seo et al., 2016). Similarly, blue light-exposed Cobb broiler chicks showed a significant increase in interlukien-1β (IL-1β) compared to those exposed to warm light (Mohamed et al., 2014). In the same respect, Guo et al., (2018) found an improved a-Naphthyl-acetate esterase and increased antibody production in broilers exposed to intermediate or low-intensity blue lights. Soliman and Hassan, (2019) reported also that blue light colour-exposed broiler chickens showed a significant increase in anti-Newcastle antibody titer as well as a highly remarkable decline in total bacterial count (TBC), and total Enterobacteriaceae count compared to red and white-exposed groups. On contrary, Sharideh and Zaghari, (2017) traced the effect of light emitting diodes with different colour temperatures on immune responses of male broiler and reported that warm-white light was the most suitable to provide the optimum level of immunity.

In conclusion light colour temperature has a pronounced effect on immune status of layers particularly Egyptian Fayoumi chickens. These findings recommend using blue light for better immune status in poultry farms to predict the most susceptible risk time for disease incidence and to build up an efficient management protocol. More studies are needed to investigate the effect of light colour temperature on other chicken breeds. Expression profile of other immune genes is also needed to understand their regulation mechanisms.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

A. Ateya conceived, designed the experiment, performed the gene expression and wrote the manuscript; H. EL-Emam collected samples, contributed to doing the gene expression and writing of the manuscript. U. Abou-Ismail, I. El-Araby, and M. Fouda analyzed data and contributed to writing of the manuscript.

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Research article
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Microbiological evaluation of some Egyptian fermented dairy products

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ABSTRACT: This study was carried out to evaluate the microbiological quality of plain and fruit yoghurt and rayeb. Sixty samples of yoghurt (30 plain &30 fruit) and 60 samples of rayeb (30 plain &30 fruit) were randomly collected from different supermarkets in Kafr El-Sheikh city. The obtained results revealed that the pH values of plain and fruit yoghurt were significantly higher than those of plain and fruit rayeb. *Staphylococcus aureus* counts were $4.33\times10^2 \pm 0.42\times10^2$ and $4.0\times10^2 \pm 0.57\times10^2$cfu/g, in fruit yoghurt and plain rayeb, respectively. Only two samples of plain yoghurt and fruit rayeb contained *S.aureus* with counts of $9.0\text{x } 10^2 \pm 1.0\times10^2$ and $1.1\times10^3 \pm 0.1\times10^3$cfu/g, respectively. Coliforms were only detected in plain and fruit yoghurt with counts of $8.82\times10^4 \pm 0.81\times10^4$ and $5.4\times10 \pm 1.04\times10$ cfu/g, respectively. E.coli was isolated from plain and fruit yoghurt with incidence rates of 73.3 and 6.7%, respectively. All *E.coli* isolates from plain yoghurt were serologically identified as, O91: H21, O26: H11, O121: H7, O163: H2, O114: H4, O128: H2 O111: H2, and O86. Fruit yoghurt had only O_{111}:H_2. Yeast and molds were also found in the four dairy products with high frequencies for *Penicillium species*.The two virulence genes, enterotoxin gene A and Shiga toxins 2, were detected in three isolates of *S. aureus* and *E. coli* by multiplex PCR. With these results, we could conclude that yoghurt and rayeb samples collected from Kafr El-Sheikh city supermarkets create a health threat to consumers. Therefore, the application of hygienic measures and sanitary practicesis necessary to produce high-quality fermented products to assure consumer's safety.

Keywords: Fermented dairy products, *S. aureus*, Coliforms, *E.coli*, yeast, mold.

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INTRODUCTION

Fermented milk products are a crucial supplement to people's daily diet and supply necessary components for growth and better health (Uccello et al., 2012). Among these dairy products, yoghurt comes on the top for its higher nutritive value as it is rich in fat, lactose, protein, and, calcium. It is easily digested and has an inhibitory effect against harmful bacteria in the intestine. These bacteria can harm the intestines, thereby reducing intestinal motility, autointoxication, and, inflammation (Khan et al., 2008). Rayeb is a curdled skim milk made in Egypt. It can be prepared by heating fresh cow milk in a hot oven for 2 hrs and then the milk was left at room temperature for a period of time ranged from 1 to 4 days based on the temperature until the milk curdles formed at the bottom underneath the fat (Benkerroum and Tamine 2004). As a fermented dairy product, Rayebis rich in vital elements with a higher nutritional value (Samet-Bali and Attia 2012). Both yoghurt and rayeb could be provided either plain (without fruits) or with fruits.

These dairy products could be contaminated with various microorganisms, such as bacteria, molds, and yeasts during manufacturing, handling, and distribution. In such a case, people consumed these contaminated dairy products would suffer from food poisoning. People also do not purchase spoiled products, thereby leading to economic losses for dairy manufacturers (Weerasekara et al., 2010). Enterotoxigenic *S. aureus* pose a safety hazard for consumers, and such existence in dairy products could be used for risk assessment of such products (Zouharova and Rysanek, 2008). S. aureus is one of the most prevalent food poisoning bacteria. The presence of this bacterium in dairy products indicates the unsanitary handling of the products at an improper temperature and time conditions (Collins et al., 2010; Huang et al., 2001). Among the five known *S. aureus* enterotoxins (SEA, SEB, SEC, SED, and SEE), SEA plays a key role in food-poisoning outbreaks world-wide (Argudin et al., 2010). These SEs are heat stable (remain active even after the death of *S. aureus* by cooking heat) and withstand the effect of digestive enzymes (Presscott et al., 2012).

The presence of any gram-negative bacteria such as coliforms group in commercially produced yogurt indicates post-pasteurization contamination or pasteurization failure (Hervert et al., 2017). According to the National Agency of Food and Drug Administration Control (NAFDAC), there can be neither *E. coli* nor coliforms in each 100 ml yoghurt sample (Mbaeyi-Nwaoha et al., 2012). Coliforms are regularly utilized as an indicator of milk products quality (Yabaya and Idris, 2012). The presence of *E. coli* in dairy products indicates fecal contamination of these products (Singh and Prakash, 2008).Diarrhoeagenic *E. coli* (DEC) are the main cause of diarrhea and classified depending on their virulence factors into enterohaemorrhagic *E. coli* (EHEC), which is a subgroup of shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC),enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), enteropathogenic *E.coli* (EPEC),enteroaggregative *E. coli* (EAEC), and necrotoxic *E. coli* (NTEC) (Vilchez et al.., 2009). Pathogenic *E. coli*, especially STEC, is also responsible for foodborne diseases which can cause bloody diarrhea, hemolytic-uremic syndrome, and thrombotic thrombocytopenic purpura (Altalhi and Hassan, 2009;Kuyucuoglu et al., 2012;Paton and Paton, 1998). The main route of *E. coli* infection is the oral route through the consumption of contaminated food and dairy products (Pearce et al., 2006).

Yeasts and molds also participate in spoilage of yoghurts because acidic pH of yoghurts can create an appropriate environment for yeasts and molds growth (Fleet and Mian, 1987). Therefore, the existence of yeasts and/or molds in yogurt indicates unsanitary procedures in production or packaging (Arnott et al., 1974). Yogurts supplemented with sugar is more prone to yeast growth (Lourens-Hattingh and Viljoen, 2001).

This study aimed to assess the microbiological quality of yoghurt and rayebin Kafr El-Sheikh city through the identification of *S. aureus*, coliform bacteria, enteropathogenic *E. coli*, yeast and molds, in addition to detection of virulence genes of *S. aureus* and *E.coli* isolates.

MATERIAL AND METHODS

All experiments were carried out in microbiology laboratories in Animal Health Research Institute and Faculty of Veterinary Medicine, Kafrelsheikh University.

Samples collection

Sixty samples of yoghurt (30 plain & 30 fruit) and 60 samples of Rayeb (30 plain &30 fruit) were purchased from different supermarkets in Kafr El-Sheikh province. All collected samples were within the expiry date printed on the label. After purchasing, they

were placed in an insulated iceboxand transported quickly to the Lab for further examination.

Determination of pH

The determination of pH of samples was performed using electrical pH meter (Bye model 6020, USA) according to the method described by Omokaro and Telema (2014).

Microbiological examination

Serial dilutions were prepared from all samples according to APHA, 2004. *S. aureus* was isolated using Baird-Parker agar medium and enrichment culture medium Giolitti-Cantonias previously described (Singh and Prakash, 2008). Pure *S. aureus* culture was characterized by the following biochemical tests: coagulase, catalase, oxidase, mannitol, hemolysis, and fermentation of glucose, lactose and sucrose (MacFaddin, 2000). Coliforms were counted using 3 tubes MPN technique on Lauryl Tryptose broth (Verma et al., 1999). *E.coli* were isolated using Eosin Methylene Blue agar (EMB, Oxoid, England) following the protocol of De Boer and Heuvelink, (2000).The isolated *E. coli* were serotyped using rapid diagnostic *E.coli* antisera sets (DENKA SEIKEN Co., Japan) as previously detailed (Kok et al. 1996).Yeast and mold were counted using Sabouraud Dextrose Agar medium supplemented with 0.05 mg/ml chloramphenicol (Bailey and Scott, 1998). Identification of isolated mold species was done as previously described (Raper and Fennel 1965; Raper and Thom 1969; Samson 1979; Samson et al. 1976; Zycha et al.1969).

Multiplex polymerase chain reaction (PCR)

Bacterial genomic DNA was extracted from *S. aureus* and *E.coli* isolated from plain yoghurt using QIAamp DNA mini kit following the manufacturer's instruction and as previously described (Allam et al., 2019).Multiplex PCR was used to detect the presence of virulence genes of *S. aureus* and *E. coli* using specific primers (Table 1). A total PCR reaction volume of 30 µl was used. This mixture included 15 µl 2x Master mix (Emerald Amp GT), 5µl DNA, 1 µl from each forward primer and reverse primer (20 pmol), 8µl nuclease free water. The thermal cycling conditions were: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 40 sec for *stx1* and *stx2* of *E. coli* and 57˚C 40 sec for *S. aureus* enterotoxin genes, extension at 72°C for 40 sec, followed by a final extension at 72°C for 10 min. A volume of 20µl of each PCR product was used in gel electrophoresis.A 100bp DNA ladder was used as a marker for PCR products. This gel contained 1.5 % agarose gel and 0.5 µg/ml ethidium bromide (for staining).The gel was photographed by gel documentation equipped with UV transilluminator.

Statistical analysis

Data were presented as a mean ± standard error of the mean (SEM) and significance was set at $P < 0.05$. Statistical analysis was achieved using either One-way ANOVA or unpaired t-test. The post-hoc test, Tukey's Honestly Significant Difference was used to determine the difference between the groups using GraphPad Prism 7.

Table.1. Oligonucleotide primers sequences

	Gene	Sequence	Amplified product (bp)	References
S. aureus	Sea	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	102	Mehrotra et al., 2000
	Seb	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	164	
	Sec	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG	451	
	Sed	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC	278	
	See	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	209	
E. coli	Stx 1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614	Dipineto et al., 2006
	Stx 2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	

RESULTS AND DISCUSSION

The obtained results showed that the pH values of plain and fruit yoghurt were significantly (P<0.001) higher than those of plain and fruit rayeb (Table 2). However, no significant difference was noticed between either plain and fruit yoghurt or plain and fruit rayeb (P>0.05). Similar pH values were obtained by Osman (2015) for plain and strawberry fruit yoghurt (4.77±0.03 and 4.37±0.02, respectively) and rayeb samples (4.47±0.03). However, El-Ansary (2014) reported lower pH values for yoghurt samples (4.06 ± 0.068). Higher pH of yoghurt might be due to the absence of accurate system of culture dosage, whichmostly influences yoghurt acidity (Abrar et al., 2009).

Table 2. The pH values in examined samples

Samples	pH values
Plain yogurt	$4.51 \pm 0.0^{5\,a}$
Fruit yogurt	$4.43 \pm 0.0^{4\,a}$
Plain rayeb	$4.21 \pm 0.0^{3\,b}$
Fruit rayeb	$4.16 \pm 0.0^{2\,b}$

Data were expressed as mean ± SEM. Means carrying different superscript letters [a (the highest value), b (the lowest value)] in the same column differed significantly at P<0.001.

According to the standards of Egyptian Organization for Standardization and Quality Control fermented each 1 g of dairy products should be free from *S. aureus* (EOSQC, 2005). The microbiological analysis results revealed the presence of *S.aureus* in plain yoghurt, fruit yoghurt, plain rayeb, and fruit rayeb with incidence rates of 6.67, 20, 13.33, and 6.67%, respectively (Table 3). Samet-Bali et al. (2016) reported a similar prevalence of *S. aureus* in rayeb (13.33%).However, Atef et al., (2017), El-Ansary, (2014), and El-Leboudy et al., (2017) found a highernumber of *S. aureus* (25% in rayeb, 42% in yoghurt, and 85% in Rayeb, respectively). On the other hand, AbdEllatif et al., (2016) and Bachir and Benattouche, (2013) could not isolate *S. aureus* from any examined yoghurt samples. S. aureus count was significantly (P<0.01) higher in plain yoghurt, and fruit rayeb than in fruit yoghurt and plain rayeb (Table 3). However, no significant difference was observed between either plain yoghurt and fruit rayeb or fruit yoghurt and plain rayeb (P>0.05). Higher *S. aureus* count in yoghurt ($5.5\times10^4\pm3.94\times10^3$cfu/g) was reported by El-Ansary, (2014). In general, the higher prevalence of *S. aureus*in these dairy products might be due to the unhygienic practices during production and processing (Salvatierra et al., 2004). Presence of *S. aureus* in fermented dairy products indicates contamination from food handlers through hand or arm lesions caused by *S. aureus*. Therefore, dairy products could be contaminated through contact, coughing and sneezing (Hussain 2010).

It is obvious from Table 3 that coliforms were only detected in plain and fruit yoghurt with incidence rates of 100% and 13%, and counts of $8.82\times10^4 \pm 0.81\times10^4$ and $5.4\times10 \pm 1.04\times10$ cfu/g, respectively. Plain yoghurt exhibited a significantly higher coliforms count than fruit yoghurt.Lower coliforms prevalence of 58% and 40% with a count of $5.6\times10^4\pm3.68\times10^3$ and $5.02\times10^2\pm0.57\times10^2$cfu/g, were reported in plain yoghurt by El-Ansary (2014) and Osman (2015), respectively. In contrast, El-Leboudy et al., (2017) reported that the prevalence and count of coliforms in rayeb samples was 100% and $1.65\times10^3\pm2.69\times10^2$cfu/g. Egyptian Organization for Standardization and Quality Control declared that each 1 g of fermented dairy products should be free from coliforms (EOSQC, 2005). High count of coliforms in yoghurt indicates unsanitary procedures, utilizing of low quality milk, inadequate preheating, post-processing cross-contamination (El Bakri and El Zubeir, 2009; Samet-Bali et al., 2016).

Table 3. S. aureus and Coliforms count in examined samples

Samples	S. aureus		Coliforms	
	NO(%)	Count	NO(%)	Count
Plain yogurt	2(6.67)	$9.0\times10^2\pm 1.0\times10^{2a}$	30(100)	$8.82\times10^4 \pm0.81\times10^{4\,a}$
Fruit yogurt	6(20)	$4.33\times10^2 \pm 0.42\times10^{2\,b}$	4(13)	$5.4\times10 \pm 1.04\times10^{\,b}$
Plain rayeb	4(13.33)	$4.0\times10^2 \pm 0.57\times10^{2\,b}$	0	0
Fruit rayeb	2(6.67)	$10.1\times 102\pm 1.1\times 10^{2\,a}$	0	0

Data were expressed as mean ± SEM. Means carrying different superscript letter in the same column differed significantly at P<0.01.

E.coli was only detected in plain and fruit yoghurt samples at prevalence rates of 73.3 and 6.7%, respectively (Table 4). This exceeded the limit set by Egyptian Organization for Standardization and Quality Control, which declared that each 1 g of fermented dairy products should be free from *E.coli* (EOSQC, 2005). The isolated strains from plain yoghurt. were serotyped to O91 : H21 (EHEC) , O26 : H11 (EHEC), O121: H7 (EHEC), O163 : H2 (EPEC), O114 : H4 (EPEC), O128: H2 (ETEC), O111: H2 (EHEC) and O86 (EPEC) at prevalence rate 12.5, 37.5, 6.3, 6.3, 12.5, 12.5, 6.3 and 6.3 %, respectively. The isolated *E.coli* from fruit yogurtwere serotyped into O111: H2(EHEC) at an incidence rate of 100%.O_{26}: H_{11} was the most frequently identified serotypes isolated from plain yoghurt and was detected with higher frequency 37.5% followed by O91: H21, O114: H4 and O128: H2 with a prevalence rate 12.5 % for each.*E. coli* O26:H11 is commonly associated with diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (Anjum et al., 2003; Bettelheim, 2003). Lower prevalence of *E.coli* (44.8%) was detected in plain yoghurt (El-Ansary, 2014). However, Atef et al., (2017) and El-Leboudy et al., (2017) reported *E.coli* number of 55% in rayeb sample and 30% in Baladi rayeb, respectively. In contrast, El-Baz, (2019) did not detect verotoxigenic *E.coli* in yoghurt. Higher number of *E.coli* in plain yogurt could be due to insufficient heating, using poor quality raw milk containing higher initial bacterial count, and unsanitary handling. The variations in *E.coli* incidence ratio and count in fermented dairy products among different studies could be attributed to the fact that the survival of *E.coli* in fermented dairy products is highly variable depending on starter cultures used, temperature of storage and pH value.

Table 4. Incidence and serotyping of *E. coli* isolated from examined samples (n=60)

Products	Plain yogurt		Fruit yogurt		
	No	%	No	%	
E.coli	22	73.3	2	6.7	Strains characterization
	Serotypes of isolated E.coli				
Serotype	No	%	No	%	
O_{91} : H_{21}	4	12.5	-	-	EHEC
O_{26} : H_{11}	12	37.5	-	-	EHEC
O_{121} : H_7	2	6.3	-	-	EHEC
O_{163} : H_2	2	6.3	-	-	EPEC
O_{114} : H_4	4	12.5	-	-	EPEC
O_{128} : H_2	4	12.5	-	-	ETEC
O_{111} : H_2	2	6.3	2	100	EHEC
O_{86}	2	6.3	-	-	EPEC

Yoghurt is a highly selective environment for the growth of yeast and mold due to its high acidity.Yeast and mold can utilize acid as a source of energy and produce alkaline products that decrease acidity and give a chance for the growth of putrefactive bacteria (Abdel Hameed, 2011). The allowed limits for yeasts and molds in fermented dairy products are less than 10 /g as stated by Egyptian standards (EOSQC, 2005). The presented data in table 5 elucidated that the yeasts were detected at incidence rates of 93.33, 26.67, 33.33, and 26.67 % with counts of $3.92\times10^4\pm0.81\times10^4$,$2.90\times10^4\pm 1.88\times10^4$, $2.30\times10^2 \pm 0.73\times10^2$, and $2.75\times10^3 \pm 1.42\times10^3$cfu/g in plain, fruit yoghurt and plain, and fruit rayeb, respectively. Yeast count was significantly higher in plain yoghurt than in other examined dairy products. Similar yeast incidence of 92.0% and count $4.2\times10^4 \pm 0.8\times10^4$cfu/gin yoghurt was reported by AbdEllatif et al., (2016). A higher prevalence of 100% with a count of $8.37\times10^5\pm0.96\times10^5$cfu/g was recorded by Osman, (2015). Lower number (40%) but with similar count ($5.6\times10^4\pm1.6\times10^4$cfu/g) was detected in plain yoghurt (Barakat, 2019). Molds were detected at incidence ratesof 66.67, 80, 100, and 100% with counts of $1.74\times10^3 \pm 0.52\times10^3$,$4.25\times10^2 \pm 0.67\times10^2$, $1.95\times10^3 \pm 0.38\times10^3$, and $8.06\times10^2 \pm 0.82\times10^2$cfu/g in plain yoghurt, fruit yoghurt, plain rayeb, and fruit rayeb, respectively (Table 5).Fruit yoghurt showed significantly lower mold count than other examined dairy products. A similar number was obtained by Osman, (2015), who reported that 100% of examined rayeb samples were contaminated with mold with an account of $4.49x10^5 \pm 0.51 x10^5$cfu/g. In addition, Samet-Bali et al., (2016) reported that 100% of rayeb samples were contaminated with yeast and mold but with a higher count of $1.34\times10^7\pm 0.23\times10^7$cfu/g. Lower mold count (28%) with a level of $1.5 \times10^4\pm0.44\times10^4$cfu/g was detected by Barakat, (2019).Amer, (2017) reported that 44 % of examined

yoghurt samples were contaminated with molds with a count of $3.92\times10^3 \pm 7.67\times 10^2$cfu/g.The presence of yeasts and molds in yoghurt indicates poor sanitary conditions (Oyeleke, 2009 and El-Malt et al., 2013).

Penicillium species were the most frequent (21.7%, 31.0, 23.6, and 33.3%) isolated mold species from plain, fruit yoghurt and plain, and fruit rayeb, respectively (Table 6). Aspergillus species were the second most prevalent mold in fermented dairy products. Similarly, Osman, (2015) also found that Penicillium species were the most frequent isolated mold species from examined plain yoghurt, flavored yoghurt, and rayeb samples. While, Barakat, (2019) found that Geotrichum species was the most frequent isolated mold species from yoghurt samples at an incidence rate 32.9%.Frequent isolation of Penicillium species from dairy products could be due toability of these species to grow within wide ranges of pH (2 to 11), water activity value (0.620 to 0.995), temperature (-10 to 60 °C) and nutrient limitations (Pitt and Hocking 2009).

Results obtained from multiplex PCR revealed positive results for only enterotoxin A (*sea*) virulent gene (102 bp) in *S. aureus* isolates 1, 2, and 4 (Fig.1). Consistent with these results, Mohamed and Mazyed (2015) also detected *sea* gene in one isolate form three examined plain yoghurts.These results suggest that the PCR assay is a fast and highly sensitive technique for the determination of enterotoxins genes in *S.aureus* isolates (Anvari et al., 2008). Multiplex PCR for detection virulence genes in *E.coli* isolates revealed that only *stx2* gene (779 bp) was detected in isolates 2, 3, and 4 (Fig. 2). Our results agreed with Neven et al., (2017) who also detected *stx2* in *E.coli* isolated from plain yoghurt.Stx proteins produced by *E.coli* are one of the main causes of enteritis in humans (Tyler, et al. 2005). Virpari et al. (2013) reported that out of 80 *E. coli* isolates from milk and dairy products12 (15 %) and 18 (22.50%) isolates were positive for *stx1* and *stx2* gene, respectively. Detection of *sea* and *stx2* does not confirm their production so RPLA technique could be used to evaluate toxin production (Vanbelkum, 2003).

Table 5. Yeast and mold count in examined samples

Samples	Yeast		Mold	
	NO(%)	Count	NO(%)	Count
Plain yogurt	82(93.33)	$3.92\times10^4 \pm 0.81\times10^{4\,a}$	20(66.67)	$1.74\times10^3 \pm 0.52\times10^{3\,a}$
Fruit yogurt	8(26.67)	$2.90\times10^4 \pm 1.88\times10^{4\,b}$	24(80)	$4.25\times10^2 \pm 0.67\times10^{2b}$
Plain rayeb	10(33.33)	$2.30\times10^2 \pm 0.73\times10^{2\,c}$	30(100)	$1.95\times10^3 \pm 0.38\times10^{3\,a}$
Fruit rayeb	8(26.67)	$2.75\times10^3 \pm 1.42\times10^{3\,bc}$	30(100)	$8.06\times10^2 \pm 0.83\times10^{2\,a}$

Data were expressed as mean ± SEM. Means carrying different superscript letterσ in the same column differed significantly at $P<0.05$.

Table 6. Incidence of identified mold species isolated from examined samples

Mold sp.	Plain yogurt		Fruit yogurt		Plain rayeb		Fruit rayeb	
	No	%	No	%	No	%	No	%
Penicillium sp.	10	21.7	18	31.0	26	23.6	20	33.3
A. niger	6	13.0	2	3.4	14	12.7	6	10.0
A. fumigatus	2	4.3	2	3.4	8	7.3	0	0
A. flavus	8	17.4	8	13.8	4	3.6	8	13.3
A. versicolor	2	4.3	0	0	2	1.8	4	6.7
A. ochraceus	2	4.3	0	0	2	1.8	2	3.3
A. terreus	0	0	8	13.8	8	7.3	0	0
A. ruber	0	0	2	3.4	0	0	0	0
A. nidulans	0	0	2	3.4	0	0	0	0
A. clavatus	0	0	0	0.0	0	0	0	0
Mucor sp.	8	17.4	2	3.4	10	9.1	6	10.0
Rhizopus sp.	2	4.3	0	0	8	7.3	0	0
Fusarium sp.	2	4.3	6	10.3	4	3.6	6	10.0
Thamnidium sp.	2	4.3	6	10.3	12	10.9	6	10.0
Trichothecium sp.	0	0	2	3.4	0	0	0	0
Cladosporum sp.	2	4.3	0	0	12	10.9	2	3.3
Total No.	46	100	58	100	110	100	60	100

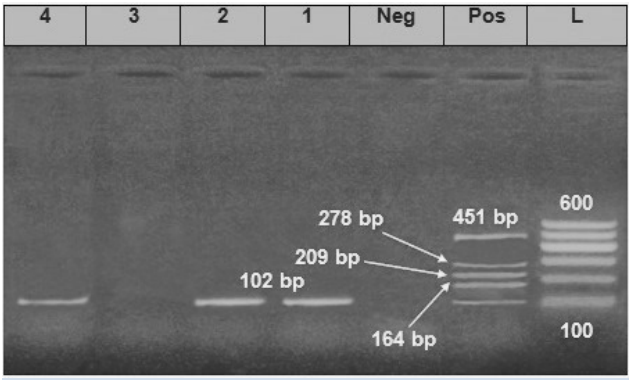

Fig.1. Agarose gel electrophoresis of multiplex PCR of *sea* (102 bp), *seb* (164 bp), *sec* (451 bp), *sed* (278 bp), and *see* (209 bp) enterotoxin genes for characterization of *S. aureus*. L: 100 bp DNA ladder; Pos: control positive for *sea*, *seb*, *sec*, *sed*, and *see* genes, Neg: Control negative; Lanes 1, 2 and 4: Positive *S. aureus* strains for sea, lane 3: negative for sea.

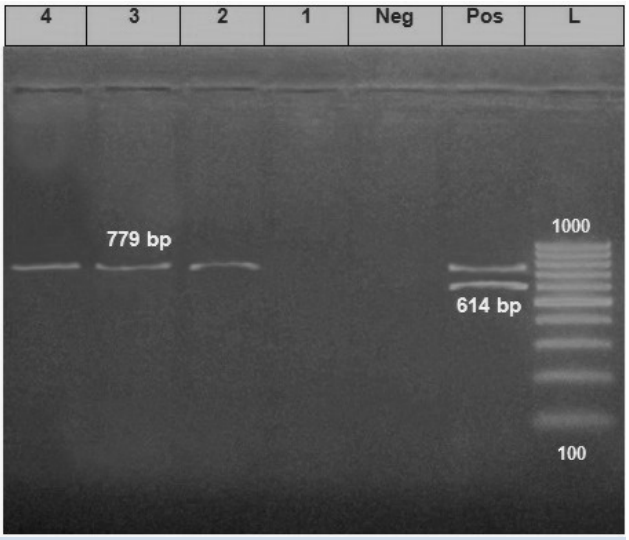

Fig.2. Agarose gel electrophoresis of multiplex PCR of *stx1* (614 bp) and *stx2* (779 bp) genes for characterization of *E. coli.* L: 100 bp DNA ladder; Pos: control positive for *stx1* and *stx2*genes, Neg: Control negative, Lanes 2, 3 and 4: Positive for *stx2*, lane 1: negative for *stx2*

CONCLUSIONS

Yoghurt and rayeb samples collected from Kafr El-Sheikh city supermarkets create a health hazards to consumers. They were contaminated, at different degrees, with *S. aureus*, coliforms, *E.coli*, yeasts, and molds. Moreover, enterotoxin A and shigatoxin 2 genes were detected in some examined isolates of *S. aureus* and *E.coli*.Presence of these micro-organisms in dairy products indicates poor sanitary procedures during the production and processing.The results of this study warrant the need to undertake safety measures during handling, manufacturing, and distribution of fermented dairy products to avoid potential threats for consumers safety.

CONFLICT OF INTEREST

The authors certify that they have no affiliations with any organization or entity with financial or non-financial interest in the subject matter or materials discussed in this manuscript.

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Research article
Ερευνητικό άρθρο

Correlation of a quantitative and a semi-quantitative method for proteinuria detection in chronic kidney disease in dogs

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ABSTRACT: Proteinuria can arise in various physiologic and pathologic conditions. Persistent proteinuria without any abnormalities detected in urine sediment is indicative of chronic kidney disease and has great diagnostic value as it is used for the categorization of the patient on IRIS (International Renal Interest Society) staging system. There are several techniques for urine protein measurement including the semi-quantitative/qualitative (urine dipstick, sulfosalicylic acid turbidimetric test and Heller's reaction test) and the quantitative tests (urine protein to creatinine ratio (UPC) and microalbuminuria assay).The purpose of this study was to correlate the semi-quantitative Heller's reaction test for proteinuria detection, with the UPC in urine samples from 89 dogs with chronic kidney disease. The non-parametric *Spearman's correlation coefficient* was used to correlate Heller's reaction test with UPC in urine samples from dogs with chronic kidney disease in proteinuria detection. Correlation analysis revealed a statistically significant positive and moderate correlation between the Heller's reaction test and UPC ($r(89)=0.510$, $p<0.0001$) which was slightly improved when USG>1010 ($r(72)=0.541$, $p<0.0001$)(urine specific gravity).Heller's reaction test might be a useful alternative to detect proteinuria when UPC is not available in the clinical setting, however it cannot be used interchangeably with UPC for the IRIS sub-staging of chronic kidney disease (CKD).

Keywords: dog, Heller's reaction test, proteinuria, UPC, urinary system

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INTRODUCTION

Proteinuria can result from a variety of proteins excreted or lost into the urine. Albuminuria, the presence of albumin in urine, is the main contributor to overt proteinuria and has the greatest clinical significance in dogs and catsas it seems to be a multifactorial and frequent finding (Sink and Weinstein, 2012; Grauer, 2011; Lyon et al., 2010). Proteinuria can arise in several different physiologic and pathologic conditions, but persistent proteinuria associated with inactive urine sediment is consistent with chronic kidney disease (Grauer, 2011). Detection of proteinuria includes both screening routine analysis and use of advanced laboratory methods Sink and Weinstein, 2012). Detection and measurement of proteinuria is essential,in order to classify the patients using the IRIS staging system (International Renal Interest Society), to administer the appropriate medication, to prevent further kidney damageand to establish the prognosis.

A urine dipstick colorimetric test is the typical first-line screening test for the detection of proteinuria and/or albuminuria (Stockham and Scott, 2008). The protein test methodology used for dry reagent strips is able to detect albumin better than other proteins (Strasinger and DiLorenzo, 2008). However, false positive reactions for protein are common and limit the test's utility (Grauer et al., 2004). Semi-quantitative tests are frequently used to confirm positive reactions for protein on a urine dipstick test (Grauer, 2011; Lyon et al., 2010). The most commonly reported semi-quantitative measuring methods are the urine dipstick and the sulfosalicylic acid test (SSA) (Sink and Weinstein, 2012). Heller's reaction test is a turbidimetric test, quite simple to perform using a test tube, a small amount of nitric acid and equal amount of urine sample (Figure 1), which should be laid slowly over the nitric acid layer (Figure 2). The two liquids (nitric acid and urine) should not be mixed during the procedure (Figure 3). Heller's reaction test is a qualitative method (Figure 4). The test is positive when urine reacts with nitric acid and a white ring of variable thickness forms in the tube (Medaille and Brien-Marshal, 2008). The quantitative methods include urine protein to creatinine ratio (UPC) and microalbuminuria assays (Sink and Weinstein, 2012). The UPC has become the gold standard test for detecting proteinuria and should be run on any patient with evidence of proteinuria tested by urine dipstick or positive SSA (Herley and Langston, 2012). The UPC, performed on a single random urine sample, is closely correlated to the 24-hour urine protein quantification (Le Vine et al., 2010; Adams et al., 1992; Monroe et al., 1989).

Figure 1. Materials needed for Heller's reaction test (tube, nitric acid, urine sample).

Figure 2. Put a small amount of nitric acid (approximately 1ml) in the tube.

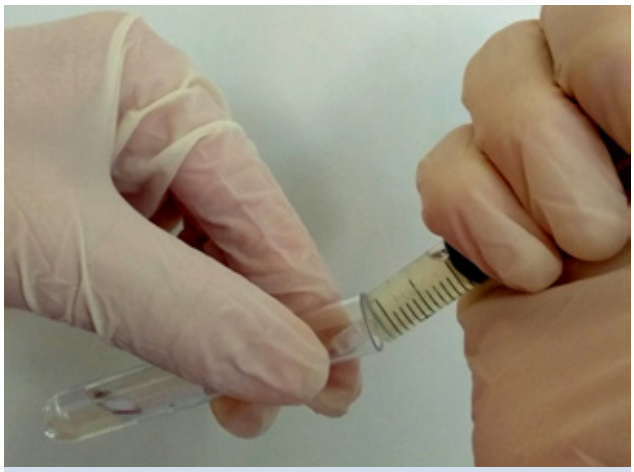


Figure 3. Add equal amount of urine in the tube by layering the urine sample above the nitric acid, caution should be given in order not to mix the samples.

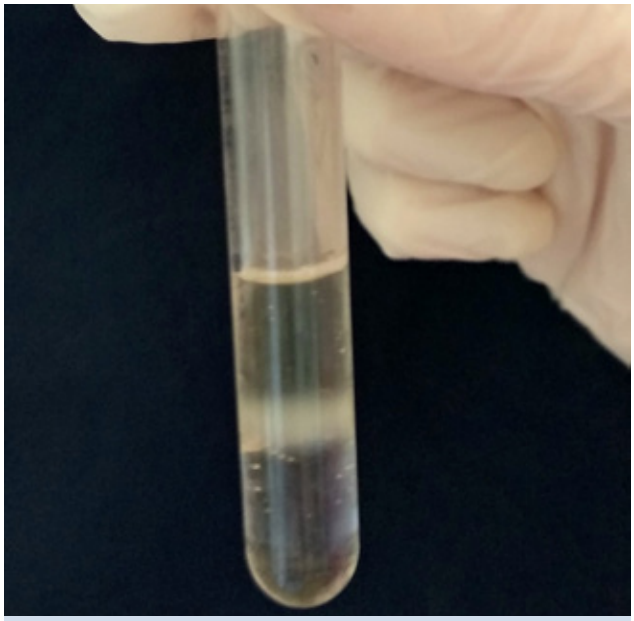


Figure 4. Urine proteins react with nitric acid and the white ring appears (positive Heller's reaction test).

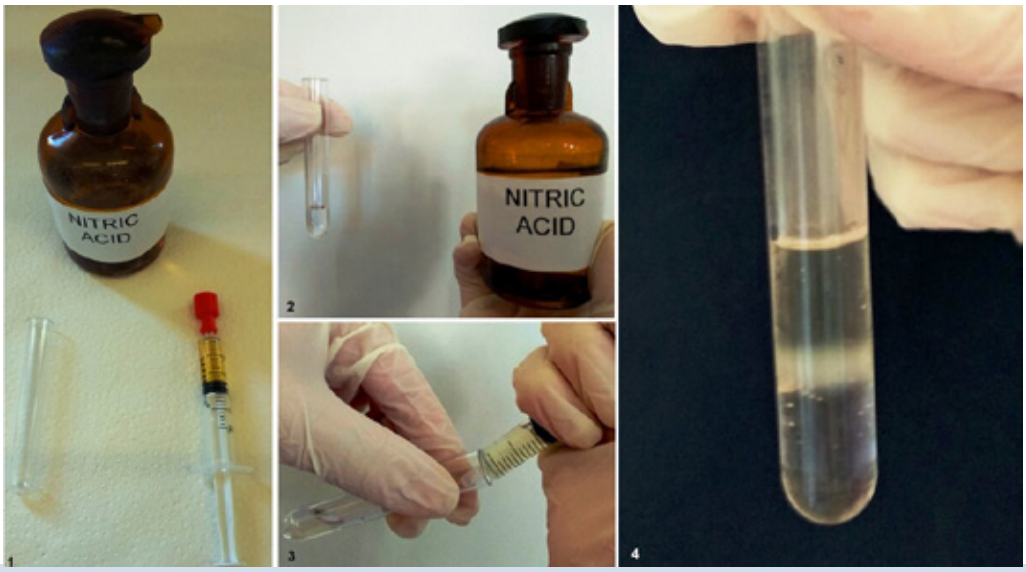
Despite the availability of tests detecting proteinuria, occasionally quantitative methods, which are more precise, cannot be performed in a clinical setting. In these cases, a semi-quantitative test could be used as an alternative practical method to detect proteinuria.

The purpose of this study was to correlate the semi-quantitative Heller's reaction test and the quantitative UPC test in detecting proteinuria in 89 dogs with chronic kidney disease (CKD) and to evaluate the utility of a semi-quantitative test when UPC is not available.

MATERIALS AND METHODS

This retrospective study involved the medical records of 89 dogs admitted to the Companion Animal Clinic of Aristotle University from May 2011 to December 2014. Urine samples from 89 canine patients with chronic kidney disease were included in this study. Patients with clinical (dysuria, pyuria, hematuria, pyometra, vaginitis, prostatitis) or laboratory evidence (active urine sediment, positive urine culture test) of lower urinary or genital tractinfection were excluded from the study. Urine samples collected via free catch using a sterilized container or cystocentesis were included for analysis. For those dogs, whose urine was collected via free catch, appropriate cleaning of the external genitalia was performed prior to voiding. A complete urinalysis was performed in all samples including measurement of urine specific gravity, urine dipstick colorimetric test, Heller's reaction test, microscopic evaluation of urine sediment, urine protein/creatinine ratio (UPC) and urine culture. Urinalyses were performed in the Companion Animal Clinic, UPC measurements were performed in the Diagnostic Laboratory, School of Veterinary Medicine, Aristotle University and the urine cultures were performed in a private veterinary diagnostic laboratory (Vet Analyseis Lab, Larisa, Greece). Urine specific gravity was measured in a refractometer (American Optical Leica TS Meter, American Optical Co, Massachusetts USA) compensated for temperature. The urine dipsticks used were the Combi Screen 10 SL PLUS, Analyticon, Germany. Urine protein/creatinine ratio was measured in Vital Lab Flexor E, (Spankeren, The Netherlands) spectrophotometric analyser. The coefficient of variation (CV) (%) at room temperature for mean urine protein concentration of 8.8, 19.2 and 82.4 mg/dL was 9.5, 4.35 and 3.49, respectively while for the mean urine creatinine concentration of 20.64, 41.80 and 64.60 mg/dL it was 8.2, 4.6, and 9.98, respectively. UPC was performed in the supernatant urine samples (after centrifugation). Both urine analyses and Heller's reaction test were done within 15 minutes from sample collection while UPC measurement was completed 2 hours after sample collection.

Due to the nature of the Heller's reaction test (qualitative), we adapted the quantification of the test (Athanasiou et al., 2014) in order to study the correlation between the two methods. The quantification of Heller's reaction test was based on the thickness of the white ring. In the negative test no white ring formation was observed. Positive tests weregraded using a 4-scale classification system based on the height of the white ring measured with a ruler. According to this system, Heller +1 was defined as white ring of 1mm, Heller +2 as a white ring of 2mm, Heller +3 as a white ring of 3mm and Heller >+3 as a 4mm white ring (Figures 1-4).



Figures 1-4. Procedure of Heller's reaction test.

Due to the nature of the variable Heller (discrete measurements), the non-parametric *Spearman's correlation coefficient* was used in order to study the correlation between UPC and Heller concentrations. In order to assess a better correlation between the two methods, the dogs were subdivided in two groups based on urine specific gravity (USG), the first group of dogs with USG > 1010 and the second group with USG ≤ 1010. Since 1010 was the mean value of isosthenuria, it was set as the cut-off value.

Statistical analyses were performed using the R environment (R core team, 2013, Vienna, Austria).

RESULTS

The study population consisted of 89 patients belonging in 18 different breeds. The majority ofanimals (42/89)(47.1%) were mixed breed dogs. Thirty-six (40.4%) were middle-aged dogs (>5-10 years old for small breeds and >3-7 years old for large breeds), 24/89 (26.9%) were young adult dogs and 26/89 (29.2%) were old dogs. Only 3 young (<1 year) dogs (3.3%), diagnosed with chronic kidney disease, were enrolled in the study. The median age of the population was 66 months (5.5 years) (range from 5 months to 204 months). Most of the study population was male intact dogs (49/89)(55%), followed by 20 female neutered (22.4%), 15 female intact (16.8%) and 5 male castrated dogs(5.6%). Table 1 shows the results of Heller's reaction test in the study population. Table 2 shows the results of UPC ratio after categorization of cases as non-proteinuric (UPC: 0-0.2), borderline proteinuric (UPC:0.21- 0.5) and proteinuric (UPC> 0.5), according to the IRIS substaging system (IRIS staging of CKD modified 2019). Based on the results, 8/89 dogs (8.9%) had a negative Heller's reaction test (no proteinuria). Based on UPC measurement, 7/89 dogs (7.8%) did not have proteinuria. The remaining 82/89 dogs (92.1%) had either borderline proteinuria (7/89)(7.8%) or prominent proteinuria (75/89) (84.2%) (UPC>0.5) (Table 2). The median UPC value was 3.67 (range from 0 to 61.5).

Table 1. Heller's reaction test in 89 dogs with chronic kidney disease that presented in Companion Animals Clinic from 2011 to 2014

Heller's reaction test	Number of dogs (Total 89)
0	8
+1	10
+2	25
+3	40
>+3	6

Table 2. UPC results in 89 dogs with chronic kidney disease that presented in Companion Animals Clinic from 2011 to 2014

UPC	Number of dogs (Total 89)
0-0.2	7
0.21-0.5	7
>0.5	75

Data analysis showed a statistically significant positive and moderate correlation betweenUPC and Heller's reaction test ($r(89)=0.510$, $p<0.0001$) when all 89 samples were used. The correlation was slightly improved ($r(72)=0.541$, $p=0.0001$) in samples with USG>1010. In samples with USG≤ 1010 no

significant correlation was observed, (r(17)=0.3208, p=0.209).

DISCUSSION

When UPC is not readily available, Heller's reaction test might be a useful alternative to detect proteinuria for urine samples with USG>1010. Semi-quantitative and quantitative methods have been compared in previous studies, in order to find alternative methods for quick and accurate assessment of proteinuria (Lyon et al., 2010; Garner and Wiedmeyer, 2007; Mardell and Sparkes, 2006; Welles et al., 2006). In a previousstudy, qualitative, semi-quantitative and quantitative methods have been compared in order to assess albuminuria in urine samples of clinically healthy dogs and cats (Lyon et al., 2010). In particular, the results of urine dipstick, sulfosalicylic acid (SSA) test and UPC were compared to those of an albumin-specific ELISA, to show that albuminuria detection should be interpreted cautiously when urine dipstick, SSA test and UPC are used interchangeably due to the high percentage of false- positive results when trace or greater considered a positive result (Lyon et al., 2010). According to the authors, the false-positive rate decreased when trace reactions were excluded in both analyses (9.3% and 11.5% for the urine dipstick and SSA test, respectively) and the specificity was increased to 98.9% for the urine dipstick and to 99% for the SSA test, when both trace and 1+ reactions were excluded. In contrast with our study, the results of this work had not been influenced by the urine specific gravity, probably because only healthy animals were evaluated; however, they mention that the exclusion of trace reactions can increase the specificity of both methods. In the current study, all urine samples were moderately to severely proteinuric and the low urine patients specific gravity (due to CKD) influenced the correlation of the two methods. The two methods were positively and moderately correlated when USG>1010 and thus Heller's reaction test can be used as a quick alternative in urine samples of dogs with CKD. In another study, there was a good correlation between results of urine dipstick, SSA test and UPC and a point-of-care microalbuminuria immunoassay (Garner and Wiedmeyer, 2007). However, in this study, samples that had mildly positive urine dipstick results (trace or 1+), there was a high false-positive rate (69%), indicating that the use of a urine dipstick to detect albuminuria in dogs had poor diagnostic value at lower protein or albumin concentrations (Garner and Wiedmeyer, 2007). Similar to our findings, correlation of diagnostic methods for proteinuria detection was shown to be more sensitive in urine samples with USG>1010 (Garner and Wiedmeyer, 2007). In our study, no significant correlation was found between Heller's reaction test with UPC, in urine samples with USG ≤1010, indicating that in much diluted urine, Heller's reaction cannot estimate proteinuria accurately. The findings of the current study were in parallel with the results of previous studies comparing two different methods of proteinuria detection where a positive correlation of the two methods was demonstrated. As Heller's reaction test is not a commonly-used qualitative test for proteinuria, this is the first study that correlated Heller's reaction test with the quantitative UPC test in dogs with CKD.

Urine samples were collected by cystocentesis or free catch, based on a previous study, in which there was a strong association between UPC values regardless of the sample collection method (Beatrice et al., 2010).

Proteinuria has been incriminated as an independent mediator of progression of renal disease (Walls, 2001; Remuzzi, 1999; Burton and Harris, 1996). There was a study showing that UPC>1 in canine patients with chronic kidney disease at the time of initial diagnosis of CKD can cause uremic crisis and death sooner than in patients with UPC<1 (Jacob et al., 2005). Thus, it is essential to detect proteinuria in canine patients with CKD as soon as possible.

The nature of the study and the usage of urine samples collected via free catch despite the appropriate cleaning of the external genitalia prior voiding were the main limitations.

This was the first study in which Heller's reaction test (a qualitative method), an easy, quick and practical method for proteinuria detection, was correlated with the quantitative UPC method in dogs with CKD. However, it cannot be used interchangeably with UPC especially in diluted urine (USG<1010). Nevertheless, it might be a good first-choice test to detect proteinuria in urine samples with USG>1010, when quantitative UPC method is not available in the clinical setting.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Research article
Ερευνητικό άρθρο

Ultrasonographic findings of intrascrotal testicular torsion at the early stage in a rabbit model

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ABSTRACT: Intrascrotal testicular torsion (ISTT) cases are rarely seen in companion animals, harming both testicular functions. These are considered reproductive emergency cases and need acute surgical intervention. In this research, early scrotal ultrasonographic findings were assessed in a rabbit model. Unilaterally, complete clockwise torsion was inducted in the left testicles of eight healthy pubertal male rabbits. The right testicle of each animal was considered as control. B-mode and Doppler ultrasonography (USG) was performed preoperatively (-1st) and postoperatively (2,4,6, and 24th) hours (h). At the first two hours, swelling, cyanosis, hypothermia, and pain symptoms were observed in all rabbits (100%). Reactive hydrocele was sonographically detected at 2-h (12.5%; 1/8), 4-6, and 24-h (50%; 4/8), respectively. At 2nd h, hyperechogenic whirlpool sign was remarkable in transversal and sagittal scans of the spermatic cord in all cases (100%). At 6th h, increased echogenicity of the spermatic cord and testicular parenchyma were also observed in all torsed testes (100%). Except for the scrotal capillary vessels in the distal part of torsion, testicular and epididymal blood flow were absent in all rabbits (100%). Increased pulsatility index (PI) at the second h and decreased resistance index (RI) at the 4th and 6th h in the control group ($P<0.05$) was observed. At the end of the study, RI was increased at 24th h in both groups ($P<0.05$). The mild hydrocele and whirlpool signs are clear and remarkable diagnostic findings of ISTT cases in rabbits. By twisting at the spermatic cord, increased echogenicity and absence of blood flow from the twisted area to the distal part of the spermatic cord help the differential diagnosis. The torsion causes the increasing RI in contralateral testicular perfusion after 24 hours of the event. In conclusion, testicular echogenicity, whirlpool signs, visibility of the blood flow, and its spectral waveform features are useful measurements in diagnosing ISTT cases. Moreover, the time of torsion and prognosis may also be estimated.

***Keywords*:** Testicular torsion, ultrasonography, whirlpool, rabbit model.

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INTRODUCTION

Intrascrotal testicular torsion (ISTT) is causedby self-torsion of the testes and is a urological emergency in infants and young males, requiring acute surgical intervention to avoid ischemia necrosis (Schneck and Bellinger, 2007; Kapoor, 2008). In dogs, spermatic cord torsion is a rare condition and can be related to cryptorchidism and concomitant testis neoplasia (Hulse, 1973; Pearson and Kelly, 1975; Young, 1979; Laing et al., 1983; Mibayashi et al., 1990; Feldman and Nelson, 1996; Quartuccio et al., 2012). In many reports, mostly abdominal pain, the cases have appeared into cryptorchidic young male dogs with abdominal pain, skin alopecia, weight loss (Pearson and Kelly, 1975; Mibayashi et al., 1990; Hecht et al., 2004; Quartuccio et al., 2012; Carr et al. 2015 Mostachio et al., 2007; Howser and Vinayak, 2018). Intrascrotal cases are much more rarely seen than intraabdominal ones thatare often reported in canine and feline literature (Zymet, 1975; Young, 1979; Crivellenti et al., 2012 Giuliano, 2013; Villiotti et al., 2018). Tunica vaginalis of the descended testicle does not sufficiently support conjointly to the scrotum (Guerra et al., 2008; Djahangirian et al., 2010). Although this rare pathology is described as an idiopathic condition regardless of age and race; trauma, excessive physical activity, and rupture of the scrotal ligament are associated as etiologic factors in normally descended testicles (Melikoğlu et al., 1992; Hoşcan et al. 2012; Villiotti et al., 2018).

This acute and emergent disease causes sudden/great pain with scrotal edema, subsequent necrosis of the gonad (Feldman and Nelson, 1996), and contralateral organ damage. They are thus releasing acrosome enzymes, neuroendocrine/vasomotor responses, and dramatic perfusion changes (Melikoğlu et al., 1992; Hoşcan et al., 2012). Therefore, quick and definitive clinical diagnosis plays a vital role in the surgical procedure pathway, up to the necropsy report. Ultrasonography (USG) is a routineexam to diagnose and differentiate testicular torsion from other pathologies with similar symptoms. Increased echogenicity and absent blood flow in the distal part of torsed tissue describe the torsion cases (Pinto et al., 2001; Bartlett, 2002; Hecht et al., 2004). Besides these signs, the remarkable changes in the spermatic cord's form like that whirlpool, snail shell, target, storm are very definitive in acute ISTT cases. This change is reported as the most specific and sensitive sign on Grayscale (Vijayaraghavan 2006). However, no reports about this finding in the testicular cases' veterinary literatureare available, except for some mesenteric disorders (Spekabow et al., 2010; Arronson, 2016). In the light of earlier literature, this experimental study aimed to evaluate the B-mode and Doppler scans' imaging features during the first 24 hours of acute testicular torsion for pet animals' diagnostic purposes.

MATERIAL AND METHODS

This study was conducted with ethical approval of Adnan Menderes University, Local Ethics Committee of Animal Experiments (ADU-HADYEK), 64583101/2017/018. During this study, eight five-month-old male New Zealand healthy rabbits were used. All the animals were fed ad libitum in individual wire-mesh cages under controlled conditions of heat (18-24 °C) and light (14 h light, ten h dark).

The left testicles were defined as the experimental side in all the animals. Animals were anesthetized with an intramuscular injection of 35 mg/kg ketamine (Alfamine %10 - Ege Vet®), and 5 mg/kg xylazine (Alfazyne %2 - Ege Vet®). Following aseptic preparation, the left testis was delivered via a longitudinal scrotal skin incision. Testicles were twisted 360° in a complete clockwise direction and sutured by 3/0 vicryl sutures to the scrotum, according to the experimental procedure reported in previous studies (Acar, 2005; Hoşcan, 2012). Right testicles without any application were served as an internal control.

During the torsion procedure, B-mode and Pulsed wave Doppler USG of testicles was performed with an 8.0 MHz microconvex probe (Mylab 30-Esaote®, Genova, Italy) preoperatively (-1^{sth}h) and postoperatively (2, 4, 6, and 24thh). In B-mode USG, longitudinal and transverse scans of testicles were obtained to detect acute changes in spermatic cord and testicles (increased echogenicity, hydrocele, whirlpool sign reflected its tortuosity) depending on the duration of the event. After B-mode scans, Color and Pulsed Doppler USG were performed to evaluate the vascular supply of the gonads. Pulsatility index (PI) and resistance index (RI) measurements were recorded in spectral traces of the arteria testicularis in the proximal part of the twisted area of test groups and symmetrical regions control group. All measurements were generated using the manual mode after obtaining a minimum of three consecutive convenient artery wave images.

The data were evaluated using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) package program. In comparing the data in Group 1 (Twisted) and Group 2 (Con-

trol), the normally distributed data were evaluated by paired t-test. To determine the group's time-dependent changes, the data were assessed by repeated two-way analysis of variance. The data in the table, graphics, and results section are expressed as mean ± standard deviation. Statistical significance was accepted as $p < 0.05$.

RESULTS

At the first two hours of the induction, it was observed that the left testes were localized on the proximal part of the scrotum, and the swelling, cyanosis, hypothermia, and pain symptoms were observed in all rabbits (100%).

Reactive hydrocele findings were detected as an anechogenic area surrounding testicles without particles reflecting hyperechoic brightness. These findings were recorded at 2nd h examination as 12.5% (1/8); 50% (4/8) at 4-6, and 24th h USG exams, respectively (Figure 1). The increase of the echogenicity in the left spermatic cord and torsed testicular parenchyma was remarkable and easily visible. Moreover, the absence of blood flow was in the torsed left testis at bilateral comparatively scans (Figure 2).

In all rabbits (100%), a hyperechogenic whirlpool sign was seen in transverse scans (Figure 3). Accordingly, twist signs in longitudinal scans of the spermatic cord after 2-h (Figure 4). Especially in longitudinal scans, hyperechogenicity of the distal part of the spermatic cord (D) was prominent when comparing the proximal portion (Figure 4). At 6th h, increased echogenicity of the spermatic cord and testicular parenchyma was also observed in all torsed testes (100%). Except for the scrotal capillary vessels in the distal part of torsion, during Doppler scans in both dimensions, there is the absent blood flow of testicular and epididymis parenchyma.

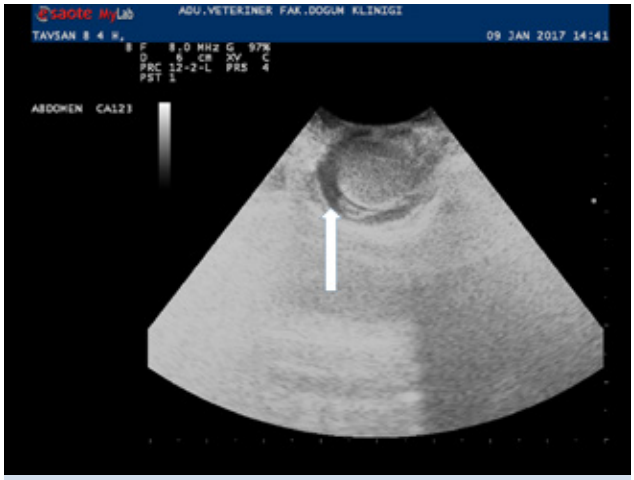

Figure 1: Mild reactive hydrocele at torsion testicle

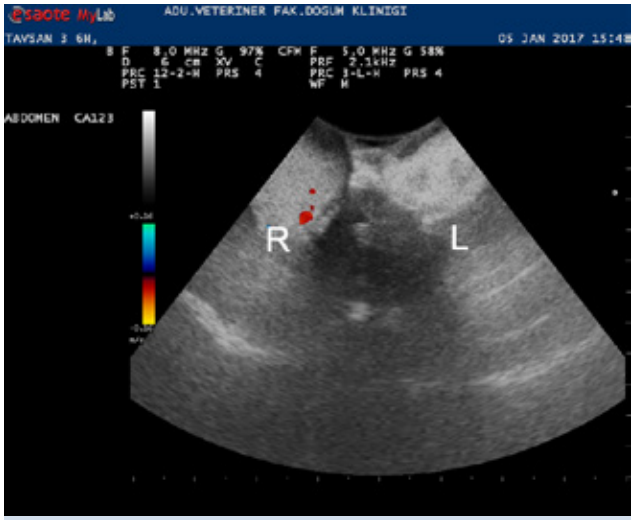

Figure 2: Increased echogenicity and absent flow in the left testicle (L) comparing with right testicle (R)

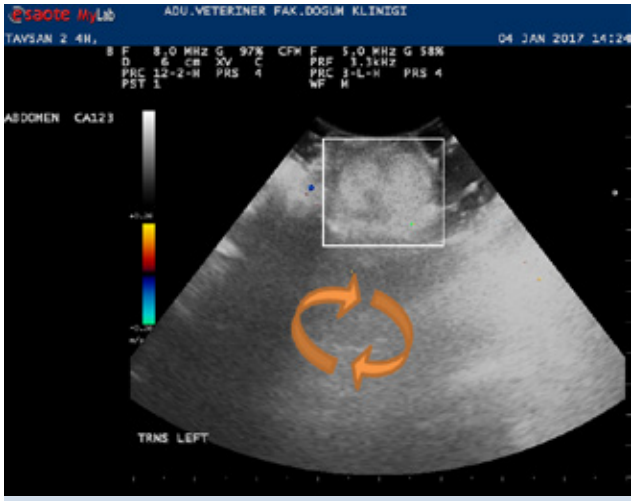

Figure 3: Whirlpool sign - transversal scan

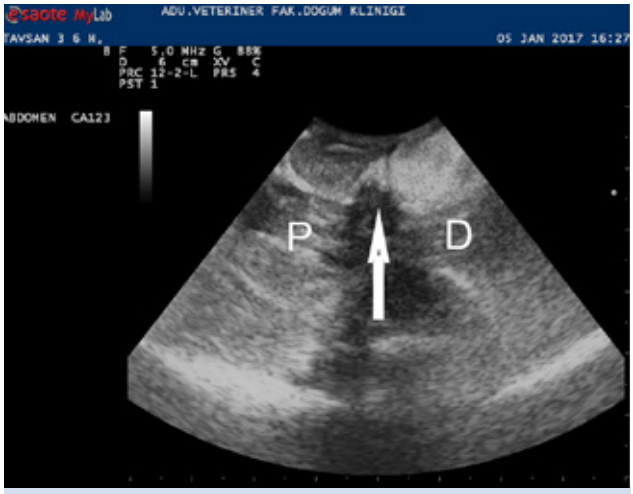

Figure 4: Twisting sign (arrow), absent flow, and increased echogenicity in the distal part (D) comparing with the proximal part of torsion (P) - at longitudinal scan

During the spectral Doppler examination, there was no asymmetry, reverse of diastolic flow, or other possible pathologic findings in traces. The mean of the PI and RI of the blood flow of arteria testicularis in groups were presented in Table 1. There was no significant difference between the groups than at the same time exams (P>0.05). However, when related to the time-dependent changes in the groups, PI values were higher at the 2nd h in the control group (Figure 5) (P<0.05). Resistance index was lower at the 4th and 6th in the control group (P<0.05), but also increased at 24th h in both groups (Figure 6) (P<0.05).

Following the last examinations, a castration operation was done in all animals under general anesthesia. Macroscopic findings revealed that the enlarged left testicles had blackened coloration and generalized hemorrhage in all torsed testicular necropsy material (100%).

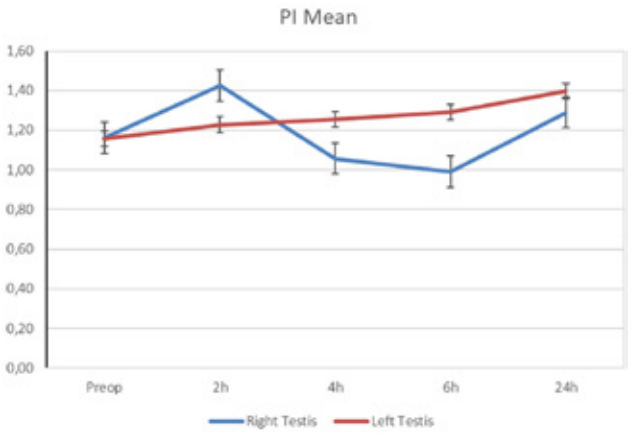

Figure 5: The mean PI of both study groups

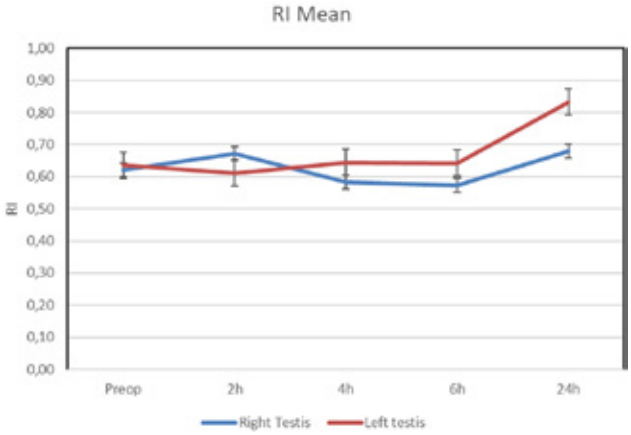

Figure 6: The mean RI of both study groups

Table 1: The mean of the PI and RI of the blood flow of arteria testicularis in study/control groups

Doppler parameters	Preoperatively	2 h	4 h	6 h	24 h
PI Torsion	1.15±0.09	1.22±0.09	1.25±0.10	1.29±0.14	1.39±0.13
PI Control	1.16±0.06	1.42±0.14*	1.05±0.10	0.99±0.11	1.28±0.09
RI Torsion	0.63±0.03	0.61±0.02	0.64±0.02	0.64±0.03	0.83±0.10*
RI Control	0.62±0.02	0.67±0.03	0.58±0.02*	0.57±0.03*	0.67±0.03*

Values are represented as mean ± SD. * P < 0.05.

DISCUSSION

The clinical and physical signs of testicular torsion can mimic those seen in cases of epididymo-orchitis (Berman et al., 1996). Therefore, different scrotal USG techniques may help differentiate acute intrascrotal pathologies (Vijayaraghavan, 2006; Cassar et al., 2008). Long-term ischemic condition in tissues results in irreversible defects on the male's reproductive capability. If the surgical detorsion of a complete torsed testis is not performed by 4-6 hours or more, infarction and necrosis of the gonads are inevitable (Harisinghani et al., 2019). Ferreira (2000) reported that the first 2 hours is the critical period of ischemia survival in canine germ cells. This studyproved the most specific findings of Grayscale and applicability of Spectral Doppler examinations in the diagnosis of ISTT on the rabbit model.

In ISTT cases, fluid collection in the scrotum by venous obstruction is the most common finding, which is more apparentafter 4 hours in Grayscale imaging (Sirivastava, 2017; Harisinghani et al., 2019). In the orchitis or other infectious condition in patients, this sign appears as a hematocele or pyocele (Berman et al., 1996). In our study, reactive hydrocele was one of the first seen complications in Grayscale caused by compression of the testicular blood flow. As the result of an aseptic experimental induction, an anechoic fluid accumulation surrounding the torsed testicles was seen in the scrotal sac. The amount of this fluid was variable and could not be measured. In our study, 4 hours after the torsion, the visibility rates of hydrocele sign increased to 50% from 12.5% previously reported in the literature. Besides, in half of the test group, hydrocele was still detected during 6, and

24th h examinations. Regarding that, the first 4 hours are most critical for salvaging, and a mild-moderate hydrocele sign can be thought of as an alerting sign for "delayed case" for complete torsion cases.

Another specific data of torsion cases is the whirlpool sign in the twisted spermatic cord mass on Grayscale (Vijayaraghavan, 2006; Cassar, 2008). The flow in the whirlpool mass vessels helpsto differentiate incomplete torsion cases (Vijayaraghavan, 2006). In our study, a whirlpool sign was recorded in all animals immediately after induction. As time goes by, the half part of the whirlpool got brighter (Figure-4). Moreover, absent flow and increased echogenicity were in the distal portion of the spermatic cord detected. By evaluating the whirlpool sign's sagittal scans, the torsion's direction could be detectable in some rabbits (Figure 4). In the evaluation of vascularity, the absence of blood flow in testis brings out more than 1800 torsion (Mevorach et al., 1991; Berman et al., 1996; Vijayaraghavan, 2006; Cassar, 2008; Howser and Vinayak, 2018; Villioti et al., 2018). As previously reported in the literature, we also observed the absence of blood flow toward the torsed testicle in the presented study. Regarding our observations, practitioners should be able to differentiate the capillary flow on the scrotal wall during Color Doppler scans.

The usefulness of spectral Doppler USG has been proven in previous studies (Middleton et al., 1989; Lerner et al., 1990). Testes with partial torsion can cause variable spectral patterns (increased, similar, or decreased amplitude) of the intratesticular arterial waveform relative to the contralateral testicle (Cassar et al., 2008). Nevertheless, the changes of the intratesticular and spermatic arterial waveforms are not in the veterinary literature up to the authors' knowledge. In the present study, spectral Doppler examination of the intratesticular blood flow was impossible in the test group because of the experimentally induced complete torsion in our rabbit model. Nevertheless, it was able to see the significant changes in the twisted spermatic cord's proximal part. These changes were noticed between the contralateral testes, related to the torsion hours' duration, contralateral. Acute response to torsion was detected as the high PI in the contralateral side at 2nd h. On the torsion side, nonsignificantly, we recorded a linear increase in PI.

The mean RI is described as 0.62 (0.48 - 0.75) for healthy males, but this is not a reference value in diagnosis in partially torsion cases (Middleton et al., 1989). Although the high RI is considered suspicious for the partial testicular torsion, researchers pointed out that Pulsed Doppler analysis should be performed from the different parts of the testicles for proper diagnosis due to its subtle variations (Cassar et al., 2008). In this study, there was only one area (proximal part of the twisted portion) having the possibility of performing USG, and no difference was between the study groups. Regarding the RI variation in our study's results, the lower RI at 4thand 6th h in the torsion group and high value at 24thh in controls were similar to those described in the literature. These RI trends are hard to explain due to the rarity of reported literature of similar cases in the rabbit. Based on the referred healthy RI limits, although it is impossible to catch any testicular resistance difference during the first six hours,tissue damage can be detected sonographically with a high RI level 24thh. In the subsequent studies, by performing the other exams between 6-24thh, it can be possible to determinehigh testicular resistance threshold time.

CONCLUSION

In conclusion, the Gray- Color and Doppler USG scans are valuable tools in diagnosing the ISTT cases even in small-size pet animals. Testicular echogenicity, whirlpool signs, blood flow detection, and its spectral waveform features are useful measurements in this small animal practice's emergency case. Based on these parameters, the time of torsion and prognosis may also be estimated.

CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Antimicrobial resistance rates in commensal *Escherichia coli* isolates from healthy pigs in Greek swine farms

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ABSTRACT: Antibiotic resistance undermines the possibility to effectively treat bacterial diseases in humans and animals and it is one of the major global threats for the future. Antimicrobial resistance among commensal *Escherichia coli* (*E. coli*) of swine is important because it may constitute a reservoir of antimicrobial resistance genes that could be transferred to pathogenic bacteria. This study aimed to estimate the prevalence of antimicrobial resistance among faecal *E. coli* from healthy weaning and growing pigs in Greek farms.

From 14 farms that were enrolled to this study, 390 isolates of *E. coli*, 160 from growing pigs and 230 from weaning pigs had been derived. Isolates were tested for susceptibility to 19 antimicrobials belonging to 10 different classes, using disk diffusion method. Extremely high resistance rates were observed for streptomycin, tetracycline, doxycycline, trimethoprim-sulphomeathoxazole, and for the penicillins, ampicillin, ticarcillin and piperacillin. All isolates were susceptible in the combination of a penicillin and β-lactamase inhibitors, in aztreonam and extended-spectrum cephalosporins. The vast majority of the isolates (87%) were multi drug resistant (MDR) and the most common MDR patterns showed resistance in three to four antimicrobial classes. Twenty different antibiotic resistance profiles were observed, the most prevalent was chloramphenicol-trimethoprim/sulphomeathoxazole-tetracycline-doxycycline-streptomycin-ampicillin-ticarcillin-piperacillin (CHL-SXT-TET-DOX-SMN-AMP-TIC-PIP) accounting 44% of the isolates. In each farm one or two AMR profiles were predominating accounting 64-100%, while the antimicrobial resistance index (ARI) was estimated to 0.39 ranging from 0.13 to 0.48 among the studied farms. To analyse the differences observed between the farms, additional information about the antibiotic consumption and the level of biosecurity in the farms is necessary. These findings indicate that resistance to a broad range of antimicrobials was prevalent among faecal *E. coli* isolates of pigs on studied farms, and that this constitutes a potential reservoir for resistance genes that could spread to gut pathogens.

***Keywords*:** Antibiotic resistance, *E. coli,* commensal flora, pigs

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INTRODUCTION

The major part of their usage is for treatment of diseases, and as such they have become an essential part of the food-animal husbandry (Marshall and Levy, 2011). Antimicrobial resistance (AMR) undermines the possibility to effectively treat bacterial diseases in humans and animals and it is considered as one of the major global threats for the future. The main driver of resistance is the unjustifiable use of antibiotics, which may cause a selection pressure favouring resistant bacteria and their spread in human as well. Resistant bacteria that emerge among food producing animals can spread to humans, along the food production chain (Silbergeld et al., 2008). Susceptibility testing of commensal intestinal *Escherichia coli (E. coli)* from healthy animals is commonly used as indicator for the occurrence of resistance in animal populations. European Union has established surveillance programmes since 2014 (ECDC, 2018; Tadesse et al., 2012).

Antimicrobials are used in animal husbandry for therapeutic reasons, for preventive treatment or prophylaxis, for metaphylaxis or control treatment and as growth promoters however, were banned in EU from 2006 according to European Regulation 1831/2003. The pig production is considered to be amongst the sectors with the highest use of antimicrobials in intensive animal husbandry (McEwen and Fedorka-Cray, 2002). Prophylactic use of antimicrobials to prevent infections was a common practice in pig farms, especially in stressful periods that predispose for infectious diseases. Therapeutic treatments are also administered in feed, although producers also treat individual animals.

Commensal *E. coli* are defined as bacteria isolated from healthy animals without known virulence (toxic, adhesive, invasive) attributes playing a role in a specific disease caused by *E. coli*. Commensal strains of *E. coli* as versatile residents of the intestine are also repeatedly challenged by antimicrobial pressures during the lifetime of their host. As a consequence, commensal strains acquire the respective resistance genes, and/or develop resistant mutants in order to survive and maintain microbial homeostasis in the lower intestinal tract. Thus, commensal *E. coli* strains can be regarded as indicators of antimicrobial load on their hosts (Szmolka and Nagy, 2013).

In the present study, we aimed to estimate the prevalence of antimicrobial resistance among commensal *E. coli* from healthy weaning and growing pigs in Greek farms and to describe the AMR phenotypic profiles.

MATERIALS AND METHODS

Sampling

Field samples were fecal samples collected at the farm with the permission of the owner of the pig herd and the Official Veterinary Surgeon. The participating farms were purposefully selected from farrow-to-finish operations that had at least 50 sows Selection was performed from the Greek identification and registration system for livestock (OSDE). A key inclusion criterion was the willingness of the farmer to cooperate at the initiation of the survey. A further inclusion criterion consisted in the absence of other livestock animal species (e.g. cattle, poultry) bred by the selected farms, so that interference of resistance selection due to antibiotic use for these animals was excluded. Fourteen farms located in Central Greece and designated with capital letters (A, B, C etc.) were selected and were categorized as small sized farms <400 sows and big sized farms with 400 or more sows. All animals that have been sampled were healthy and showed no sign of disease.

Samples were taken individually by each animal's rectus with the use of sterile swabs. Samples were kept refrigerated in until analysis within one to four days after sampling. A minimum set of information on the date, location and sample source was collected and submitted to the central database designed for managing of the study. Samples were taken from pigs during the weaning period (3-5 weeks of age) and from pigs during the growing period (16-17 weeks of age); one pig per cage was sampled. From the first four farms (A, B, C, D) 35 animals were sampled 20 weaners and 15 growers. From the remaining 10 farms 25 animals were sampled 15 weaners and 10 growers. Sampled pigs were not treated with antimicrobials during the last month.

Isolation and identification

Samples were inoculated in Tryptone Bile X- Glucuronide Agar (Oxoid, CM0945) and incubated aerobically at 44 ± 0,5 °C for 24 ± 2h. Blue or blue green colonies were determined as *E. coli.* One colony from each dish was picked up randomly and tested for positive indole test for further confirmation.

Antimicrobial susceptibility testing

All isolates were tested for susceptibility against

19 antimicrobials belonging to 10 different classes, using disk diffusion method. For the Quality Control *E. coli* ATCC 25922 was used, while the interpretation and the evaluation of the results was performed according the CLSI (M100S-29th Edition/2019) guidelines. An isolate was considered "resistant" if resistance or intermediate resistance was observed for at least one antimicrobial agent tested. The antibiotics(BIO-RAD), tested were: chloramphenicol (CHL, 30 μg), trimethoprim & sulphomeathoxazole (SXT, 1,75/23,75 μg), nalidixic acid (NAL, 30 μg), ciprofloxacin (CIP, 5 μg), piperacillin (PIP, 100 μg), ticarcillin (TIC, 75 μg), ampicillin (AMP, 10 μg), gentamicin (GMN, 10 μg), tobramycin (TM, 10 μg), ceftazidime (CAZ, 30 μg), ceftriaxone (CRO, 30 μg), cefotaxime (CTX, 30 μg), cefpodoxime (CPD, 10 μg), aztreonam (ATM, 30 μg), amoxicillin-clavulanic acid (AMC, 10/20 μg), ticarcillin-clavulanic acid (TCC, 75/20 μg, doxycycline (DOX, 30 μg), tetracycline (TET, 30 μg) and streptomycin (SMN, 100 μg). These antimicrobials were chosen because they represent a variety of antimicrobial types. To calculate the rate of resistant isolates per 100 we performed the following calculation

$$\%\ rate = \frac{\text{Number of resitant isolates}*100}{\text{Number of tested isolates}}$$

Resistance was categorized according to %rate as extremely high (%rate>70%), very high (%rate: >50 to 70), high (%rate >20 to 50), moderate (%rate >10 to 20), low (%rate >1 to 10), very low (%rate 0.1 to 1) and rare (%rate <0.1). Multidrug resistance was defined as previously described (Magiorakos et al., 2012).

We quantified the resistance level by means of antimicrobial resistance index (ARI) which is calculated as the number of antimicrobials against which resistance is detected divided by the total number of antimicrobials tested. For these analyses intermediate results were considered resistant (Hinton et al., 1985). The ARI can vary from 0 (0%), when the strain is (fully) susceptible to every tested antimicrobial agent, to 1.00 (100%) when the strain is (pan-) resistant to all tested antimicrobial agent classes (Catry et al., 2005, Catry et al., 2016)

RESULTS

Overall, 390 *E. coli* strains were isolated; 160 strains were isolated from growing pigs and 230 from weaning pigs.

Overall, extremely high rates of resistance were found for streptomycin (100%), doxycycline and tetracycline (94%), trimethoprim-sulphomethoxazole (93%), ampicillin and ticarcillin (89%), and piperacillin (81%), whereas very high resistance rates were found for chloramphenicol (69%). Moderate resistance rates were found to gentamycin (12%) and trimethoprim (12%) and low to nalidixic acid (6%) and ciprofloxacin (6%). Resistance to cephalosporins, aztreonam and combinations of amoxicillin or ticarcillin with clavulanic acid were not detected. Differences were observed in the resistance rates for each antimicrobial among the 14 farms however most of the strains from each farm shared the similar resistant profiles. Table 1 presents the number and percentages of *E. coli* isolates from pigs found resistant to the antibiotics used in this study.

Resistance to at least one or more antibiotics from three different antimicrobial classes (MDR strains) exhibited 340/390 (87%) isolates while 246/390 (63%) exhibited resistance to at least one antimicrobial from four different classes. Detailed characteristics of the resistance per farm are shown in Table 2.

Table 3 summarises the antimicrobial resistance (AMR) patterns among the 390 *E coli* isolates. Twenty different patterns were identified among the 390 isolates presenting resistance from two to 12 different antimicrobials. The predominant AMR profile was CHL SXT TET DOX SMN AMP TIC PIP, accounting 173 (44%) of the isolates. The second and third most prevalent were the SXT TET DOX SMN AMP TIC PIP and the CHL SXT TET DOX SMN GEN TM AMP TIC PIP accounting 60 (15%) and 40 (10%) of the isolates respectively.

We observed one to five different AMR profiles in each farm. However, there was a predominant profile for each farm that ranged from 32-100% among isolates. Interestingly the two most prevalent profiles in each farm ranged from 64-100%. The antimicrobial resistance index (ARI) was estimated in 0.39 for all the 390 tested isolates ranging from 0.13 to 0.48 (Table 4).

Table 1: Antimicrobial resistance among ***E. coli*** isolates from 14 pig farms, Greece, 2014-2015 (N=390)

Farm	N	CHL		SXT		TET		DOX		SMN		GEN		TM		AMP		TIC		PIP		AMC		TCC		CPD		CRO		CAZ		CTX		ATM		NAL		CIP	
	Total	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
A	35	35	100%	34	97%	35	100%	35	100%	35	100%	21	60%	21	60%	35	100%	35	100%	33	94%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
B	35	35	100%	35	100%	35	100%	35	100%	35	100%	0	0%	0	0%	35	100%	35	100%	35	100%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
C	35	35	100%	35	100%	35	100%	35	100%	35	100%	7	20%	7	20%	35	100%	35	100%	33	94%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
D	35	33	94%	34	97%	35	100%	35	100%	35	100%	9	26%	9	26%	35	100%	35	100%	35	100%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
E	25	0	0%	24	96%	25	100%	25	100%	25	100%	0	0%	0	0%	6	24%	6	24%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
F	25	25	100%	0	0%	25	100%	25	100%	25	100%	0	0%	0	0%	25	100%	25	100%	15	60%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
G	25	25	100%	25	100%	25	100%	25	100%	25	100%	5	20%	5	20%	25	100%	25	100%	21	84%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	14	56%	14	56%
H	25	25	100%	25	100%	25	100%	25	100%	25	100%	4	16%	4	16%	25	100%	25	100%	24	96%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	6	24%	6	24%
I	25	0	0%	25	100%	2	8%	2	8%	25	100%	0	0%	0	0%	2	8%	2	8%	2	8%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
J	25	25	100%	25	100%	25	100%	25	100%	25	100%	0	0%	0	0%	25	100%	25	100%	25	100%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
K	25	25	100%	25	100%	25	100%	25	100%	25	100%	0	0%	0	0%	25	100%	25	100%	23	92%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	4	16%	4	16%
L	25	2	8%	25	100%	24	96%	24	96%	25	100%	1	4%	1	4%	23	92%	23	92%	22	88%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
M	25	6	24%	23	92%	25	100%	25	100%	25	100%	0	0%	0	0%	25	100%	25	100%	24	96%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
N	25	0	0%	24	96%	25	100%	25	100%	25	100%	0	0%	0	0%	25	100%	25	100%	23	92%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Total	390	271	69%	359	92%	366	94%	366	94%	390	100%	47	12%	47	12%	346	89%	346	89%	315	81%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	24	6%	24	6%

CHL: Chloramphenicol, SXT: trimethoprim & sulphomeathoxazole, NAL: nalidixic acid, CIP: ciprofloxacin, PIP: piperacillin, TIC: ticarcillin, AMP: ampicillin, GMN: gentamicin, TM: tobramycin, CAZ: ceftazidime, CRO: ceftriaxone, CTX: cefotaxime, CPD: cefpodoxime, ATM: aztreonam, AMC: amoxicillin-clavulanic acid, TCC: ticarcillin-clavulanic acid, DOX: doxycycline, TET: tetracycline and SMN: streptomycin.

Table 2: Resistance to at least one antimicrobial from different classes among *E. coli* isolates from 14 pig farms, Greece, 2014-2015 (N=390)

	Resistance to antibiotic classes												
	1 class		2 classes		3 classes		4 classes		5 classes		6 classes		Total
Farm	N	%	N	%	N	%	N	%	N	%	N	%	N
A	0	0	0	0	0	0	15	43	20	57	0	0	35
B	0	0	0	0	0	0	35	100	0	0	0	0	35
C	0	0	0	0	0	0	28	80	7	20	0	0	35
D	0	0	0	0	3	9	23	66	9	26	0	0	35
E	1	4	18	72	6	24	0	0	0	0	0	0	25
F	0	0	0	0	25	100	0	0	0	0	0	0	25
G	0	0	0	0	0	0	8	32	15	60	2	8	25
H	0	0	0	0	0	0	15	60	10	40	0	0	25
I	21	84	4	16	0	0	0	0	0	0	0	0	25
J	0	0	0	0	0	0	25	100	0	0	0	0	25
K	0	0	0	0	0	0	21	84	4	16	0	0	25
L	0	0	3	12	19	76	3	12	0	0	0	0	25
M	0	0	2	8	17	68	6	24	0	0	0	0	25
N	0	0	1	4	24	96	0	0	0	0	0	0	25
Total	22	6	28	7	94	24	179	46	65	17	2	1	390

Table 3: Antimicrobial resistance profiles among *E. coli* isolates from 14 pig farms, Greece, 2014-2015 (N=390)

Antimicrobial resistance profile	N	%
CHL SXT TET DOX SMN AMP TIC PIP	173	44.36
SXT TET DOX SMN AMP TIC PIP	60	15.38
CHL SXT TET DOX SMN GEN TM AMP TIC PIP	40	10.26
SXT TET DOX SMN	22	5.64
SXT SMN	21	5.38
CHL SXT TET DOX SMN AMP TIC PIP NAL CIP	18	4.62
CHL TET DOX SMN AMP TIC PIP	16	4.10
CHL TET DOX SMN AMP TIC	10	2.56
SXT TET DOX SMN AMP TIC	8	2.05
CHL SXT TET DOX SMN AMP TIC	4	1.03
CHL SXT TET DOX SMN AMP TIC NAL CIP	4	1.03
CHL SXT TET DOX SMN GEN TM AMP TIC	3	0.77
CHL SXT TET DOX SMN GEN TM AMP TIC PIP NAL CIP	2	0.51
SXT SMN AMP TIC PIP	2	0.51
TET DOX SMN AMP TIC PIP	2	0.51
CHL TET DOX SMN GEN TM AMP TIC PIP	1	0.26
SXT SMN AMP TIC	1	0.26
SXT TET DOX SMN GEN TM AMP TIC PIP	1	0.26
TET DOX SMN	1	0.26
TET DOX SMN AMP TIC	1	0.26

*CHL: Chloramphenicol, SXT: trimethoprim & sulphomeathoxazole, NAL: nalidixic acid, CIP: ciprofloxacin, PIP: piperacillin, TIC: ticarcillin, AMP: ampicillin, GMN: gentamicin, TM: tobramycin, CAZ: ceftazidime, CRO: ceftriaxone, CTX: cefotaxime, CPD: cefpodoxime, ATM: aztreonam, AMC: amoxicillin-clavulanic acid, TCC: ticarcillin-clavulanic acid, DOX: doxycycline, TET: tetracycline and SMN: streptomycin.

Table 4: Antimicrobial resistance index (ARI) and proportions of most prevalent and the two most prevalent antimicrobial resistance profiles among 390 *E. coli* isolates from 14 pig farms, Greece, 2014-2015

Farm	Isolates tested (N)	ARI	Number of different profiles	Isolates in the most prevalent profile		Isolates in the two most prevalent profiles	
				N	%	N	%
A	35	0.48	5	19	54	32	91
B	35	0.42	1	35	100	35	100
C	35	0.44	3	28	80	32	91
D	35	0.44	4	23	66	32	91
E	25	0.23	3	18	72	24	96
F	25	0.35	2	15	60	25	100
G	25	0.49	5	8	32	16	64
H	25	0.46	4	14	56	20	80
I	25	0.13	3	21	84	23	92
J	25	0.42	1	25	100	25	100
K	25	0.43	3	19	76	23	92
L	25	0.36	5	19	76	21	84
M	25	0.37	4	17	68	23	92
N	25	0.36	3	22	88	24	96

DISCUSSION

E. coli represents an important aerobic organism in the gut of pigs and other vertebrates, living in symbiosis with its host. We observed high rates of resistance to commonly used antimicrobials and high rates of MDR strains. We observed diversity among the predominant resistance profiles and the antimicrobial resistance patterns in each farm, interestingly; one or two AMR profiles were predominating in each of the studied farms.

We observed high rates of resistance to ampicillin, doxycycline, tetracycline, trimethoprim-sulfamethoxazole, amoxicillin and streptomycin and low rates to nalidixic acid and ciprofloxacin; resistance to cephalosporins or aztreonam was not detected. In accordance to our results, of the *E. coli* isolates from healthy grower-finisher pigs in Canada, resistance was most commonly found to tetracycline (66.8%), sulfamethoxazole (46.0%) and streptomycin (33.4%) (Rosengren et al., 2008). In general, analysis of resistance patterns confirms an increased resistance of isolates to older, frequently administered antibiotics such as streptomycin, chloramphenicol, sulfamethoxazole, and tetracycline (Kang et al., 2005; Österberg et al., 2016; Rosengren et al., 2008; Zhao et al., 2005). In Greece Valiakos et al (2016) reported findings similar to ours such as high rates of resistance to ampicillin, amoxicillin and tetracycline but lower to sulfonamides. According to EFSA report for fattening pigs, the highest overall 'microbiological' resistance levels observed in EU were to tetracycline (54.7%), sulfamethoxazole (44.2%), ampicillin (39.3%) and trimethoprim (35.3%) with resistance to cefotaxime 1.4% and to ceftazidime 1.3%. MDR resistance was lower in EU (38%) compared to our study, however there was considerable variation between reporting countries in the proportion of isolates which were MDR (EFSA., 2018).

We observed diversity among the predominant resistance profiles and the antimicrobial resistance patterns in each farm although the vast majority of the strains from each farm shared the same profile. We believe that this is possibly due to the use of certain antimicrobials in these farms as there is a direct relationship between AMR and antimicrobial use as suggested also from ECDC (ECDC, 2018). Common classes of antibiotics used in pig production vary across countries. Overall, penicillins and tetracyclines class are the most commonly used antibiotic in pigs, (Legakul et al. 2019) and this can partially match with our findings concerning resistance. Antimicrobial usage in food animals contributes to the development and the spread of resistant microorganisms in the environment but there is also a considerable variation in the antimicrobials consumption all across Europe in terms of differences in antimicrobials used in pig farms (Akwar et al., 2008; Carmo et al., 2017; ECDC, 2017; Garcia-Migura et al., 2014; Gibbons et al., 2016). Animal demographic characteristics, farming practices, veterinarians' and farmers' education are factors which contribute to these variations (Carmo et al., 2017) and can explain the variations in AMR

patterns in different farms.

Although antimicrobial growth promoters have been forbidden in the EU since 2006 (Castanon, 2007), antimicrobials can be used, apart from the direct treatment of diseases, in control treatment called metaphylaxis and this can explain the wide use of antimicrobials in animal husbandry. However, antimicrobials are usually administered to all animals of the group or to the herd; consequently group level use of antimicrobials is the most important way of antimicrobial administration (Callens et al., 2012). We suggest that the predominance of one or two AMR profiles can be partially explained due to similarities in used antimicrobials in our study in farms, as Greek farmers use specific antimicrobials in each farm for prophylaxis or metaphylaxis despite the fact that all the farmers did not mention such use of antimicrobials in the farms.

CONCLUSIONS

The observed high level of resistance to tetracyclines, sulfamethoxazole, ampicillin and trimethoprim in *E. coli* may reflects extensive usage of these antimicrobials in the studied farms. According to EMA's 9th ESVAC report that documents the sales of veterinary antimicrobial agents in 31 European countries for the year 2017, for Greece among the most commonly used antimicrobials in farm animals include Tetracyclines with 47,7 mg/PCU, Penicillins with 18,6 mg/PCU and Sulfonamides with 8,3 mg/PDU.

Moreover, we observed one or maximum two AMR profiles predominating in each of the studied farms which again indicates slightly different use of antimicrobials among farms. Antimicrobial usage in food animals contributes to the development and the spread of resistant microorganisms in the environment, prudent uses of antimicrobials is crucial for avoiding spreading resistance to the community.

CONFLICTS OF INTEREST

None to declare

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Research article
Ερευνητικό άρθρο

The Role of Poultry Farms and Wild Birds During 2016-2017 Avian Influenza Epizootic in Europe

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ABSTRACT: Avian influenza is a contagious viral disease, affecting several species of birds, and poses a significant public threat. During 2016-2017, there were 2,224 high pathogenic avian influenza outbreaks in Europe, which led to the destruction of 9,663,770 birds, and 72 low pathogenic avian influenza outbreaks in 5 European countries, which caused 276,584 bird fatalities. The majority of the epidemics were in wild and backyard birds, except for France and Hungary, where the majority of outbreaks were in duck-goose farms. Notably, there were a total of 100 outbreaks in turkey farms and 37 outbreaks in layer farms, while in broiler farms there were only 10. It is indisputable that wild birds are natural hosts and reservoirs for all types of avian influenza viruses. However, the role of poultry farms on the AI intra-country epidemiology has not been fully clarified. Based on the official reports of OIE for AI, this study indicates that poultry farms, especially fattening turkey and layer chicken farms, are high-risk factors concerning the introduction of the disease into an area and its spread into other poultry farms.

Keywords: Avian influenza, epizootic, Europe, wild birds, poultry farms

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INTRODUCTION

Avian influenza (AI) is an infectious viral disease of substantial importance, due to the virus' rapid spread and high mortality rates in poultry flocks and its possible transmission to mammals, including humans, usually following close contact with infected poultry. During the last century, viruses originated, either entirely or partly, from avian influenza A viruses, caused most of the human influenza pandemics (Acha and Szyfres, 2003, Fouchier and Munster, 2009, Kuchipudi et al., 2014).

Besides, AI has a tremendous economic impact, due to trading restrictions and embargoes imposed on infected areas or countries. The control measures, which are enforced by legislation in the European Union as well as in other countries worldwide, usually involve the implementation of stamping-out policies with defined restriction zones and severe trade implications (Aldous et al., 2010). The cost of 2016-2017 AI epizootic in Europe is immense, although difficult to be estimated. The last devastating AI epizootic in the USA had a dramatic impact on the country's poultry sector, in both economic terms and the number of birds' losses. In particular, the cost was estimated to 1.3 billion American dollars and resulted in the death of more than 50 million birds, either by cause of the disease itself or after the stamping-out policy (Ramos et al., 2017). Two main global pandemics characterize the epizootiology of AI in the 21st century. Notably, the first epizootic started in 2004 and concluded in 2012, peaking in 2006, while the second was observed in 2013 and concluded recently (OIE Situation Report, 2018).

Most avian influenza viruses (AIVs) cause only mild disease in poultry and are called low pathogenic avian influenza viruses (LPAIVs). Highly pathogenic avian influenza viruses (HPAIVs) evolve from H5 and H7 LPAI viruses after their introduction and circulation in poultry flocks. HPAI viruses can kill up to 90-100% of the flock, and cause epizootics that may spread rapidly, devastate the poultry industry and result in severe trade restrictions (OIE Avian Influenza Review, 2016).

It is well known that wild birds are the main vehicle for AIVs long distance spread, such as between countries since they are natural hosts and reservoirs for all types of avian influenza viruses (Fouchier and Munster, 2009, Hill and Runstadler, 2016). However, the intra-country AI epidemiology and the role of poultry farms have not been fully clarified. Therefore, the objective of this study is to highlight the role of poultry farms and wild birds during the 2016-2017 AI epizootic in Europe as well as to estimate the number of bird losses per country and totally in Europe.

MATERIALS AND METHODS

This study registers and categorizes AI outbreaks in several European countries, based on the official reports of epidemiological data submitted by those countries to the World Organisation for Animal Health (OIE) between January 1, 2016, and December 11, 2017. The data from each country were summarized and classified according to birds' species, farming system, number of outbreaks and number of destroyed birds. The study is in full compliance with all relevant codes of experimentation and legislation.

RESULTS

During this period, there were 2,224 HPAI outbreaks in 29 European countries, which led to the death of 9,986,136 birds, as a result of either disease mortality or stamping-out measures (Table 1). The top 10 countries, in terms of bird losses, were Italy, Hungary, France, Germany, Poland, Bulgaria, Sweden, The Netherlands, UK and Czech Republic. The top 10 countries, in terms of the number of outbreaks, were France, Hungary, Germany, Romania, Poland, Italy, Switzerland, Bulgaria, Czech Republic and The Netherlands.

The majority of the outbreaks were in wild and backyard birds, except France and Hungary, where the outbreaks were mostly observed in duck-goose farms. It is worth mentioning that among 10 European countries whose reports came from farms of Galliformes birds, there were 100 HPAI outbreaks in fattening turkey farms and 37 in layer farms, while in broiler farms there were only 10 outbreaks, closely related to turkey and/or duck-goose farms. More specifically, in Italy, there were 40 HPAI outbreaks in turkey farms, 16 in layer farms and 4 in broiler farms in a total of 60 HPAI outbreaks. In Germany, there were 49 HPAI outbreaks in turkey farms, 4 in layer farms, 2 in turkey breeder farms and 1 in a breeder farm in a total of 56 HPAI outbreaks. In France, there were 6 HPAI outbreaks in layer farms, 5 in broiler farms, 2 in breeder farms and 1 in a turkey farm, while there were 34 outbreaks in birds' farms, as the breeding category in the reports was unclear, in a total of 48 HPAI outbreaks. In Hungary, there were 6 HPAI outbreaks in turkey farms, 4 in layer farms and 1 in a broiler farm in a total of 11 HPAI outbreaks. In The Netherlands,

there were 3 HPAI outbreaks in layer farms and 2 in breeder farms in a total of 5 HPAI outbreaks. In the UK, there were 3 HPAI outbreaks in turkey farms and 1 in a breeder farm, in a total of 4 HPAI outbreaks. In Sweden, there were a total of 2 HPAI outbreaks in layer farms. Finally, in Greece and Luxemburg, there was one reported HPAI outbreak in a layer farm, and in Austria in a turkey farm. Overall, regarding HPAI outbreaks of Galliformes, 52.91% were in turkey farms, 19.58% in layer farms, 5.29% in broiler farms, 3.17% in broiler breeder farms and 1.06% in turkey breeder farms.

Table 1. HPAI outbreaks in poultry farms, backyard flocks and wild birds in European countries during 2016-2017 Avian Influenza Epizootic.

Country	Broiler farms	Layer farms	Breeder farms	Turkey farms	Turkey breeder farms	Duck/goose farms	Backyard farms	Multi-farms	Wild birds	Unidentified	Total outbreaks	Destroyed birds
France	5	6	2	1	0	358	16	19	55	56	556	1,413,897
Hungary	1	4	0	6	0	195	30	0	67	3	308	1,972,341
Germany	0	4	1	49	2	11	19	6	200	0	292	1,192,215
Romania	0	0	0	0	0	0	46	0	90	0	136	1,452
Poland	0	0	0	0	0	0	25	0	71	39	135	1,008,896
Italy	4	16	0	40	0	5	15	3	14	0	99	2,803,589
Switzerland	0	0	0	0	0	0	0	0	94	0	94	0
Bulgaria	0	0	0	0	0	0	19	0	14	43	77	582,188
Czech Rep.	0	0	0	0	0	3	33	2	34	0	72	74,928
Netherlands	0	3	2	0	0	5	0	0	59	0	69	230,049
Slovakia	0	0	0	0	0	0	9	0	60	0	69	210
Denmark	0	0	0	0	0	0	1	1	51	0	53	69
Sweden	0	2	0	0	0	0	3	1	37	0	43	258,894
Austria	0	0	0	1	0	0	0	0	33	0	34	21
UK	0	0	1	3	0	0	5	2	19	0	32	106,603
Serbia	0	0	0	0	0	0	4	0	21	0	25	243
Croatia	0	0	0	0	0	0	8	0	11	3	22	492
Slovenia	0	0	0	0	0	0	0	0	21	0	21	0
Belgium	0	0	0	0	0	0	0	0	5	13	18	4,861
Finland	0	0	0	0	0	0	0	1	16	0	17	29
Greece	0	1	0	0	0	0	5	0	9	0	15	81
Spain	0	0	0	0	0	1	0	0	2	9	12	9,880
Ireland	0	0	0	0	0	0	0	0	9	0	9	2
Lithuania	0	0	0	0	0	0	0	0	5	0	5	0
Luxemburg	0	1	0	0	0	0	0	3	0	0	4	787
Bosnia and Herzegovina	0	0	0	0	0	0	1	0	2	0	3	51
Republic of North Macedonia	0	0	0	0	0	0	1	0	1	0	2	1,992
Cyprus	0	0	0	0	0	0	0	0	1	0	1	0
Portugal	0	0	0	0	0	0	0	0	1	0	1	0
Total	10	37	6	100	2	578	240	38	1,002	166	2,224	9,663,770
Total %	0.45	1.66	0.27	4.5	0.09	25.99	10.79	1.71	45.05	7.46	100	

Source: OIE, Immediate notifications and follow-up reports of highly pathogenic avian influenza

Table 2. LPAI outbreaks in poultry farms, backyard flocks and wild birds in affected European countries during 2016-2017 Avian Influenza Epizootic.

Country	Subtype	Broiler farms	Layer farms	Breeder farms	Turkey farms	Turkey breeder farms	Duck/goose farms	Backyard farms	Multi-farms	Wild birds	Unidentified	Total outbreaks	Destroyed
Germany	H5N1, H5N2, H5N3	0	0	0	0	0	2	5	1	0	0	8	3,009
The Netherlands	H5N2, H7N9	0	0	0	0	0	0	0	0	0	3	3	103,143
France	H5N1, H5N2, H5N3, H5N8, H5N9, H7	0	0	1	0	2	40	0	1	0	13	58	126,216
Denmark	H5N2, H7N7	0	0	0	0	0	2	0	0	0	0	2	4,416
UK	H5N1	0	0	0	0	0	0	0	0	0	1	1	39,800
Total		0	0	1	0	2	44	5	2	0	17	72	276,584

Source: OIE, Immediate notifications and follow-up reports of low pathogenic avian influenza

The dominant HPAI virus subtype in Europe during the 2016-2017 epizootic was H5N8. Nevertheless, other AI subtypes were also observed during that period. The H5N5 subtype was detected in Croatia, Czech Republic, Germany, Greece, The Netherlands, Serbia and Slovenia in wild birds and fattening turkey farms. H5N9, H5N1 and H5N2 subtypes were identified in France, in duck/goose farms, broiler farms, guinea fowl farm, multi-species farm and a hatchery. The H5N6 subtype was detected in a backyard farm in Greece and The Netherlands, in two outbreaks in wild birds, while the H7N7 subtype was found in Italy, in a turkey and a layer farm.

During the same period, there were also 72 LPAI outbreaks in 5 European countries, which led to the death of 277,752 birds (Table 2). France was the country with the highest number of birds' losses, followed by The Netherlands, UK, Denmark, and Germany. Regarding the number of LPAI outbreaks, France was the country with the highest number, listing 58, followed by Germany with 8 outbreaks, The Netherlands with 3, Denmark with 2 outbreaks and the UK with 1 outbreak. The majority of LPAI outbreaks were reported in duck-goose farms (44/72 outbreaks). In breeder and turkey breeder farms were observed only 3 LPAI outbreaks, in France while there were no outbreaks in broiler, layer and turkey farms. The dominant LPAI virus subtype in Germany, The Netherlands, France, and Denmark was H5N2, which was responsible for the death of 96,598 birds in backyard, breeder, duck-goose farms and game bird farms. The H5N1 subtype was responsible for the death of 96,349 birds of duck-goose farms and multi-species farms in the UK, France, and Germany. The H5N3 subtype was the cause of death of 17,921 birds in backyard, turkey breeder and duck-goose farms in Germany and France. Besides, France was the only country where the H5N8 and H5N9 subtypes of LPAI virus were detected in duck-goose farms. Similarly, The Netherlands was the only country where the H7N9 subtype was observed, but the affected population was not identified.

DISCUSSION

AIVs pose significant threats to avian and human health and cause severe damage to the poultry industry, concerning mortality, welfare, economic losses, and trading restrictions (Kuchipudi et al., 2014, Ramos et al. 2017). Wild birds, and especially those belonging to the orders Anseriformes (for instance waterfowl, ducks, geese, swans) and Charadriiformes (gulls, terns and shorebirds), are considered the natural reservoir hosts of the AIVs (Acha and Szyfres, 2003, Swayne, 2006). The findings of our study fully support the above mentioned results since the majority of infected wild birds during the 2016-2017 AI epizootic in Europe were ducks, geese, swans, gulls, terns and shorebirds.

Migratory birds, during their long migratory journeys, exchange viruses with other populations at staging, stopover, or wintering sites (Georgopoulou and Tsiouris, 2008, Gill et al., 2009). Shorebirds and gulls

contribute more to the AI transmission over more extensive geographic distances than ducks and geese. In particular, gulls and shorebirds play a crucial role in linking the continents, as they undertake long-distance migration, and allow a more direct route of virus transmission (Gill et al., 2009, Hill and Runstadler, 2016). In contrast, waterfowl are non-migratory birds, which fly and reside over land, stop at wetlands for food and supply, thus contributing to interspecies transmission and spread of AIV on the inside (Miller et al., 2005, Gill et al., 2009, Hill and Runstadler, 2016).

Based on the official reports of OIE during 2016-2017 AI epizootic, there were 72 outbreaks of LPAI viruses, the majority of which prevailed in duck-goose farms in France. H5N2 was the dominant LPAI virus subtype of the epizootic, while other subtypes, such as H5N1, H5N3, H5N8-H5N9, H7N7 and H7N9 were reported as well. In cases of LPAI viruses transmission from wild birds to poultry, there are three scenarios. The first scenario, the most common among the three, is the termination of the viruses' circulation. The second is the preservation of the viruses' circulation. Finally, the third scenario is the evolution of the viruses into HPAI, such as H5 or H7. However, once an AIV has been transmitted and efficiently circulated to poultry, it can rarely be re-adapted in wild birds (Swayne, 2006).

HPAI and LPAI viruses have been frequently isolated in commercial poultry farms of chickens, turkeys, ducks, geese, and ratites, as well as in pigeons and cage birds. However, the sensitivity and the clinical manifestation of the disease in birds vary between avian species. For example, pigeons appear to be relatively resistant to infection compared to poultry (Swayne, 2006). Similarly, the high mortality in chickens and simultaneously the absence of clinical disease in ducks after AIV challenge, indicate that there are differences in the innate immune response among avian species (Kuchipudi et al., 2014).

An interesting observation during the 2016-2017 AI epizootic, according to the official reports of the World Organization for Animal Health, was the type of Galliformes farms which were mostly affected by HPAI viruses. In particular, 52.91% of the outbreaks occurred in turkey farms, 19.58% in layer farms, 5.29% in broiler farms, 3.17% in breeder farms and 1.06% in turkey breeder farms.

Regarding that, the higher sensitivity of fattening turkeys to AI compared to that of broiler chicks could be the reason for the higher prevalence of AI outbreaks in turkey farms (Aldous et al., 2010). In particular, turkeys were 10-fold more susceptible to infection than chickens, with the cases concerning turkey farms being 4.5% of the total cases, while in broiler farms the rate was 0.45%. Likewise, turkeys are being raised for a more extended productivity period and under less strict biosecurity measures since occasionally they have outdoor access, e.g. for scratching and grazing. Similarly, layer hens are being raised for even more than a year productive period, and biosecurity gaps are more frequent since layer farms have more visits (egg transports, feed) and equipment exchange (egg trays, egg trolleys, egg tray crates), thus the infestation degree concerning layer farms equals 1.66%. These management practices increase the risk of contact with both wild birds and mechanical carriers and contribute to the higher prevalence of AI outbreaks in turkey and layer farms. Taking all the above into account, we could conclude that turkey and layer farms constitute a critical risk factor for the introduction of the disease into an area and its spread into other poultry farms.

Additionally, according to the official reports of OIE during 2016-2017, the majority of AI outbreaks were in duck-goose farms. The main reason for that higher prevalence is probably the outdoor farming system and the notably direct contact of them with the wild birds. Furthermore, the relative resistance of these species to AIV infections could lead to the shedding of large amounts of the virus to the environment and the spread of the disease to the other birds of the farm (Kuchipudi et al., 2014).

During the conduction of the present investigation, it became clear that OIE is keeping and recording a very detailed file regarding animal diseases, and that cannot be accomplished without the close collaboration of every country. It is obvious that evaluating the exact size of an endangered population is not always easy to determine, and every so often even the type of the epidemiological unit itself. This kind of work seems to be even more difficult amid a pandemic, especially in the case of a disease such as AI, whose spread can occur rapidly and dramatically. That can be seen quite clearly in the reports of countries such as Poland and Bulgaria that reported a large number of "unidentified cases". Likewise, France reported both a substantial amount of unidentified cases and plenty of outbreaks in birds' farms, without the specification of the breeding system. In this way, the study

and statistical processing of the results is made difficult, as the data from the reports seem incomplete, mainly regarding the bird species, thus not allowing a more thorough epidemiological investigation of the outbreaks.

The majority of the OIE reports claimed that the AIV infection was a result of contact, direct or indirect, between wild birds and poultry (OIE Situation Report, 2017). In particular, outbreaks linked to the dominant H5N8 subtype in Europe indicated that the sources of the infection were the contact with wild species or with infected animals when grazing or watering, through fomites (such as humans, vehicles, feed), the introduction of new live animals, via airborne spread, and, finally, via unknown or inconclusive routes. Genetic analyses of the European viruses indicate that they were introduced via wild birds to northern and central Europe from Asia (El-Shesheny et al., 2017).

According to another OIE Situation Report for avian influenza, published on 18/09/2017, HPAI H5N8 caused 277 outbreaks in poultry, 51 in wild birds and led to the destruction of 427,081 poultry. HPAI H5N8 subtype is a complex reassortant virus carrying genes from A (H5N1) as one of its parental viruses. It was firstly reported in China in 2010, while later on, following further virus evolution via reassortment, several outbreaks with HPAI virus A (H5N8) viruses occurred in aquatic migratory birds, chicken, geese and ducks in China, Japan, Republic of Korea and Europe (Hill et al., 2015, El-Shesheny et al., 2017). The HPAI virus A (H5N8) virus pathogenicity varies among avian species. Particularly, during infection, mass mortality is observed in turkeys and chickens, while it has yet to be correlated with acute illness or excessive mortality rates in mallards (Adlhoch et al., 2014). The transmission of avian influenza A(H5N8) virus to humans has not been reported so far worldwide, and no human cases have been reported (Brown et al., 2017).

The epidemiologic investigation of outbreaks linked to the H5N1-H5N2 subtypes in France claimed that the source of infection either was the introduction of new live animals or was unknown/inconclusive. Based on the OIE reports on AI in 2017, the HPAI H5N1 is enzootic in Asia and Africa and causes outbreaks in poultry and sporadic human infections. Since wild birds are most frequently implicated in the transmission, all affected countries should focus on applying biosecurity measures and on avoiding contact of wild birds with poultry. HPAI H5N1 led to 17 ongoing worldwide outbreaks in poultry and the destruction of 131,168 birds, whereas HPAI H5N2 led to 182 ongoing outbreaks in poultry and 1 in wild birds and therefore led to the destruction of 1,617,816 birds (OIE Situation Report, 2017).

Greece and The Netherlands were the only European countries to be reported with the H5N6 subtype of AIV. However, this subtype is considered the result of genetic reassortment from the H5N8 European subtype (OIE Update on Avian Influenza, 2018). H5N6 affected three countries worldwide and caused 358 outbreaks in poultry. The data shows that it has outnumbered the H5N8 AIV, concerning the amount of poultry destroyed after the application of control measures by the veterinary authorities (25,096,648 birds). The Asian lineage H5N6 causes severe clinical signs in poultry and associated mortality. Migratory birds could be the vectors of HPAI H5N6 transmission outside Asia. According to reports of WHO since 2014, this group of H5N6 viruses has been related to 24 laboratory-confirmed outbreaks of human infection, including 7 deaths in China (WHO, 2020).

The Netherlands was the only European country where outbreaks associated with H7N9 AIV, which were detected in ducks and neighboring broiler farms. Although the source of the infection was unknown or inconclusive, OIE stands by the fact that live bird markets pose a significant risk factor for H7N9 virus spreading among poultry and from poultry to humans. This virus, based on phylogenetic analysis, is not the same as the China H7N9 virus that has a significant impact on poultry and human infections in Asia. Since its origin in 2013, the H7N9 virus remained LPAI in poultry mainly in China but caused over 1,562 human infections (as of September 13, 2017). However, OIE reported recently that the LPAI H7N9 virus has mutated to an HPAI H7N9 virus. According to OIE Situation Report for Avian Influenza on 18/09/2017, the H7N9 virus is the cause of 22 additional worldwide outbreaks and led to the destruction of 831,087 birds.

Finally, yet importantly, the H5N5 virus affected several European countries. The source of the infection was either the contact with wild species or unknown/inconclusive. According to the OIE update on AI in 2018, H5N5 AIV affected only 2 countries worldwide and is the cause of 2 ongoing outbreaks concerning wild birds. There were no outbreaks in poultry, and no poultry was destroyed due to the control measures.

CONCLUSION

Poultry farms play a crucial role in the spread of AI into an area, although wild birds are known to be the source for the infection of domestic poultry and humans. The introduction of AI in poultry farms leads to severe damages and trade restrictions, which have a global impact on the market. Therefore, it is of grand necessity to eliminate the exposure of poultry to wild birds through a plethora of preventive measures, such as strict biosecurity measures and rearing restrictions. Still, the particular type of poultry farms should be considered for the implementation of biosecurity protocols, since fattening turkey and layer chicken farms pose a crucial risk factor for an AI outbreak. The establishment and application of surveillance systems, diagnostic methods for early detection of AIV and harmonized restriction and control measures are only some fundamental actions that need to be administered to restrain the distribution, prevalence and introduction pathways into Europe and for the control of AI.

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Research article
Ερευνητικό άρθρο

Evaluation of blood omega-3 and omega-6 levels in healthy female dogs and female dogs with mammary tumours

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ABSTRACT: The study was designed to determine the blood levels of omega fatty acids, which have an important role in the etiology of mammary tumours, in healthy and female dogs with mammary tumour. The study was carried out in 9 female dogs with histologically confirmed mammary tumour and a control group with 9 healthy female dogs without clinical mammary neoplasia. 10 ml cephalic blood samples were collected by using a 21G x 1.5´´ blood collecting needle into anticoagulated tubes before the surgical removing of the mammary masses. Mastectomy was performed in all female dogs with mammary tumours and all the mammary specimens were sent to laboratory for histopathological examination. According to histopathological diagnosis results, all of the tumours were found to be malignant. Omega-3 levels were found to be higher in healthy female dogs ($p<0.001$) whereas omega-6 levels were higher in female dogs with mammary tumour ($p<0.001$). These observations support the notion tha thigh levels of omega-3 fatty acids might proove to have a protective role on mammary tumor formation in female dogs, while increased levels of omega-6 fatty acids may be related to an increased mammary tumor risk. This difference between omega-3 and omega-6 levels was found to be caused mainly by Eicosatrienoic acid. It is concluded that omega fatty acids mayplay an important role in the biological mechanism of mammary tumour in female dogs.

Keywords: female dog, mammary tumour, omega-3, omega-6.

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INTRODUCTION

Mammary tumours are important for canine health as they are encountered very commonly in female dogs. Reports indicate that among all mammalian species, female dogs present with the highest incidence of mammary tumours. Compared to other reproductive organs, the mammary glands are five times more prone to the development of tumours. Mammary tumours can be localized to a single gland or can be observed simultaneously in all mammary glands. In female dogs, approximately 40% of all mammary tumours occur in the inguinal mammary glands, and 60% occur in the thoracic and abdominal glands. Nearly 50% of all mammary tumours are of malignant character. Definitive diagnosis is based on the histopathological examination of an incisional or excisional biopsy (Bostock, 1986; Reddy et al., 2009).

Diet is suggested to have a major role in etiology of mammary neoplasia (MacLennan and Ma, 2010). Essential fatty acids (EFAs) are polyunsaturated fatty acids (PUFAs), which are not synthesized in the body, and therefore need to be ingested with food. Essential fatty acids are classified under two groups, namely, omega-3 (ω-3) fatty acids and omega-6 (ω-6) fatty acids (Gültiken and Vural, 2004; Zatsick and Mayket, 2007). The principal ω-3 fatty acids include linolenic acid (LNA), eicosapentaenoicacid (EPA), docosahexaenoicacid (DHA) andstearidonic acid (SDA) (Gebauer et al., 2005). Omega-3 fatty acids are found in vegetable oils (linseed oil, walnut oil), seeds, green-leafy vegetables, beans and nuts, and fatty fish. Furthermore, the principal ω-6 fatty acids include linoleic acid (LA), arachidonic acid (AA) and gamma-linoleic acid (GLA).Omega-6 fatty acids are found in maize oil, sunflower seed oil, soybean oil, cottonseed oil,safflower oil, peanut oil, and margarine, as well as in the liver, brain, and red meat and poultry meat (Lasekan and Ney, 1990; Greenly, 2002).

Essential fatty acids may contribute to formation of mammary neoplasia (MacLennan and Ma, 2010). In the extensive meta-analyze study of Fay et al. (1997), it was shown that ω-6 fatty acids promoted the tumour development substantially in the rodents. Rose et al. (1995) reported that diets containing linoleic acid supplemented with eicosapentaenoic acid or docosahexaenoic acid led to lesser tumour growth and lung metastasis compared to the diets with only linoleic acid in the athymic nude mice. In an *in vitro* study, arachidonic acid, a member of the ω-6 fatty acids, was shown to increase the proliferation of endothelial cells derived from human breast carcinomas and trigger formation of vessel-like structures(Pla et al., 2008). On the contrary, in the prepubertal rats, exposure to low-fat ω-3 PUFA diet caused reduction in the mammary tumorigenesis (Olivo and Hilakivi-Clarke, 2005). Exogenous supplementation of ω-3 fatty acid docosahexaenoic acid (DHA) in human breast cancer cells downregulates Her-2/neu expression and decrease tumour growth rate (Menendez et al., 2005). Sonnenschein et al. (1991) showed that high-fat diet had protective effects on the mammary tumour risk in dogs. But this study didn't have any detailed information on which type of fatty acid are present in the diet due to the scarce data about dog food preparations. Obesity or overweight might be associated with more aggressive tumours (Costa-Santos et al., 2019). This relationship between obesity/overweight is resulting from the activation of insulin/IGF-1 pathway, high level of pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) and their influence on adipocytokines (Lim et al., 2015). The level of plasma insulin-like growth factor-I (IGF-I), known to have a regulatory role in tumour development, has been reported to decrease in mammary tissue with an increase in the ratio of omega-3 fatty acids to omega-6 fatty acids (Zhu et al., 2011).

Supplementation for long period than three months with fish oil, rich in omega-3 fatty acids, can control dyslipidemia in dogs. It is expressed that dyslipidemias are possible factors related with accelerated tumor genesis (Costa-Santos et al., 2019). Major changes occured in our diet since the Agricultural Revolution began 10.000 years ago (Simopoulos, 1999). Especially, type and amount of essential fatty acids in the diets changed dramatically (Eaton and Konner, 1985). In today's industrialized societies, diets include more ω-6 fatty acids and less ω-3 fatty acids (Eaton and Konner, 1985; Simopoulos, 1999). Due to the increased production of industrial food and the altered nutrient composition of animal feedstuffs, various food such as red meat, fish and poultry meat have become significantly poorer in ω-3. Therefore, the dietary intake of ω-3 fatty acids has fallen below that of ω-6 fatty acids (Candela et al., 2011).

It is indicated that food rich in ω-6 triggers the production and release of oestrogen, the increased levels of which are known to induce tumour growth, in adipose tissue in human (Nagata et al., 2007).Therefore, it is suggested that reducing the dietary intake of ω-6 fatty acids could decrease the prolactin-binding capacity of tumours and thereby, slow down tumour

growth in rats (Cave and Erickson-Lucas, 1982).Increasing the ratio of omega-3 fatty acids toω-6 fatty acids in the diet is considered an important tool in reducing the risk of mammary tumours and preventing their post-excision recurrence (Rose, 1997).

The ω-6 fatty acid most used by tumour cells is reported as AA (MacLennan and Ma, 2010). Linoleic acid, which is an ω-6 fatty acid, is converted to AA, a precursor of eicosanoids, when metabolized in the body. A high level of dietary intake of ω-6 fatty acids increases the level of AA in the body, and thereby, increases the production of pro-inflammatory eicosanoids such as prostaglandins, which are synthesized from AA (Gebauer et al., 2005). Malignant tumour cells are reported to synthesize a higher level of eicosanoids than benign tumour cells, and eicosanoids are known to be capable of accelerating tumour growth. As eicosanoids synthesized in the body are produced from EFAs, the eicosanoid level of the tumour and host are both affected by the type and level of EFAs in the diet (Cave, 1996).

The EFA least used by mammary tumour cells is EPA, anω-3 fatty acid, which is used in the treatment of certain types of cancers (Ward and Singh, 2005). In the body, by means of various desaturase isoforms, LA is converted to AA, and LNA is first converted to EPA and then to DHA. As ω-3 fatty acids and ω-6 fatty acids compete for desaturase enzymes, an increased consumption of LNA, EPA and DHA decreases the production ofAA (Cowing and Saker, 2001). The two main compounds required for eicosanoid synthesis are AA and EPA. Eicosanoids synthesized from AA (AA-derived eicosanoids) have properties opposite to those of EPA-derived eicosanoids. As AA and EPA compete for cyclooxygenases (COX) and lipoxygenases (LOX), they lead to the synthesis of eicosanoids with opposing properties. Generally, while AA-derived eicosanoids show a pro-inflammatory effect,EPA-derived eicosanoids have an anti-inflammatory effect (Cowing and Saker, 2001).

The study was designed to determine the blood levels of omega fatty acids, which may play an important role as modifiers of breast cancer risk, in healthy female dogs and female dogs with mammary tumors.

MATERIALS AND METHODS

Material

The study material comprised 18 female dogs of various breeds. Study group was created with German Shepherd (n:4), Terrier (n:3), Setter (n:2); and control group was composed by German Shepherd (n: 2), Terrier (n:3), Beagle (n:1), mix (n:3) breed. Nine of which were admitted to the veterinary clinic with signs of a mass structure (lump) in the mammary gland and were clinically diagnosed with mammary tumour, and the other 9 of which were healthy female dogs. Of the female dogs diagnosed with mammary tumour, four were eight years old, one was nine years old, and the remaining four were aged 10 years or older. The healthy female dogs were selected among animals older than 5 years of age. Reproductive history of the female dogs with mammary tumours and healthy female dogs include status of ovariohysterectomy, age at first estrus, number of full-term pregnancy, age at first pregnancy, hormonal treatment to prevent or inhibit estrus. Female dogs diagnosed with mammary tumors, 7 were not neutered and 2 were neutred previously and these animals had been neutered after the age of five. All healthy dogs for control group were selected from neutered and these animals had been neutered before the age of three. Any animals were not treated with the hormones to prevent or inhibit the estrus. All of the dogs are owned by the second owners. Therefore, no definite information about when the first estrus took place. None of the dogs in control group gave a birth except from one which had a dystocia in its first parturition. Only this dog had an ovariohysterectomy operation as an surgical treatment of dystocia. Of female dogs with mammary tumours, only two of them gave birth. The rest of them didn't have pregnancy. All animals were fed with homemade and dry commercial diet. Amount of commercial diet fed to the dogs are based on brand recommendation. Homemade meals are not main part of diet, generally given to the dogs as a reward per day. Body condition score (BCS) was performed according to nine-point BCS system (Laflamme, 1997). All animals were in the normal body weight (4-5/9).

Mammary tumors were found in inguinal mammary glands in 5 female dogs in which 3 female dogs had tumor on only one mammary gland while 2 female dogs had tumors on two mammary glands. Axillar mammary gland tumors were detected in 3 female dogs in which 2 female dogs had tumors on only one mammary gland while one female dog had tumors on two mammary glands. In one female dog multiple masses detected, one tumoral mass on axillary gland and two masses on inguinal gland.

Methods

Informed client consent was taken before the clinical examination of the dogs. Prior to clinical examination, the medical history including the location of the mammary masses, history of ovariohysterectomy, housing and general nutrition conditions of the female dogs was recorded on an inspection form. Ten-ml whole blood samples were collected by cephalic venepuncture by using 21G x 1.5´´ blood collecting needle from both the animals diagnosed with mammary tumour, prior to surgery, and the healthy animals, into anticoagulated tubes, and were stored during two months at -20 ◦C until being analyzed for fatty acids. The masses suspected of being mammary tumours were excised by mastectomy, and were transferred to the laboratory for histopathological examination after fixed with formalin. Any biopsy procedure was not performed before surgery to the tumoral mass.

Following atropine sulphate(Atropin 0.2%, Vetaş, Turkey)(0.45 mg/kg, sc) application for preanesthetic,general anesthesia was performed by xylazine hydrochloride (Basilazin %2, Bavet, Turkey) (2 mg/kg, im) and ketamine hydrochloride (Ketasol 10%, Richter Pharma AG, Austria)(10 mg/kg, im) combination. After general anesthesia routine surgical procedure was applied. Female dogs which had multiple masses on mammary gland were undergone unilateral total mastectomy (all tumor masses, mammary glands, inguinal and axillary lymph nodes were removed).Female dogs which had even single or more masses on inguinal or axillary gland were undergone unilateral partial mastectomy (primer tumoral mass, connected mammary glands and lymph nodes were removed).

Histopathological Method

The mammary masses, belonging to the 9 animals suspected of having mammary tumours, were firstly fixed in 10% formalin solution. The tissue samples were processed in an automated processor (Leica TP 1020), such that they were dehydrated through alcohol and xylolseries, embedded in paraffin and hardened on a cryoconsole (Hestion TEC 2800 Embedding Center), and sliced into5-µm-thick sections using a Leica RM 2135 rotary microtome. The sections were deparaffinised in xylol, passed through a series of graded alcohols (100%, 96%, 80%,70%), and stained with haematoxylin-eosin (H&E) (Luna, 1968). The sections were examined under a light microscope (Olympus BX35), and were photographed with a digital camera (Olympus SC 180).Tumour classification was based on the criteria described by Michael et al. (2017).

Fatty Acid Analysis

Ten-ml blood samples taken from all of the animals into anticoagulated tubes containing sodium EDTA were transferred to the laboratory under cold chain. The tubes were added 50 ml of diethyl ether, mixed on a shaker for 4 h, and centrifuged at 2000 rpm for 5 min. Blood fat was extracted from the supernatant. Fatty acid methyl esters were produced modified methods of Kocak et al. (2016). For this purpose, 4 ml of 2% methanolic NaOH was added to each sample (approximately 50-70 µl blood fat) for saponification at 95 °C for 2-3 min. Subsequently, 5 ml of 14% methanolic BF_3 was added, and the samples were maintained at 95 °C for 5 min. Next, the samples were added 2 ml of n-heptane, maintained at 95 °C for 1 min, added 4 ml of saturated NaCl, and centrifuged at 1000 rpm. Later, 1.5 ml-portions of the resulting supernatant were transferred into vials and analyzed by gas chromatography.

Fatty acid methyl esters were analyzed by gas chromatography (Trace 1300, Thermo Scientific, USA) using a flame ionization detector (FID). The FID temperature was adjusted to 260 ^{0}C. Cyanopropylpolysilphenylene-siloxane (length: 60 m, diameter: 0.25 mm × 0.25 µm, film thickness: 0.25 µm) (TR-FAME, Thermo Scientific, USA) was used in the column. The continuous flow rate of the carrier gas, helium, was set as 1.5 ml/min. The dry airflow rate was set as 300 ml/min, and the flow rate of hydrogen was programmed as 30 ml/min. The injection volume was 1 µl and the injection was performed in split form. The injector temperature was 250 ^{0}C. The oven temperature was initially set to 100 ^{0}C for 3 min, and was increased up to 240 ^{0}C at a rate of 4 ^{0}C/min. The unknown peaks in the chromatogram were identified by comparison with the standard FAME Mix (Chem-Lab, Belgium) of known composition, to determine the fatty acids.

Statitstical Analysis

The values obtained in fatty acid analyses were evaluated with Student's t-test using the Statistical Package for Social Sciences (SPSS, version 14.0) software.

RESULTS

The fatty acid composition of blood is shown in Table 1. Sum and ratios of blood fatty acids are shown in Table 2.

Table 1. Fatty acid composition of blood (Means ± SE)

Fatty acids (%)	Groups		P
	Control (n=9)	Tumour (n=9)	
Capriylic acid	0,013±0,001	0,133±0,071	0,184
Capric acid	0,079±0,002	0,165±0,040	0,075
Undecylic acid	0,012±0,001	0,019±0,004	0,326
Lauric acid	0,062±0,014	0,116±0,019	0,047
Tridecylic acid	0,012±0,001	0,047±0,007	0,001
Myristic acid	0,468±0,051	0,583±0,040	0,089
Myristoleic acid	0,057±0,009	0,086±0,017	0,200
Pentadecylic acid	0,156±0,018	0,106±0,014	0,045
Pentadecanoic acid	0,047±0,018	0,036±0,017	0,669
Palmitic acid	14,892±0,957	15,395±0,845	0,699
Palmitoleic acid	1,732±0,313	1,629±0,211	0,783
Heptadecanoic acid	0,367±0,031	0,284±0,048	0,189
Stearic acid	7,395±0,527	11,269±0,812	0,003
Oleic acid	19,734±1,22	18,711±1,013	0,525
Linoleic acid ω 6	26,469±1,412	25,260±1,457	0,566
Ɣ- Linolenic acid ω 6	0,750±0,127	0,490±0,069	0,025
Linolenic acid ω 3	0,209±0,067	0,102±0,015	0,109
Arachidic acid	0,647±0,103	0,362±0,057	0,022
Paullinic acid	0,266±0,034	0,311±0,071	0,604
Eicosadienoic acid ω 6	0,364±0,203	0,152±0,041	0,272
Eicosatrienoic acid ω 6	0,225±0,102	10,443±0,861	<0,001
Eicosatrienoic acid ω 3	11,980±0,973	0,230±0,043	<0,001
Arachidonic acid ω 6	0,692±0,410	0,151±0,090	0,173
Eicosapentaenoic acid ω 3	0,395±0,124	0,371±0,076	0,866
Heneicosanoic acid	0,280±0,046	0,226±0,031	0,337
Erucic acid	12,408±1,034	11,606±0,552	0,480
Docosadienoic acid ω 6	0,176±0,085	0,338±0,156	0,478
Docosahexaenoic acid ω 3	0,371±0,050	0,382±0,085	0,917
Lignoceric acid	0,798±0,037	0,710±0,121	0,544
Nervonic acid	0,064±0,009	0,391±0,143	0,059

Significant level was accepted as P<0,05

Table 2. Sum and ratios of blood fatty acids (Means±SE)

Fatty acid (%)	Groups		P
	Control (n=9)	Tumour (n=9)	
ΣSFA	23,892±1,272	29,136±1,042	0,005
ΣMUFA	34,678±1,174	33,053±1,167	0,347
ΣPUFA	41,428±1,235	37,809±1,305	0,066
ΣUFA	76,107±1,272	70,863±1,042	0,005
Σ PUFA/ ΣSFA	1,772±0,114	1,320±0,078	0,004
ΣUFA/ ΣSFA	3,254±0,185	2,472±0,124	0,002
Σ ω 6	28,471±1,449	36,722±1,298	0,001
Σ ω 3	12,748±0,962	0,984±0,146	<0,001
Σ ω 6/ Σ ω 3	2,344±0,226	42,296±4,166	<0,001

Significant level was accepted as P<0,05
SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids

Histopathological Findings

Histopathological examination demonstrated that each of the nine mammary masses were malignant tumours. Four of the cases were diagnosed with mixed carcinoma (Fig. 1). In these cases, histopathologically, the malignant epithelial fusion, observed in the form of irregular tubules, was supported by a fibrovascular stroma. This mass contained sporadic non-atypical cartilaginous formations and mesenchymal tissue composed of benign fusiform myoepithelial cells.

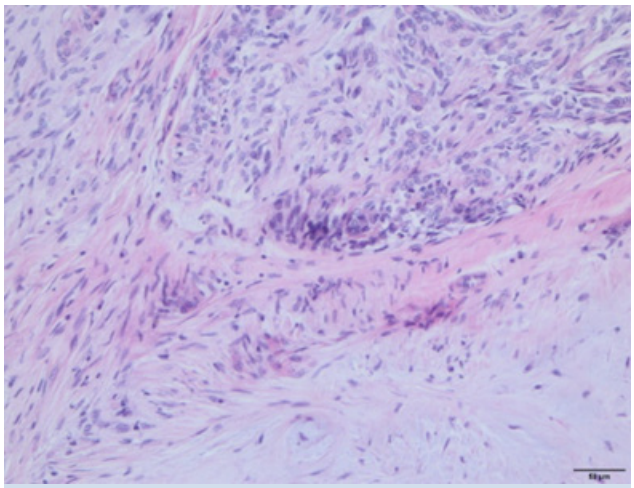

Fig.1. Mixed carcinoma

Three of the cases were diagnosed with tubular carcinoma (Fig. 2). These malignant tumours contained tubular and gland-like structures. The tubules were lined by a single layer or two layers of nuclear pleomorphic cells with an eosinophilic cytoplasm and hyperchromatic nucleus. The intertubular spaces contained sporadic aggregates of fibroblasts and inflammatory cells, including plasma cells, macrophages and lymphocytes.

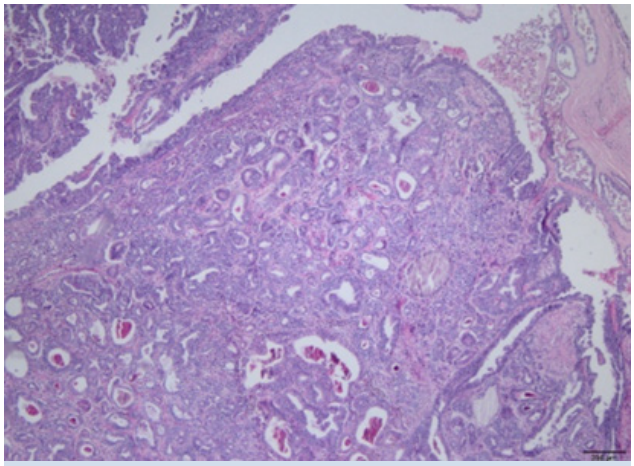

Fig.2. Tubular carcinoma

One case was diagnosed with malignant mesenchymal tumour (fibrosarcoma) (Fig. 3).This malignant tumour originated from the interstitial stroma of the mammary gland and was characterized by the proliferation of interwoven fusiform cells. The neoplastic cells had indistinct cell borders, a small amount of eosinophilic fibrillary cytoplasm, and an oval elongated nucleus.

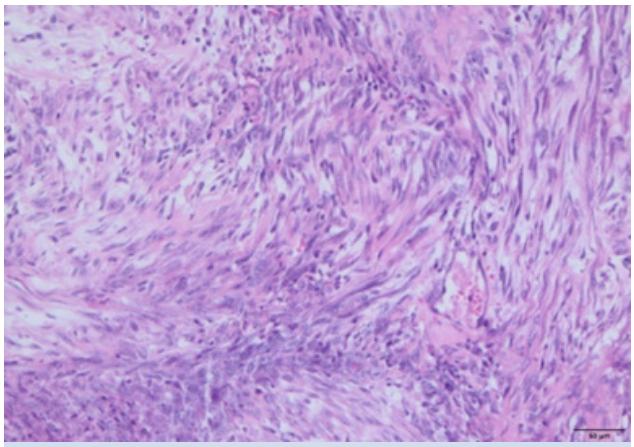

Fig.3. Malignant mesenchymal tumour (fibrosarcoma)

One case was diagnosed with carcinoma and malignant myoepithelioma (Fig. 4).The tumour contained both epithelial and myoepithelial components of malignant character. The cells, which varied from cuboidal to columnar in shape, formed irregular tubules. Furthermore, the interstitium contained atypical cells with an oval-fusiform vesicular nucleus and eosinophilic cytoplasm. These cells were surrounded by a small amount of basophilic fibrillary material (myxoid matrix), which was also observed within the cytoplasm of some of the cells.

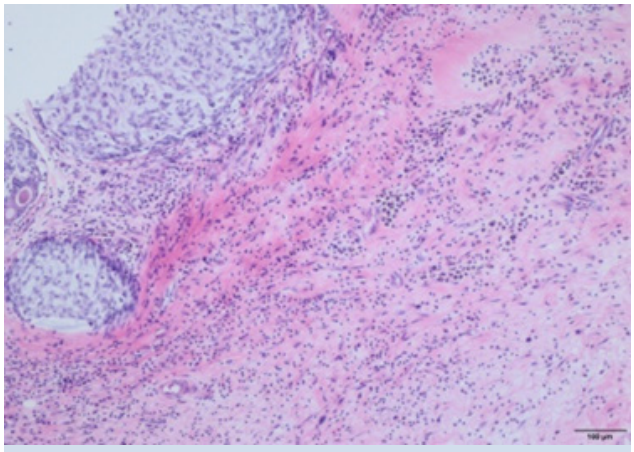

Fig.4. Carcinoma and malignant myoepithelioma

DISCUSSION

It has been reported that the serum fatty acid profile of an individual is a reflection of the fatty acid composition of his/her diet (Cohen et al., 1993), and that the ratio of ω-3 fatty acids to ω-6 fatty acids in the serum is an indicator of the level of dietary intake of these fatty acids (Yang et al., 2014). Thus, the measurement of serum LA levels is suggested as a reliable method of determining the dietary intake level of LA (Dougherty et al., 1987). Söderhjelm (1962) reported that linoleic acid supplementation caused substantial

changes in the level of the fatty acid in the serum in dogs. In a study examining the effect of dietary intake of fatty acid on serum fatty acid levels, it was shown that fatty acid supplementation can be used to change serum and cutaneous fatty acid composition of dogs. Sunflower (78% 18:2n6) supplementation to Beagle puppies increased serum linoleic acid (18:2n6) levels after three weeks. In the control group in which no supplementation of fatty acid are made, there is no significant change in serum fatty acid profile throughout the study period (12 weeks) (Campbell and Dorn, 1992). Therefore, it may be inferred that serum linoleic acid levels is a reflection of dietary fatty acid intake. In the present study, serum fatty acid levels were determined in both healthy female dogs and female dogs with mammary tumour (Table1).

In a study carried out in mice, Rose et al. (1995) determined that primary tumour growth was delayed and lung metastasis was less in animals given feed that was supplemented with ω-3 fatty acids(EPA and DHA), compared to animals given feed containing 8% of ω-6 fatty acids (LA). The researchers attributed this result to the inhibitory effect ofω-3 fatty acids on tumour eicosanoid biosynthesis.

There is a limitation in our study concerning diet. We couldn't exact amount of diet, preparation or variety of homemade food. However, due to the local eating habits, it is possible that dogs had been fed with red meats. Alenza et al. (1998) stated that homemade meals and high intake of red meat are associated with the risk for mammary tumour in female dogs. This contrasts with our results. In our study, both groups are fed with mainly homemade food.

Given the impact of diet on the development of mammary tumours in humans, diet-related factors have been well scrutinized. Multiple factors, including among others, advanced age, neutering, undergoing ovariohysterectomy after 2-5 years of age, and progesterone treatment, all increase the risk of mammary tumour development (Benavente et al., 2016). Simon et al. (2006) has reported that mammary tumour incidence is highest in female dogs aged 8 years and older. In the present study, four of these female dogs were 8 years old, one was 9 years old, and the remaining four were aged 10 years or older. Of the female dogs with mammary tumour, only two were reported to have undergone ovariohysterectomy, and these animals had been neutered after the age of five. Similarly, the healthy female dogs were also determined to be fed mainly on homemade food, and those that were older than 5 years of age were sampled for blood.

It is reported that the most common mammary tumours in female dogs are mixed tumours (67%), and adenomas and adenocarcinomas (32%) (Gültiken and Vural, 2004). Moulton (1990) suggested that almost half of all mammary tumours are of the mixed type, and 37% are adenocarcinomas. Furthermore, Fidler and Brodey (1967) indicated that of all mammary tumours, 50-65% are mixed tumours, 25-40% are carcinomas, and the remaining are either hyperplasic structures, adenomas or myoepitheliomas**.** In the present study, all of the mammary masses were confirmed to be malignant tumours. Four were diagnosed as mixed carcinomas (Fig.1), 3 as tubular carcinomas (Fig.2), 1 as fibrosarcoma (Fig.3), and 1 as carcinoma and malign myoepithelioma (Fig.4).

The inhibitory effect of ω-3 fatty acids on mammary tumours is explained by these EFAs supressing the generation of arachidonic acid-derived prostanoids (prostaglandin E2), which are responsible for inflammatory response, cell growth, apoptosis, angiogenesis and metastasis. It is reported that ω-3 fatty acids increase apoptosis and decrease the rate of mitosis in the mammary gland. It is also hypothesised that ω-3 fatty acids alter membrane stability in tumour cells (Habermann et al., 2010). Huerta-Yepezet et al. (2016) reported that omega-3 fatty acids show an antiangiogenic effect by inhibiting the synthesis of several angiogenic mediators, including vascular endothelial growthfactor (VEGF), platelet-derived growth factor (PDGF) and prostaglandin E2 (PGE2). Fabian et al. (2015) indicated that, EPA and DHA, which increase with arachidonic acid and belong to the family of ω-3 fatty acids, reduce the risk of mammary tumour development by decreasing the level of pro-inflammatory lipid derivatives, inhibiting the synthesis of nuclear factor-κB-induced cytokines, and reducing growth factor receptor signals due to cell membrane changes. In the present study, it was determined that serum ω-3 fatty acid levels were higher in the healthy female dogs, compared to those diagnosed with mammary tumour ($p<0.001$) (Table2). Besides, omega-6 level were found higher in female dogs with mammary tumour. The difference between ω-3 and ω-6 levels on mammary tumors was found to be caused mainly by Eicosatrienoic acid (Table 2).

Tumours consist of different cell types with stem-cell character having capability of self-renewal, differentiation and high-tumorigenic activity. Also, these cells are responsible for lower sensivity to chemotherapy and radiotherapy. These cells are term as cancer stem cells (CSCs) or tumor-initiating cells (TICs)

which are a contributing factor in initiation, recurrence and metastasis of tumours (Clarke et al., 2006; Nguyen et al., 2012). In canine mammary tumours, CSCs can be detected by using a sphere-forming assay. CSCs derived from canine mammary cancer lines are of high tumorigenic activity in immune deficient mice. CSCs also have ability to resist to anticancer drugs. So, CSCs show stem-cell like features (Michishita et al., 2011; Pang et al., 2011). In canine mammary adenocarsinoma cell culture study, DHA levels in sphere-forming cells (CSCs) were found to be low and variable (Michishita et al., 2011). It was stated that EPA and DHA inhibit the ability of self-renewal and decrease the survival of CSC via apoptosis (Erickson and Hubbard, 2010; Yang et al., 2013).

The main components for the production of eicosanoids are AA and EPA which is produced by metabolizing omega-6 and omega-3 fatty acids respectively. However AA and EPA, leads to the production of eicosanoids which has opposite effects. Typically, AA leads to the emergence of eicosanoids that stimulate mammary tumors, while EPA causes the emergence of eicosanoids which has preventive effects on mammary tumors (Rose, 1997; Cowing and Saker, 2001; Gebauer et al., 2005). It has been reported that ω-6 fatty acids increase the synthesis of AA, and thereby, accelerate cell division, supress the immune system, and enhance the growth, development and spread of tumours (Abou-El-Ela et al., 1989). It is suggested that a diet rich in ω-6 fatty acids disrupts the balance between cell proliferation and apoptosis in the mammary gland, and induces cell proliferation (Solanas et al., 2010). Moreover, it is indicated that eicosanoids produced from ω-6 fatty acids are involved in tumour angiogenesis, and thereby, facilitate the growth and metastasis of tumours (Rose, 1997). In a study carried out in mice (Lasekan et al., 1990), 16 weeks of dietary supplementation with safflower oil, known to be rich in ω-6,was observed to increase the incidence of mammary tumours and 74% of the cases were diagnosed with adenocarcinoma.

Based on the significant decrease they observed in the mammary tumour incidence of mice given EPA- or DHA-supplemented feed with an ω-3:ω-6 fatty acid ratio of 1:1.8, Noguchi et al. (1997) suggested that increasing the ω-3:ω-6 ratio of the diet could provide protection against the development of mammary tumours. In another study conducted by Rose et al. (1996), dietary ω-3 supplementation was observed to inhibit the local recurrence and metastasis of mammary tumours in mice, the mammary tumours of which were surgically excised. Literature reports suggest that rather than the level of ω-3, the ratio of ω-3 to ω-6 is important in relation to mammary tumour development, and a ratio ranging from 1:1 to 1:2 would provide protection against the growth and development of mammary tumours. In another study, Abou-El-Ela et al. (1989) demonstrated that food with anω-3: ω-6 ratio of 1:2 reduced the formation of mammary tumours induced by 7-12 dimethylbenzanthracene (DMBA). On the other hand, in the opposite situation, in the event of an increased ω-6:ω-3 ratio, it is reported that tumour incidence increases due to a decrease in the level of DHA among membrane phospholipids (Pettersen, 2012). In a research conducted by Yang et al. (2014) on 8,331 mammary tumour cases among 274,135 participants, the association of the ω-3:ω-6 fatty acid ratio with the risk of mammary tumour development was investigated, and it was determined that an increase of 1/10 in the ω-3: ω-6 ratio reduced mammary tumour risk by 6%. In their study in mice, Fabian et al. (2015) ascertained that increasing the total ω-6:ω-3 ratio in feed to >1 decreased the incidence and multiplicity of mammary tumours by 20-35%. In the present study, it was determined that the blood ω-6:ω-3 ratio was lower in the healthy female dogs, compared to those diagnosed with mammary tumour ($p<0.001$) (Table 2). This result supports the correlation suggested for the blood ω-6:ω-3 ratio and mammary tumour incidence in several literature reports (Abou-El-Ela et al., 1989;Rose et al., 1996; Noguchi et al., 1997; Pettersen, 2012; Yang et al., 2014; Fabian et al., 2015).

Mammary tumours generate a high level of ω-6 metabolites, such as prostaglandin E2, which are known to place immune functions under risk and induce tumour growth. On the other hand, ω-3 metabolites have been shown to prevent tumour growth. Increased levels ofω-3 are reported to have a potential anticarcinogenic effect exerted through the inhibition of the generation ofω-6 metabolites (Simonsen et al., 1998). Simonsen et al. (1998) reported that in mammary tumour cell cultures, increasing the ratio of ω-3 fatty acids to ω-6 fatty acids decreased the generation of prostaglandin E2 by the tumours cells, and thus, showed a negative correlation with mammary tumour development. In another study carried out in mice, Sasakiet al. (1998) determined that the PGE2 concentration in the tumour tissue was significantly lower in the group with a blood ω-3: ω-6 ratio of 1.03, compared to the group with an ω-3: ω-6 ratio of 0.01. Simopoulos (1991) determined thatω-3 fatty acids reduced the metastasis of mammary tumours to the lungs in mice. The results of Simo-

nopoulos (1991) agree with our results stating that the lower ω-3 fatty acids in tumour groups.

In an investigation on the impact of diet on mammary tumour development in mice, Wirfalt et al. (2002) determined that high dietary intake levels ofω-6 fatty acids increased the incidence of mammary tumours. Rose et al. (1991) assigned 58 female mice to two equal groups, and provided one group with feed that was added 23% of maize oil rich in ω-6 fatty acidsand the other group with feed that was added 5% of maize oil for a period of 15 weeks. At the end of the first week of the study, the researchers injected tumour cells into the mammary tissue of the mice, and at the end of the study period, the necropsies performed on the animals demonstrated that in the group given feed containing 23% of maize oil, 27 of the animals had developed tumours, whilst in the group given feed containing 5% of maize oil, 21 of the animals had developed tumours. Furthermore, in the group fed on a high-oil diet, out of the 27 animals with mammary tumour, 18 presented with lung metastasis, whilst in the group fed on a low-oil diet, out of the 21 animals with mammary tumour, 8 presented with lung metastasis. In result, the researchers determined that the incidence of mammary tumours was high in the mice fed on a diet rich in ω-6 fatty acids, and thereby, concluded that this type of a diet could also increase lung metastasis. In another study carried out in mice, Rose et al. (1995) determined that primary tumour growth was delayed and lung metastasis was less in animals given feed that was supplemented with ω-3 fatty acids(EPA and DHA), compared to animals given feed containing 8% of ω-6 fatty acids (LA). The researchers attributed this result to the inhibitory effect ofω-3 fatty acids on tumour eicosanoid biosynthesis.

Sonnenschein et al. (1991) showed that high-fat diet had protective feature on the mammary tumour risk in dogs. But this study have some shortcomings, as no detailed information on which type of fatty acid are involved in the diet, are presented due to the scarce data about dog food preparations. Costa-Santos et al. (2019) reported that a relationship between dyslipidemia and tumor aggressiveness. Dyslipidemia is a substantial factor in the tumour formation (Aravani et al., 2018). Higher triglyceride, VLDL, albumin, globulin and lactate levels were observed in the aggressive carcinomas in overweight/obese dogs. Fish-oil supplementation didn't change the abovementioned parameters, but glucose, total protein, globulin levels increased, leading to alter metabolic parameters in cancer patients (Costa-Santos et al., 2019). In this study, the detection of higher blood ω-6 levels ($p < 0.001$) in female dogs with mammary tumors and higher serum ω-3 levels ($p < 0.001$) in healthy female dogs support the opinion that fatty acid levels may be associated with mammary tumor risk.

CONCLUSIONS

In result, ω-3 was determined at higher levels in the blood of the healthy female dogs whereas ω-6 was detected at higher levels in the female dogs diagnosed with mammary tumour.This suggest that omega fatty acids may be an important factor in the biological mechanism of canine mammary tumour development. We consider that the obvious differences between blood level ofω-3 and ω-6 might be caused by Eicosatrienoic acids. Further research should be done to understand the exact mechanism of how ω-3 and ω-6 contribute to development of female dog mammary tumours.

CONFLICT OF INTERESTS STATEMENT

The authors declare that there is no conflict of interests regarding the publication of this article.

ANIMAL RIGHTS STATEMENT

All the experimental procedures followed in this study were approved by the Animal Care and Use Committee of Hatay Mustafa Kemal University, Hatay, Turkey via letter no. 2017/5-1.

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Research article
Ερευνητικό άρθρο

Assessing the Impact of using biofloc system with different feeding rates on Nile Tilapia (*Oreochromis niloticus*) Performance

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ABSTRACT: Biofloc technology is a technique of enhancing water quality in aquaculture through balancing carbon and nitrogen in the system. Randomly designed 3×1 factorial treatments for 90 days was conducted;to assess the effects of biofloc technology in indoor tanks for Nile tilapia (*Oreochromis niloticus*); on the growth performance, digestive enzyme activity, hematology, immune response, intestinal morphometry and chemical composition of Nile tilapia and flocs. In addition, to distinguish the best feeding; through utilizing different feeding rate 0.5%, 1% and 1.5% rate; with zero water change (biofloc technique; BFT) and their impact on aquatic animal rearing. Fish were reared in nine fiber-glass indoor tanks (2 m^3/ each); three replicates / treatment (feeding rate) with 100 fish / tank. Diets were offered twice / day. Results showed that values of water parameters were optimal for tilapia culture and the recorded ammonia, nitrite and nitrate concentrations are created through nitrification process in the BFT system. BFT protein increased positively with increased feeding rate, while BFT lipid and carbohydrates negatively decreased. Growth performance and feed utilization efficiency were significantly improved by increased feeding rates however; the best food conversion rate was recorded in 0.5% feeding rate treatment.There was negative relationship between crude protein and fat contents in fish body; with the highest crude protein content in 1.0% feeding rate treatment. The overall improvement in hae-matological and serum biochemical parameters reflects the positive effect of biofloc system on the physical condition and immune response of tilapia.The total intestinal length and intestinal villi heights were significantly increased with decreased feeding rate; with highest length in 0.5% feeding rate treatment. In conclusion, using BFT in tilapia rearing with 0.5% feeding rate, had beneficial effects on maintaining good water quality, improving feed utilization and growth performance, increasing fish body protein content, physical and immune response promotion as well as increasing the absorptive capacity of the intestine. Subsequently, BFT offers not only economic benefits for fish farmers and safe product for consumers but also, for the sustainability of the fish environment.

***Keywords*:** Biofloc, feeding rates, hematology, Nile tilapia, performance

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INTRODUCTION

Aquaculture is a fundamental industry for supporting the world's interest of seafood protein and will play a much increasingly significant role as the worldwide population continues to increase (Jackson, 2007). To adapt to the issue of lack in protein food supplies, which is mainly located in the developing countries, the current worldwide growth rate of the aquaculture business (8.9-9.1% per year since the 1970s) is highly required (Subasinghe, 2005; Gutierrez-Wing & Malone,2006; Matos et al., 2006). Recently, tilapia has become the sparkling star of aquaculture with beginning of several farms to compensate the progressive consumption rate (Fitzsimmons, 2005; FAO, 2012).The Egyptian aquaculture sector is the largest producer of cultivated fish in Africa and the third largest worldwide producer of cultivated tilapia after China and Indonesia (FAO, 2016; Fitzsimmons, 2016).

Intensive aquaculture constitutes the intelligent key to keep up the tilapia production (FAO, 2012). Intensive aquaculture industry faces two noteworthy issues; (i) Water quality deterioration caused by pollution from waste products (Piedrahita, 2003) (ii) expanded cost burden of artificial feed. Feed is the greatest portion of working expense of aquaculture systems additionally, availability of ingredients limit the growth of fed aquaculture. So as to make aquaculture completely effective, there is an urgent demand to create innovation that will increase financial, environmental sustainability and lessen feed cost and improve production (Sharma *et al.,* 2015).

Recently, biofloc technology (BFT) is considered as a more eco-friendly and sustainable technique for use in zero-water exchange culture systems (Avnimelech, 2007; Azim & Little, 2008; De Schryveret al., 2008; Crab et al., 2009). Utilization of biofloc technology (BFT) offers a solution for both last problems. The system depends on the fact of lower speculation and maintenance costs together with incorporating the possibility to reuse feed together with regular domestic wastewater treatment systems (Timmons & Ebling, 2007). Microbial biomass is developed on fish excreta resulting in evacuation of these undesirable components from the water. The major driving force is the intensive growth of heterotrophic bacteria (De Schryver *et al.,* 2008).

Biofloc can acclimatize the nitrogen wastes and recycle it into microbial protein, where the last made by means of floccules of bacteria that attracts other organisms as micro/macro invertebrates, filamentous organisms fungi, ciliates, flagellates, rotifers, nematodes, metazoans and detritus which known as flocs. Bacterial flocs contribute to diminishing the requirements for artificial food for fish species such as tilapia, carp and shrimp where, it's considered as optional feed source, according to its composition and nutritional value (Wasielesky *et al.,* 2006; Emerencia*no et al.,* 2012). Biofloc combines the expulsions of nutrients from the water with encourage growth of microbial biomass (heterotrophic bacteria) which consume ammonia for growth leading to diminishing the pond water exchange. Subsequently, few investigations have examined different feeding management under biofloc system (Sharma et al., 2015; Lara et al., 2017). However, there are several advantages of a biofloc system for aquaculture. Nevertheless, there are some practical disadvantages of implementing a BFT system to culture fish includes the additional requirement of organic carbon delivery to maintain a C:N ratio above 10 and relatively high energy costs associated with intense mixing and aeration to prevent active bioflocs from settling out of suspension and to meet the additional biological oxygen demand (BOD) caused by elevated microbial respiration. Excessive suspended solid concentration in the rearing environment can also clog the gills of fish, resulting in growth and welfare depression (Luo *et al.,* 2014). Moreover, the most obvious disadvantage is the need for high oxygenation and hence high energy cost in order to keep the fish as well as the microbiotas in optimal condition, any prolonged power failure in the scale of minutes is highly lethal to the biofloc system. Besides; a biofloc system is slow to develop as it may take more than 4 weeks for the nitrifying bacterial community to establish (Thong & Yong, 2014). Hargreaves & John (2013) stated that increased energy requirement for mixing and aeration as well as reduced response time because water respiration rates are elevated.

The current experiment was conducted to assess the effects of biofloc technology in indoor tanks for Nile tilapia (*Oreochromis niloticus*); on the growth performance, digestive enzyme activity, hematology, immune response, intestinal morphometry and chemical composition of Nile tilapia and flocs. In addition, to distinguish the best feeding; through utilizing different feeding rate 0.5%, 1% and 1.5% rate; with zero water change (biofloc technique; BFT) and their impact on aquatic animal rearing.

MATERIALS AND METHODS

Ethical Approval

All handlings of fish were directed according to the guidelines for animal care and use for scientific purposes built up by the Ethics Committee of the Faculty of Agriculture, Kafrelsheikh University, Egypt (Approval Date: 18-03-2018).

Fish and experimental conditions

The experiment was performed using 900 mono-sex male Nile tilapia (*O. niloticus*) fish weighing an average of 53.45±1.49 g. They were obtained from National institute of Oceanography and fisheries (NIOF), El-Serw Research farm, Damietta Governorate, Egypt. All collected fish were accommodated in three fiberglass tanks for two weeks at the laboratory of El-Serw Research farm, Damietta Governorate. During the accommodation period, fish were fed a commercial diet (25% crude protein, Skretting Company; Egypt) at a rate of 3% of biomass, which provided twice/day of equal rations at 09:00 am and 3:00 pm to adapt the artificial diet and conditions of the trial.

After the accommodation period, randomly designed 3×1 factorial treatments were applied. The fish were randomly divided into 3 groups (treatments) of 300 fish / each group allotted into three replicates of 100 fish / each replicate. Fish were distributed into the experimental fiberglass tanks (2 m^3 in size for each) contained 2000 L of water, (100 fish/ fiberglass tanks) and were equipped with effective aeration system. Treatments are based on different feeding ratio of 0.5, 1.0, and 1.5% of fish body weight under zero water exchange; referring to T1, T2 and T3, respectively.

The experimental diets was admitted to fish twice daily at (09:00 am and 3:00 pm) for 90 days. Fish were weighed at fortnightly intervals along the experimental period and the feed amounts were adjusted by the change in live body weight. Aeration was continuously provided using an air blower (model 2BH7-520-0AH-8) made in Germany.

Assessment of water quality parameters

Water temperature and dissolved oxygen were recorded daily at one o'clock utilizing thermometer and dissolved oxygen meter (HI 9146-HANNA interment, USA). The pH values were recorded twice a week (Orion pH meter, Abilene, Texas, USA). Ammonia, nitrite, and nitrate were measured bi-weekly according to APHA (1998).

Biofloc precipitation

Subsequent to setting the fish in the tanks, providing the feed and calculating the amount of remaining ammonia, the molasses were added as a source of carbon to control the proportion among carbon and nitrogen. The amount of molasses required was determined according to De Schryver *et al.*, 2008. Using the Imhoff cone, the volume of floc on the bottom of the cone was estimated after 15 minutes of sedimentation three times weekly (Avnimelech, 2009). Suspended material was precipitated twice when floc volume reached to 20 ml L^{-1}. Naylon net 55 µ mesh size, 25 cm diameter and 80 cm length was utilized for filtering the suspended material. Thin layers of collected biofloc were exposed directly to the sunlight to diminish the moisture.

Growth indices

Random fish samples representing the whole tank (around half of the tank) were totally weighed (50 fish/each replicate) using an electronic balance.

Final body weight (FBW), weight gain (WG), relative growth rate (RGR), specific growth rate (SGR, % day^{-1}), feed conversion ratio (FCR), and protein efficiency ratio (PER) were calculated using the following equations:

WG = Final body weight (g) - Initial body weight (g)(Annet, 1985)

RGR = 100 ×(Final body weight (g) - Initial body weight (g))/ Initial body weight (g)

Specific growth rate (SGR % / day) = SGR= 100 (Ln FBW-Ln IBW)/ T

(Pouomonge & Mbonglang, 1993)

FCR = feed intake (g) /weight gain (g) (De Silva & Anderson, 1995)

PER = weight gain (g) /protein intake (g)(De Silva & Anderson, 1995).

Chemical composition of fish and biofloc

Dry matter, crude protein, crude lipid and ash contents of the fish (five fish/each tank), the experimental diets and bifloc were all performed according to AOAC (1990). Fish samples were dried in an oven at 80 ºC till steady weight than were grounded and stored at -20ºC for subsequent analysis; while, precipitate flocs were solar dried. Ash determined by incineration at 550 ºC for 4-6 h (Azim & Little, 2008). Crude protein was estimated by the micro-Kjeldahl method, %N× 6.25 (utilizing

Kjeltech auto analyzer, Model 1030, Tecator, Höganäs, Sweden) and crude fat were additionally estimated by Soxhlet extraction with diethyl ether (40 - 60 ºC).

Blood sampling and serum separation

At the end of the experiment, all fish were anesthetized using 150 mg/l MS222 (Argent Laboratories, Redmond, Washington). Blood samples were gathered from the caudal blood vessels (v. caudalis) from 12 randomly sampled fish from each group (4 fish/each replicate) using a sterile syringe (Urbinate & Carneiro, 2006). Each sample was divided into two portions; the first portion was transferred into a 2-mL sterile test tube with anticoagulant (10% ethylene diamine tetra acetate-EDTA) for haematological assay and the second portion was kept in a 2-mL plain Eppendorff tube for serum separation. Blood was left to clot at 4°C for 6 min. After that, tubes were centrifuged at 704 g/ 10 min using an Eppendorff centrifuge for serum separation. The serum was collected in Eppendorff tubes and stored at -40 °C until analyses.

Haematological analysis

Red blood cells (RBCs) and WBCs were counted immediately with a hemocytometer after dilution with Natt& Herrick's solution (Houston, 1990). For hematocrit (Hct) determination capillary tubes were filled with blood and spun in a hematocrit centrifuge at 12,000 g for 5 min and hematocrit values were read as percentage (Karimi *et al.,* 2013). For haemoglobin assay Drabkin's solution was added to blood and then solution was centrifuged (3500 g for 6 min) to remove interferents, afterwards blood haemoglobin concentration was determined with a spectrophotometer (Model RA 1000, Technicon Corporation, USA) at 540 nm using the method of Blaxhall & Daisley (1973).

The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated (Houston 1990) according to the following formulas:

MCV (fl) = 10 × (PCV per RBC)

MCH (pg) = 10 × (Hb per RBC)

MCHV (%) = 100 × (Hb per PCV)

Biochemical analysis

Serum total proteins (biodiagnostic, Egypt cat No TP20 20) was estimated colorimetricaly at wave length 550) according to Doymas *et al.,* (1981). Serum albumin (Diamond, Egypt) was estimated colorimetricly at wave length 550 nm according to Dumas & Biggs (1972). Activities of aspartate amninotransferase (AST) (biodiagnostic, Egypt cat No AS 1061 (45)) and alanine aminotransferase (ALT)(biodiagnostic, Egypt cat No AL 1031(45)) were determined calorimetrically at the wave length 540 nm, according to Reitman & Frankel (1957). Glucose level (mg/100 ml) was determined using glucose enzymatic PAP kits obtained from Bio-Merieux (France) (Trinder, 1969).Serum creatinine (biodiagnostic, Egypt cat No Cr 1251) was colorimetrically determined according to Heinegard & Tiderstrom (1973). Cholesterol (Cholesterol colorimetric assay kit CHOD-PAP method Elabscience, USA), triglyceride (T.G colorimetric assay kit GPO-PAP method Elabscience, USA) and uric acid (colorimetric assay kit uricase-POD, Spinreact, Spain at wave length 520)were determined colorimetrically according to the manufacturer's instructions using the commercial kits purchased from the Laboratory Biodiagnostics Company (Cairo, Egypt).

Intestinal Morphometry

Five fish were randomly selected from each treatment. After deep anaesthesia using 40% ethyl alcohol, the belly was dissected and specimens from anterior (hepatic loop) of the intestine were sampled. The tissue samples were fixed in Bouin's solution for 18-24 hours. After fixation, the samples were dehydrated by using ascending concentrations of ethyl alcohol (70% to absolute alcohol) then cleared in xylene and prepared for histological investigations. Sections of 4-5 µm thickness were stained with hematoxylin and eosin for morphometric analysis according to Bancroft & Gamble (2007). The length, width of intestinal villi and crypt depth in addition to goblet cells count was measured by using image analysis software (NIH, Bethesda, MD). A total of six random villi and villus-associated crypts from 5 intestinal cross-sections were selected from each and the average was calculated (±SE)

Statistical analysis

All data are presented as means± standard error (SE). Growth, hematology, blood chemistry and hormones data were analyzed using one-way ANOVA, followed by Duncan's multiple range tests (Duncan, 1955) which was used to compare differences among individual means, with statistical software SAS ANOVA procedure (statistical analysis system, 2006). A probability of 0.05 was utilized to account for the statistical difference between the means. Before the

analysis, percentage data were normalized by arc-sine-transformation.

RESULTS

Water quality parameters

Water quality parameters; pH, temperature, dissolved oxygen, ammonia, nitrite and nitrate was estimated and summarized in table 1. The 1.5% feeding rate group showed the highest values in temperature, ammonia and nitrate values. However, the 1.0% feeding rate group showed the highest values in dissolved oxygen and nitrite values. On the other side, the 0.5% feeding rate group showed the highest values in pH value.

The chemical composition of biofloc

As demonstrated in table2,the highest values of protein of biofloc were recorded in treatment with a feeding rate of 1.5%. Feeding rate 0.5 % treatment has the highest carbohydrates and ash of biofloc content. In general, fat content of biofloc was low in different feeding rate groups except for 1.0 % feeding rate.

Table 1: Physicochemical parameters of rearing water of biofloc technique at different feeding rates

	pH	Temperature	O_2	NH_4	NO_2	NO_3
0.5	7.66±0.21	26.16±1.69	6.08±0.65	0.344±0.14	0.264±0.15	0.280±0.20
1.0	7.58±0.21	26.15±1.70	6.10±0.88	0.331±0.16	0.281±0.17	0.295±0.17
1.5	7.57±0.22	26.21±1.68	5.93±0.38	0.396±0.15	0.267±0.19	0.437±0.31

Table 2: Chemical composition of biofloc at different feeding rates on dry matter base

Feeding levels (% of biomass)	Chemical composition (%)			
	Crude lipid	Crud protein	Ash	Carbohydrate
0.5	3.03±0.03^{b}	10.87±0.32^{b}	13.70±0.87^{a}	72.38±0.80^{a}
1.0	10.20±0.12^{a}	12.87±1.59^{ab}	6.10±0.23^{b}	70.83±1.62^{ab}
1.5	3.90±0.52^{b}	16.03±0.26^{a}	12.00±0.40^{a}	68.06±0.68^{b}

* Data shown are Means (±SD) in each row; different superscript letters indicate significant difference ($P \leq 0.05$)

Floc volume

The impact of different feeding rate treatments on floc volume was shown in fig.1. Floc volume was increased with the increase of the feeding rate of tilapia. The highest floc volume was recorded in1.5 % feeding rate treatment while, the lowest floc volume was recorded in 0.5% feeding rate treatment.

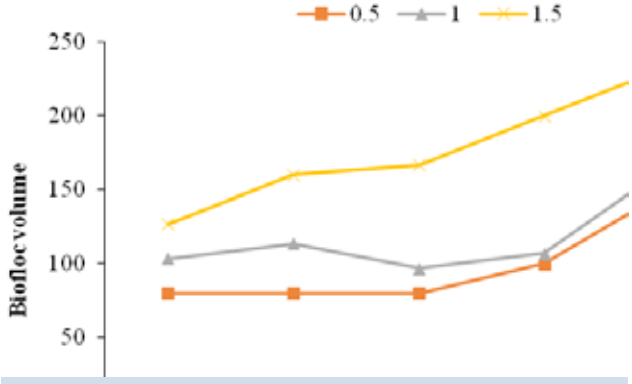

Fig. 1: Biofloc volume in different treatments throughout the experimental period

Tilapia performance

As shown in table 3, under biofloc condition, fish fed at feeding rate 1.5% of fish body weight recorded the highest significant values of growth performance parameters (FW, TWG, ADG, RGR, and SGR) and FI. However, fish fed at feeding rate of 0.5% under biofloc condition showed the best significant food conversion rate values ($P \leq 0.05$). However, no significant differences ($P \geq 0.05$) were recorded to survival rate among different treatments.

Chemical composition of tilapia fish

Proximate chemical analysis of the whole fish body is well demonstrated in table 4. Highest significant ($P < 0.05$) dry matter, fat, and energy contents were recorded in fish group fed at 1.5% feeding rate under biofloc condition. Whereas, the highest significant ($P < 0.05$) crude protein content in the fish body were recorded in 1.0% feeding rate fish group.

Hematological parameters:

Results of haematological analysis are summarized in table 5. The highest feeding rate (1.5%) fish group reflected the highest RBCs and WBCs counts, Hb concentrations, MCH and HCT %; however, 1.0 % feeding rate fish group reported the highest MCHC (%).

Blood serum biochemical parameters:

Blood serum biochemical parameters were summarized in table 6. The 1.5% feeding rate group showed the highest values in TCH, GLU and AST values; whereas, 1.0% feeding rate group showed the highest values in total protein, albumin and uric acid values. On the other side, 0.5% feeding rate group recorded the highest values in creatinine and ALT values.

Table 3: Growth performance and feed efficiency of Nile tilapia under biofloc technique at different feeding rates throughout the experimental period

Parameters	Feeding levels (% of biomass)		
	0.5	1.0	1.5
Final weight (FW, g/fish)	130.2±3.19^{b}	133.3±0.88^{b}	157.7±2.33^{a}
Weight gain (TWG, g/fish)	75.50±3.18^{b}	80.33±1.20^{b}	104.0±1.53^{a}
Average daily gain (ADG, g/fish/day)	0.84±0.04^{b}	0.89±0.01^{b}	1.16±0.02^{a}
Relative growth rate (RGR, %)	138.1±5.83^{c}	151.6±3.63^{b}	193.8±1.57^{a}
Specific growth rate (SGR, %/day)	0.96±0.03^{c}	1.02±0.02^{b}	1.20±0.01^{a}
Condition factor	2.68±0.09^{c}	2.96±0.03^{b}	3.26±0.11^{a}
Intestine length	157.5±4.33^{a}	128.5±0.87^{b}	108.0±3.46^{c}
Villi length	342.2±10.92^{a}	217.5±13.19^{b}	132.3±7.9^{3c}
Villi width	54.88±4.18^{a}	51.31±2.52^{a}	38.77±2.01^{b}
Crypt depth	17.13±1.46^{b}	25.05±3.45^{a}	14.19±0.95^{b}
Goblet cells /mm2	35.80±0.57^{a}	25.90±0.52^{b}	22.70±0.51^{c}
Intestine length:bodylength	7.74±0.43^{a}	6.57±0.23^{b}	5.11±0.04^{c}
HIS	2.32±0.17^{b}	3.50±0.65^{ab}	4.45±0.65^{a}
Feed intake (g/fish)	36.28±0.45^{d}	73.64±0.90^{c}	128.8±1.36^{a}
Feed conversion ratio	0.48±0.05^{d}	0.92±0.04^{c}	1.24±0.01^{b}
Feed efficiency (%)	207.9±5.14^{a}	109.0±4.42^{b}	80.74±1.17^{c}
Survival (%)	97.67±0.33	96.00±0.58	94.67±0.33

Data shown are Means (±SD) in each row; different superscript letters indicate significant difference (P ≤0.05).

Table 4: Chemical composition of Nile tilapia under biofloc technique at different feeding rates throughout the experimental period

Feeding rate %	Chemical composition of whole-body fish (% dry matter basis)				
	DM¶	Ash	Protein	Lipid	EC (kcal/100g)
0.5	27.72±0.28^{d}	21.59 ±0.65^{a}	64.90 ±0.33^{b}	13.51 ±0.56^{c}	493.5 ±5.59^{c}
1.0	28.90 ±0.17^{c}	17.22 ±0.35^{b}	68.65 ±0.46^{a}	14.13 ±0.80^{c}	520.6 ±5.00^{b}
1.5	31.30 ±0.21^{a}	15.57 ±0.34^{c}	64.40 ±0.67^{b}	20.02 ±0.41^{a}	552.2 ±1.61^{a}

Data shown are Means (±SD) in each column; different superscript letters indicate significant difference (P ≤0.05).
¶**DM** = dry matter

Table 5: Hematological parameters of Nile tilapia under biofloc technique at different feeding rates throughout the experimental period

Parameters	Feeding rates %		
	0.5	1.0	1.5
RBCs (×10^{6} mm^{-3})	1.57 ±0.18^{b}	1.53±0.18^{b}	1.80±0.06^{a}
Hb (g/dL)	5.55 ±0.26^{b}	5.15±0.20^{b}	7.43±0.41^{a}
Hct (%)	22.93±1.41^{b}	17.13±0.32^{c}	34.50±2.02^{a}
MCV (μ^{3})	146.7±5.46^{b}	143.8±0.72^{b}	152.0±2.40^{ab}
MCH (pg)	38.33±1.73^{b}	41.30±0.29^{ab}	43.37±1.88^{a}
MCHC (%)	25.50±0.29^{ab}	30.00±0.58^{a}	24.33±1.86^{b}
WBCs (×10^{3} mm^{-3})	24.30±1.15^{b}	23.30±1.41^{b}	36.67±1.66^{a}

Data shown are Means (±SD) in each row; different superscript letters indicate significant difference (P ≤0.05).
RBCs: Red blood cells
Hb: Hemoglobin
PCT: Hematocrit
MCV: Mean corpuscular volume
MCH: Mean corpuscular hemoglobin
MCHC: Mean corpuscular hemoglobin concentration
WBCs: White blood cells

Table 6: Serum biochemical parameters of Nile tilapia under biofloc technique at different feeding rates throughout the experimental period

Parameters	Feeding rates %		
	0.5	1.0	1.5
TCH	38.50±0.29^{c}	41.00±1.73^{c}	72.50±3.18^{a}
TG / Mg/dl	257.5±19.34	231.0±10.97	259.0±18.56
Total protein, g/dl	2.60±0.12^{ab}	3.43±0.48^{a}	2.77±0.03^{ab}
Albumin, g/l	0.80±0.06	0.90±0.06	0.83±0.03
GLU/ Mg/dl	70.00±4.04^{d}	86.00±1.15^{c}	148.0±4.04^{a}
Creatinine, mg/dl	0.33±0.07^{b}	0.30±0.00^{b}	0.30±0.06^{b}
ALT, u/l	16.00±1.53	13.67±2.19	13.00±0.00
AST, u/l	125.0±2.89	133.0±0.58	150.0±15.01
Uric acid, mg/dl	1.87±0.26^{b}	5.27±0.09^{a}	1.20±0.00^{c}

Data shown are Means (±SD) in each row; different superscript letters indicate significant difference (P ≤0.05).
TCH: Total cholesterol
TG: Triglyceride
GLU: Glucose
AST: aspartate aminotransferase
ALT: aspartate aminotransferase

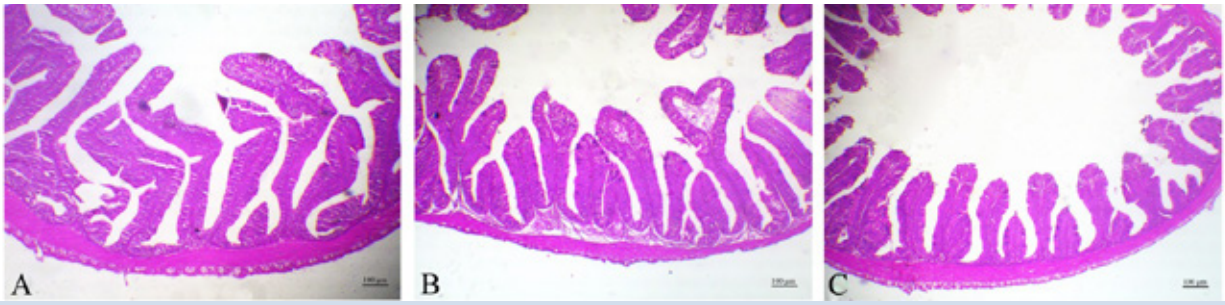

Fig. 2: photomicrograph of anterior part of small intestine of *Oreochromis niloticus* showing:A: intestinal villi of 0.5% feeding rate treated group, B: intestinal villi of 1.0% feeding rate treated group and C: intestinal villi of 1.5% feeding rate treated group. H&E, Bar=100µm

Morphometric analysis

The morphometric analysis of anterior part of intestine revealed significant increase in the intestinal villi length and width besides increase in goblet cells count in 0.5% group compared with the other groups. On contrast to the previous findings, the crypt depth of 0.5% group was significantly decreased compared with 1% group (table 1).

The results of the morphological analysis are summarized in Figure 2 and table 3. The total length of the intestine was significantly increased with decreased feeding rate level; the highest intestinal length was reported in 0.5% feeding rate fish group and the lowest intestinal length was in 1.5 % feeding rate fish group. In addition, the height of the intestinal villi in the anterior part of the intestine was significantly increased with decreased feeding rate. The treatment of 1.5% feeding rate showed the least intestinal villi length. However, the treatment of 1% feeding rate showed moderate increase in the villi length. On the other side, there was marked increase in the intestinal villi length in the treatment of 0.5% feeding rate.

DISCUSSION

Biofloc is clustered aggregations of microbial communities like phytoplankton, bacteria, and particulate organic matter. Biofloc Technology (BFT) is a new potentially revolutionary technology that is particularly productive for tilapia culture (Prajith, 2011). Under favorable economies, tilapia production using biofloc technology represents an attractive investment proposition. BFT is an environmental-friendly technique in aquaculture that controls both water quality and pathogens besides, providing microbial protein feed for the aquatic farm; so, reducing feed costs and feed conversion ratio (De Schryver *et al.,* 2008 and Abdelhamid, 2009a& b, 2019a, b & c and Kourie, 2018a). BFT leads to ammonia removal from water. The bio-flocs technology has received considerable attention because it results in low cost, high production yields, feed protein recycling, water quality, and bacterial infection control (Avnimelech, 2006; Crab

et al., 2007; Little *et al.,* 2008).

Average measured values of water parameters in the current experiment were within the optimal range suitable for tilapia culture (Boyd & Tucker, 1998, El-Sayed, 2006; & Delong *et al*., 2009). Moreover, differences in ammonia, nitrite and nitrate concentrations seem to be typical features of biofloc systems (Azim & Little, 2008 & Luo *et al.,* 2014). Nitrite and nitrate are created through nitrification in the BFT system; nitrate may experience inadequate denitrification to deliver nitrite and dissimilatory nitrate reduction to ammonia may occur (Azim *et al.,* 2008; & Wu *et al.,* 2012). Nitrite accumulation might be due to free ammonia inhibition during nitrification and denitrification (Shi *et al.,* 2011).

It was suggested that 25-30% crude protein in diets is appropriate for tilapia growth (Chou & Shiau, 1996, Jauncey, 2000). The crude lipid content was sufficient according to thedietary lipid requirement of 5-12% for tilapia (Lim *et al.,* 2009). Floc volume was increased with the increase of the feeding rate of tilapia and settled to the bottom of the tanks. This means that uptake of biofloc by the fish was insufficient to prevent its build-up and the need to remove it regularly from the system was clear. Therefore, characterization of floc and improved approaches to its removal are a pre-requisite for effective management of BFT system. Protein is increased positively with increased feeding rate, while lipid and carbohydrates negatively decreased. The results coincided with previous reports (Azim & Little, 2008; Azim*et al.,* 2008; Crab *et al.,* 2010) but disagreed with Emerenciano *et al.* (2012).

Growth performance and feed utilization efficiency were significantly improved by different feeding rates, implying a potential role of Biofloc system in mitigating stress factors and promoting fish welfare. Biofloc system substantially contributes to tilapia growth and production where wastes turned over into natural food particles such as suspended bacteria (Avnimelech, 2007; Azim & Little, 2008; Beveridge & Baird, 2000; Little *et al.,* 2008; Yuan *et al.,* 2010). The best significant food conversion rate was observed in 0.5% feeding rate where decreased amount of feed was necessary for producing one unit of fish leading consequently to production cost reduction. The results are similar to Avnimelech *et al.,* 1994 and Luo *et al.,* 2014.

In the current study, there is a negative relationship between crude protein and fat contents in the fish body. According to Xu & Pan (2012), biofloc can influence the whole body composition of cultured shrimp with increased lipid content in the whole shrimp body. Izquierdo *et al.* (2006) also found that the whole body lipid content of shrimp growing in mesocosms systems with biofloc exhibited an increasing trend. This might be due to the essential amino acids, fatty acids (PUFA and HUFA) and other nutritional elements provided by the biofloc in the BFT treatment (Izquierdo *et al.,* 2006; Ju *et al.,* 2008b).

Hematological parameters reflect the health status of fish (Harikrishnan *et al.,* 2011). The overall improvement in haematological characteristics reported in 1.5% feeding rate group, used in the current study, indicate that the biofloc system had positive effect on the physical conditions of the tilapia. The results are similar to many previous records (Öz *et al.*, 2018 &2020 a, b; Xu & Pan (2013) and disagree with some reports (Azim & Little, 2008; Souza *et al.,* 2014 and Xu & Pan, 2014). This difference might be attributed to different experimental conditions and culture species

The results of the fish serum biochemical analysis in this study reflected a significant increase in serum total proteins and Albumin in 0.1% feeding rate group. The results indicate the contribution of the biofloc system in improving the immune response of Nile tilapia (Ballester et al., 2010; Long et al., 2015). Biofloc is not only a source of additional nutrition, such as proteins, lipids, minerals and vitamins (Izquierdo *et al.,* 2006; Moss *et al.,* 2006; Ju *et al.,* 2008b; & Xu *et al.,* 2012a), but also provides abundant natural microbes and bioactive compounds such as carotenoids and fat-soluble vitamins (Ju *et al.,* 2008a), and other immune-stimulatory compounds (Crab *et al.,* 2012) that may stimulate the immune response of cultured fish.

The total intestinal length and the heights of the intestinal villi were significantly increased with decreased feeding rate. The role of intestine in nutrient digestion and absorption is well-known in herbivorous fish as tilapia (Grosell *et al.,* 2010). Moreover, the intestinal villi height and the muscular layer thickness are good indicators of a healthy intestine (Khojasteh, 2012). Therefore, the increased intestinal absorptive area with a subsequent increase in nutrient absorption and retention highlight the observed improvement in growth performance, immune response and stress resistance in Nile tilapia in the current study. German and Horn (2006) confirmed the present results; they

found that intestine lengths of herbivores were longer than those of omnivores, and these were longer than those of carnivores.

CONCLUSION

Based on the current results, it could be concluded that using biofloc technique in tilapia rearing had beneficial effects on maintaining good water quality, improving the feed utilization and growth performance, increasing fish body protein content, physical and immune response promotion as well as increasing the absorptive capacity of the intestine. Subsequently, biofloc offers not only excellent benefits for fish farmers (economically) and consumers (safe product) but also, for the sustainability of the environment (treating wastes which turned over into natural feed stuff for fish saving the artificial food cost). Further research efforts should be made to improve the biofloc uptake by the fish to prevent its accumulation.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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Research article
Ερευνητικό άρθρο

Effects of Adding *Spirulina platensis* to Laying Hen Rations on Performance, Egg Quality, and Some Blood Parameters

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ABSTRACT: This study was conducted to determine the effect of adding different levels of *Spirulina platensis* (*SP*) to laying hen diets on performance, egg quality, egg yolk color and some blood parameters. Sixty 60-week-old Lohmann LSL Classic laying hens were used in the study. The experiment was conducted in 15 subgroups with 4 hens in each cage, with 5 replications in 3 treatment groups. Three different diets were offered to laying hens; one control (based to wheat) and two supplemented with different levels (1 and 2%) of *SP*. As it was observed, the addition of *SP* to laying hen diets had no significant effect on live-weight change (LWC), egg yield (EY), egg mass, egg weight (EW), feed intake (FI), and feed conversion ratio (FCR) ($p>0.05$). Concerning the egg quality characteristics that were examined, the effects of *SP* on shell strength (SS), shell ratio (SR), shell thickness (ST), shape index (SI), albumen index (AI) and serum parameters were not statistically significant ($p>0.05$); egg yolk color characteristics (Roche Color Scale and L*, a*, b*) were affected by dietary *SP* supplementation ($p<0.01$). Addition of different levels of *SP* (1% and 2%) to laying hen diets caused a significant increase in egg yellow color values compared to the control group. According to the results of the study, the addition of 1% and 2% SP to the laying hen rations had a significant positive effect on egg yolk color, but did not cause a significant change in other parameters.

Keywords: Egg quality; Laying hen; Performance; *Spirulina platensis*; Serum parameters

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INTRODUCTION

In recent years, the latest trend in the feed sector has been towards the use of natural ingredients as alternatives to antibiotics, synthetic color agent, and other chemicals. *Spirulina platensis* (*SP*), also known as blue-green algae, one of these natural ingredients, is one of the high-quality natural feed additives that can be used in the diets of ruminants and poultry. There are two species of Spirulina that are widely used worldwide: *Spirulina platensis* and *Spirulina maxima* (Oliveira et al., 1999). *Spirulina platensis*, with its nutrient profile and high protein and carotenoid content, has been used as an excellent food source for humans and animals for hundreds of years (Mostolizadeh et al., 2020; Sharmin et al., 2020) and is commonly found in Africa, Asia, and South America (Vonshak, 2002). It is rich in unsaturated and polyunsaturated fatty acids, especially that oleic acid, linoleic acid, gamma-linolenic acid, and docosahexaenoic acid (Hue et al., 2002; Yukino et al., 2005; Habib et al., 2008; Sharmin et al., 2020).

Zeweil et al. (2016) reported that *SP* consist of 85.77% dry matter, 57.66% crude protein, 1.75% crude oil, 3.6% crude cellulose, and 9.83% crude ash. At the same time, *SP* has a protein content ranging from 55% and 70% and contains all the essential amino acids (Seyidoğlu and Galip, 2013, Seyidoğlu et al., 2017). In addition, the energy content of *SP* was determined to be 2500-3290 kcal ME/kg and its phosphorus availability was 41% (Zahroojian et al., 2013). *Spirulina platensis* is also rich in thiamine, riboflavin, pyridoxine, vitamin B_{12}, vitamin C, and carotenoids. Also, *SP* is widely used as a feed additive for the improvement of the egg yolk color in laying hens and the meat quality in broilers (Ross and Dominy, 1990). It was reported in a study that the addition of 3-9% *SP* to the diet of laying hens resulted in egg yolk colors that best represented consumer preferences (Saxena et al., 1983). This effect of *SP* on egg yolk is due to its rich content of zeaxanthin, xanthophyll, and other carotenoid pigments, especially β-carotene (Takashi, 2003; Maoka, 2011). *SP* has also been reported to fortify human organism against disorders such as diabetes and rheumatism (Parikh et al., 2001; Rasool et al., 2006). It was also stated in literature that *SP* has immune system-stimulating effects and antiviral activity (Khan et al., 2005).

Spirulina platensis has been reported to improve the health status and survival ability in poultry and enhance immune function (Qureshi et al., 1996; Kanagaraju and Omprakash, 2016). In this context, some researchers reported that the poultry fed with diets containing *SP* gained superior reproductive performance compared to the control group (Ross and Dominy, 1990; Nikodémusz et al., 2010; Mariey et al., 2012; Samia et al., 2018). It has been shown that *SP* is an effective way of improving the quality of product obtained from poultry to meet consumer preferences. For example, the total cholesterol content of eggs can be reduced by the addition of *SP* to hens diets (Sujatha and Narahari, 2011). This can mainly be attributed to the high antioxidant and omega-3 polyunsaturated fatty acid content of *SP*, which enhance the nutritional value of eggs (Rajesha et al., 2011; Sujatha and Narahari, 2011).

In another study, it was reported that the addition of *SP* to the diet increased egg yield from 87% to 96% (Ross and Dominy, 1990). Raju et al. (2005) found that the addition of *SP* by 0.05% to the diet could partially offset the negative effect of 300-ppm aflatoxin on the growth rate and lymphoid organ weight of broilers. Islam et al. (2009) reported that *SP* could help in reducing the arsenic accumulation in the tissues of ducks. Selim et al. (2018) stated that *SP* dietary supplementation at the level of 0.3% could be suggested for laying hens, and the egg yield increased in the 0.3% *SP* added group compared to the other groups. Considering the age of 60-weeks-old laying hen, the hypothesis that *SP* has no effect on laying hen of this age is the original aspect of this study. The purpose of this study was to determine the effect of adding *Spirulina platensis,* which was obtained from a commercial company, to the laying hen diets on performance, egg internal and external quality characteristics, and some blood parameters.

MATERIALS AND METHODS

In this study, sixty 60-week-old Lohmann LSL Classic laying hens were used as animal material. The average live weight of the hens was 1652.8 g. A trial was conducted with three treatment groups (5 replications of 20 hens per treatment). In study, three different diets were offered one of which was the control (without SP addition) and 2 that were supplemented with different levels (1% and 2%) of *Spirulina platensis*.

The experimental diets were prepared based on wheat-soybean meal and the nutrient needs of laying hens were formulated according to recommendations of NRC (1994). The study lasted 28 days, the feed

and water were provided to the animals *ad libitum* throughout the study, and 16 hours of lighting was applied. The composition of the experimental diets used in the study are shown in the Table 1.

Table 1. The composition of experimental diets

Ingredients	Dietary Spirulina levels, %		
	0	1	2
Wheat	60.00	63.70	62.50
Soybean meal (43.8% CP)	17.00	15.00	15.00
Sunflower seed meal (32% CP)	8.50	8.00	6.70
Spirulina (65% CP)	0.00	1.00	2.00
Vegetable oil (8800 Kcal ME/kg)	3.20	2.00	2.5
Limestone	9.10	9.10	9.10
Dicalcium phosphate, DCP	1.50	1.50	1.50
Salt	0.25	0.25	0.25
Premix	0.25	0.25	0.25
Methionine	0.20	0.20	0.20
Calculated nutrient composition			
Metabolizable energy, ME (Kcal/kg)	2770	2753	2767
Crude Protein, CP (%)	16.64	16.66	16.70
Calcium (%)	3.89	3.89	3.88
Available phosphorus (%)	0.40	0.40	0.41
L- Lysine (%)	0.76	0.75	0.76
DL-Methionine (%)	0.44	0.44	0.45

Premix (in 1 kg of the ration): vitamin A, 8.800 IU; vitamin D3, 2.200 IU; vitamin E, 11 mg; nicotinic acid, 44 mg; Cal-DPantothenate, 8.8 mg; riboflavin 4.4 mg; tiamine 2.5 mg; vitamin B12, 6.6 mg; folic acid, 1 mg; D-Biotin, 0.11 mg; colin, 220 mg; manganese, 80 mg; copper, 5 mg; iron, 60 mg; zinc, 60 mg; cobalt, 0.20 mg; iodine, 1 mg; selenium, 0.15 mg

The live weights of the animals were determined at the beginning and end of the trial. The feed intake (FI) and egg weights (EW) were measured in biweekly and the egg production was recorded daily. Using the data of egg yield (EY) and egg weight (EW), the egg mass was calculated by the following formula: Egg mass (EM) = (egg yield x egg weight)/period (day). The feed conversion ratio (FCR; feed g/egg g) was calculated by the following formula: FCR = FI (g feed/hen/period) / EM (g egg/hen/period).

The egg shell breaking strength, egg shell thickness, egg shell weight, egg shape index, yolk and albumen index, and egg yolk color criteria (Roche scale and CIELAB as L*, a*, b*) were measured in the eggs collected in the last two days of the trial. The yolk and albumen heights were determined using a digital height gauge, and yolk diameter and albumen length and diameter were determined using a digital caliper.

The following formulas were used to calculate the yolk index and albumen index: Yolk index (%) = (yolk height/yolk diameter) x 100 and Albumen index (%) = (albumen height / (albumen length + albumen width) x 100. The egg quality analyses were completed within 24 hours after the eggs were collected. The shell membrane weight (%) was calculated using the following formula: Eggshell weight (g)/egg weight x 100. The eggshell strength was measured by applying the assisted system pressure to the blunt part of the egg (Egg Force Reader, Orka Food Technology, Israel). The shell membrane thickness was calculated by averaging the values measured from three points on the eggs (2 from the equatorial region, 1 from the blunt and pointed part) using a micrometer (Mitutoyo, 0.01 mm, Japan). A colorimeter was used for the assessment of yolk color (Minolta Chroma Meter CR 400 (Minolta Co., Osaka, Japan) (Romero et al., 2002). Egg yolk color was measured with Roche color scale.

At the end of the trial, 10 hens randomly selected from each group (2 hens from each replicate) a were slaughtered and approximately 5 ml of blood was collected into the vacuum tubes with clotting activators and delivered to the laboratory by the cold chain. The serum tubes brought to the laboratory were centri-

fuged at 2700 x g for 10 minutes and then their serum were collected and put into the Eppendorf tubes. Serum cholesterol, HDL, albumin, total protein, phosphorus, calcium, uric acid, and globulin analyses were carried out using the commercial kits in the autoanalyzer device (DDS ® Spectrophotometric Kits, Diasis Diagnostic Systems Co., Istanbul, Turkey).

Data were subjected to one-way ANOVA using Minitab (2000). Duncan's multiple range tests were applied to detect diffirences among means (Duncan 1955). Statements of statistical significance are based on a probability of $p < 0.05$.

RESULTS

The effects of the addition of 1% and 2% *SP* to the laying hen rations on LWC, EY, FC, EW, EM, and FCR are presented in the Table 2. It was observed that the addition of *SP* to laying hen rations had no significant effect on LWC, EY, EM, EW, FC, and FCR ($p>0.05$).

Table 2. Effects of the rations containing different levels (1% and 2%) of *Spirulina platensis* on performance parameters of laying hens.

Parameters	Treatments			SE	*P*-Value
	Control	SPR1	SPR2		
ILW, g	1629.5	1654.0	1674.9	30.8	0.59
FLW, g	1594.9	1652.9	1670.2	38.5	0.38
LWC, g	-34.7	-1.1	-4.7	51.4	0.41
EY, %	89.1	93.8	93.1	3.4	0.60
FI, g/hen/day	120.3	116.9	118.6	1.8	0.46
EW, g	63.8	65.2	66.5	1.4	0.45
EM, g/hen/day	56.9	61.2	61.7	2.4	0.34
FCR, FC/EM	2.1	1.9	1.9	0.1	0.31

ILW: Initial live weight, FLW: Final live weight, LWC: Live weight change, EY: Egg yield, EW: Egg weight, EM: Egg mass, FI: Feed intake, FCR: Feed conversion ratio, SE: Standard error. SPR1: 1% *Spirulina platensis,* SPR2: 2% *Spirulina platensis*.

The effects of the addition of *SP* (1% and 2%) to the laying hen rations on the shell strength, shell ratio, shell thickness, shape index, albumen index, yolk index, and yolk color values (L, a, b) are shown in the Table 3. When we compared different doses of *SP* with the control group, it was found that its effect on yolk color values (L, a, b) was statistically significant ($p<0.01$). The effect of *SP* addition on yolk index was also significant ($p<0.05$).

Table 3. The effects of the rations containing different levels (1% and 2%) of *Spirulina platensis* on egg quality parameters of laying hens

Parameters	Treatments			SE	*P*-Value
	Control	SPR1	SPR2		
SS, kg	3.88	3.79	3.86	0.20	0.95
SR, %	9.32	9.59	9.28	0.14	0.31
ST, mm	0.36	0.38	0.37	0.00	0.22
SI, %	72.17	73.64	73.64	0.89	0.44
AI, %	8.34	8.76	8.12	0.25	0.23
YI, %	44.74^{a}	42.21^{b}	44.51^{a}	0.70	0.05
Color Characteristics					
Roche	2.35^{C}	9.20^{B}	11.20^{A}	0.23	<0.001
CIELAB					
L^{*}	54.24^{A}	51.44^{B}	49.87^{B}	0.66	<0.001
a^{*}	-5.84^{C}	1.34^{B}	4.92^{A}	0.27	0.002
b^{*}	20.24^{B}	34.50^{A}	35.68^{A}	1.18	<0.001

SS: Shell strength, SR: Shell ratio (% of egg weight), ST: Shell thickness, SI: Shape Index, AI: Albumen index, YI: Yolk index, SE: Standard error. SPR1: 1% *Spirulina platensis,* SPR2: 2% *Spirulina platensis*.

^{A,B,C} Differences shown with different letters in the same row are statistically significant ($p<0.01$); ^{a,b}:$p<0.05$.

The effects of the addition of 1% and 2% of *SP* to the laying hen rations on the serum cholesterol, HDL, albumin, globulin, total protein, uric acid, calcium, and phosphorus parameters are given in the Table 4. The effect of *SP* addition on serum parameters was not found statistically significant (p>0.05).

Table 4. Effects of the rations containing different levels of *Spirulina platensis* on serum parameters of laying hens

Parameters	Treatments			SE	*P*-Value
	Control	SPR1	SPR2		
Cholesterol,mg/dl	132.40	146.40	128.00	9.56	0.39
HDL, mg/dl	19.00	18.60	20.20	1.21	0.63
Albumin, g/dl	1.76	1.92	1.80	0.05	0.11
Globulin, g/dl	4.68	5.78	4.66	0.34	0.06
Total Protein, g/dl	6.44	7.70	6.46	0.37	0.06
Uric Acid, mg/dl	5.90	5.70	6.10	0.56	0.88
Calcium, mg/dl	30.60	30.04	30.54	1.00	0.91
Phosphorus, mg/dl	5.62	5.14	5.00	0.50	0.66

SE: Standard error. SPR1: 1% *Spirulina platensis,* SPR2: 2% *Spirulina platensis*

DISCUSSION

Performance parameters

According to the Table 2, the effect of 1% and 2% *SP* addition to the laying hen rations on the performance parameters was not significant (p>0.05).

In a study, the high dose of *SP* (15%) reduced the albumin content in the egg, causing a decrease in EW (Blum et al., 1975). Takashi, (2003) and Nikodemusz et al. (2010) reported that the production performances of the laying hens fed on the rations containing *SP* were positively affected. Another study reported that the addition of 10% or above *SP* to ration could suppress the growth of poultry (Ross and Dominy, 1990). In contrast, some researchers reported that *SP* positively improved the LWC and FCR of hens compared to the control group (Kharde et al., 2012; Shanmugapriya and Saravana Babu, 2014).

Mariey et al. (2012) found that 0.2% addition of *SP* to the laying hen rations had a statistically positive effect on EY, EW, EM, and FCR (p<0.05). Furthermore, Bellof and Alarcon (2013) observed that the addition of *SP* under the conditions of organic farming significantly improved the growth and carcass performances of broilers. Bonos et al. (2016) found that the addition of *SP* to the broiler rations did not cause a statistically significant difference in terms of LWC, FCR, and mortality compared to the control group. Similarly, Zeweil et al. (2016) stated that *SP* addition did not affect the LWC of broilers under temperature stress. In contrast to our findings in the trial, Michalak et al. (2020) emphasized that the effect of *SP* addition on FCR was significant.

However, Halle et al. (2009) observed a decrease in the feed intake of hens; however, the ration with higher amounts of algae did not affect the egg production. In another study, it was emphasized that the addition of *SP* did not affect the FI of quails (Hayati et al., 2020). Moreover, Omri et al. (2019) found that the *SP* addition did not affect the FI of quails, which is consistent with the finding of the present study.

Furthermore, some researchers found that the hens fed with *SP* supplemented ration achieved the highest egg production rates and FCR compared to the control group (Ross et al., 1994; Nikodémusz et al., 2010; Mariey et al., 2012). The increase in the egg weight of hens fed on *SP* ration in these studies may be associated with the heavier yolks. Accordingly, the results of the present study were similar to those found by Mariey et al. (2012), Zahroojian et al. (2013), and Selim et al. (2018). The different results in the studies may be due to the differences in the breeds and age of the laying hens.

Zahroojian et al. (2013) reported that *SP* had no statistically significant effect on EY, FI, FCR, and EW (p>0.05). Moreover, Hayati et al. (2020) stated that *SP* did not affect FCR, EW, and EY. It can be suggested that the differences in these results may stem from the inability to accurately determine the *SP* doses added to the rations, the problems regarding the housing conditions of animals, different species of animals used in the studies, the feed composition, and the production systems, etc.

Egg quality characteristics

As can be observed in the Table 3, there was no

statistical difference between adding 1% and 2% of *SP* to the laying hen rations in terms of shell strength, shell weight, shell thickness, shape index, albumen index ($p>0.05$). In contrast, the effect of *SP* supplementation was significant on the yolk index ($p<0.05$) and yolk colour "L*, a*, b*" values ($p<0.01$).

Selim et al. (2018) found that the addition of different levels of *SP* to the diet did not affect SI, eggshell percentage, albumen (%), albumen index (%), yolk weight (%), and Haugh unit ($p>0.05$); whereas the yolk color score was higher in the group in which 0.3% *SP* was added to the diet.

The yolk color scores, one of the egg quality parameters, were examined and it was found that the addition of different doses of *SP* to the laying hen rations to increase the yolk color scores compared to the control group. Based on this, the highest Roche score was found at the dose of 2%. These results were similar to those found in the previous studies on yolk color scores (Mariey et al., 2012, Zahroojian et al., 2013). Algae increase the total carotenoid and antioxidant content in the egg and, as a result, reduce egg yolk cholesterol; therefore, they have some positive effects on human health (Zahroojian et al., 2013; Al-Harthi, 2014; Park et al., 2015). According to Selim et al. (2018), the increase in yolk color due to the increase in the dose of *SP* added to the laying hen rations compared to the control group most probably reflected an increase in the yolk pigmentation, enabling the accumulation of carotenoids in the egg yolk. Omri et al. (2019) stated that the *SP* addition significantly affected the L*, a*, and b* values, the color characteristics of egg yolk.

The effect of adding different doses of *SP* to the laying hen rations on the lightness (L), one of the color characteristics of egg yolk, was found to be statistically significant ($p<0.01$). The addition of different doses of *SP* to the ration reduced the lightness (L) of the egg yolk compared to the control group (54.24). No statistical difference was observed between the administered doses ($p>0.05$). The red/green value of the egg yolk (a) ranged between -5.84 and 4.92 in the study ($p<0.01$).

Egg selection by consumers is based not only on the cholesterol content of egg yolks or fatty acid profiles but also on the color of egg yolks (Englmairova et al., 2013). The degree of pigmentation required for the eggs varies depending on the geographical regions of countries, and the golden yellow colors are generally considered more attractive (Baiao et al., 1999). The yolk color intensity varies depending on the amount of carotenoid content found in the ration. Laying hens are unable to synthesize carotenoids; so, they have to absorb them from the rations (Park et al., 2015).

Algae, plants, fungi, and some bacteria synthesize carotenoids. In this study, the yolk color intensity was achieved by adding *SP* to the laying hen rations. A researcher reported that the addition of *SP* to the ration caused an increase in the egg yolk redness and a decrease in the yellowness intensity (Omri et al., 2019). Herber and Van Elswyk, (1996) reported that the addition of microalgae product to laying hen rations increased the amount of docosahexaenoic acid and also darkened the egg yolk color, the taste of the egg did not change, and the red/green value (a) was found to be high in the color analysis, and this was due to the increase in the color quality of the egg. Mariey et al. (2012) reported that the addition of different levels of *SP* (0.1%, 0.15%, and 0.2%) to the ration increased the yolk color score compared to the control group.

Zahroojian et al. (2013) found that the highest dose (2.5%) of *SP* yielded the highest value for the yolk color score among the groups in which 1.5%, 2%, and 2.5% *SP* were added to the ration, compared to the control group. Selim et al. (2018) found that the addition of *SP* to the ration increased the yolk color score due to the increase in dose and there was a strong positive correlation between the yolk color and the ration's containing *SP* from the 42nd week to the end of the 46th week. Anderson et al. (1991) reported that the addition of *SP* to quail rations gradually increased the egg yolk color scores from the beginning to the end of the trial. Park et al. (2015) found that the addition of 0.5% and 1.0% *Schizochytrium*, a microalgae species, to the laying hen rations increased the yolk color score compared to the control group at the end of the 6-week trial.

Serum parameters

The effects of the addition of different levels of *SP* to the laying hen rations on the serum cholesterol, HDL, albumin, globulin, total protein, uric acid, calcium, and phosphorus were not found significant ($p>0.05$).

Mariey et al. (2012) found that the addition of different levels of *SP* to the diet caused a significant decrease in cholesterol of blood concentrations.

Another study concluded that the decline of algae

was associated with the docosahexaenoic acid and the ration fibers in its content (Lahaye and Jegou, 1993; Park et al., 2015). However, some researchers attributed this decline to the content of polyunsaturated fatty acids in *SP* and the inclusion of newly formed cholesterol esters in HDL (high-density lipoprotein) by omega 3 fatty acids' stimulating the LCAT (lecithin cholesterol acyltransferase) activity, an enzyme responsible for esterification of serum cholesterol (Rajaram and Barter, 1986; Vaysse-Boue et al., 2007; Ferchaud-Roucher et al., 2014). Furthermore, Chen et al. (2011) reported that docosahexaenoic acid (DHA) found in microalgae could inhibit the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase by reducing serum cholesterol concentration.

A study found that the effect of *SP* addition on total protein, albumin, and globulin was statistically insignificant, whereas its effect on cholesterol, HDL, and LDL was found to be significant (Zeweil et al., 2016). Omri et al. (2019) stated that the effect of *SP* addition on cholesterol levels in egg yolks was statistically insignificant.

Many studies assert that algae have rich mineral content, which means that these plants can improve the health of poultry and the quality of products obtained from it. Studies have shown that the *SP* applied to laying hen rations does not have any effect on the calcium and phosphorus content in the blood of the animals studied (Konkol et al., 2018). In contrast, Michalak et al. (2011) reported that macroalgae-fed chickens had higher calcium content in blood. Finally, in a study examining the effect of *SP* addition on the serum parameters of quails, Abouelezz, (2017) reported that the *SP* addition did not statistically affect the total protein, albumin, and globulin values, but the low dose decreased the free fatty acids and cholesterol more than the high dose.

CONCLUSION

In conclusion, it was observed that the addition of *SP* to the laying hen rations had no significant effect on the performance and serum parameters ($p>0.05$), and it was effective only on the color characteristics among the egg quality characteristics examined in this study ($p<0.01$). Various researchers explained that the reasons for this were related to the fact that *SP* contained high amounts of carotenoids. In the study, the addition of *SP* to the ration increased the Roche score. It can be stated that the algae used commercially in poultry rations cause an increase in the egg yolk pigmentation by accumulating carotenoids in egg yolk. Based on the findings on its nutritional profile, it can be asserted that *SP* undoubtedly appears to be a strong feed supplement for poultry. For a better understanding of its nutritional value, it should also be examined in the studies to be carried out on various poultry species. For this reason, there is a need for further studies on the use of *SP* in poultry farming and its effects on the performance and egg quality characteristics.

CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Effects of Dietary Supplementation of Propolis on Performance, Egg Quality and Blood Parameters of Layer Quails

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ABSTRACT: The aim of this study was to investigate the effect of addition of different levels of propolis to the layer quail diets on performance, egg quality and blood parameters. In the experiment, a total of 120 quails at the age of 22 weeks were randomly distributed to six treatments groups, each with four subgroups. There were 5 quails in each subgroup and the trial lasted 12 weeks. Treatments diets were prepared by adding 0, 2, 4, 6, 8 or 10 g/kg propolis to the basal diet. Body weight change, egg production, egg weight and feed intake were not affected by the addition of propolis to the diet (P>0.05). The egg mass (P<0.01) and feed conversion ratio (P<0.05) linearly improved by addition of propolis. The eggshell breaking strength (P<0.05),eggshell thickness (P<0.01) and Haugh unit (P<0.01) improved by the addition of propolis but other egg quality parameters were not affected (P>0.05).The haematological parameters of the blood were not affected of propolis to the diet (P>0.05), except for the neutrophil content, which was quadratically affected by the addition of propolis, and increased at doses up to 6 g/kg but decreased at the levels of 8 or 10 g/kg (P<0.05). The addition of propolis to the diet did not affect the serum parameters (P<0.05), except cholesterol (P<0.01) and calcium (P<0.05) contents in layer quails. The cholesterol content of serum was linearly affected by the addition of propolis and minimized at the dose of 8 g/kg. The calcium level increased when propolis was supplemented at the dose of 2 g/kg but linearly decreased with higher dose levels. According to these results, it can be said that the addition of 8 g/kg propolis to laying quail diets positively affected the egg mass, eggshell breaking strength, eggshell thickness and cholesterol concentration of serum, however, calcium metabolism was negatively affected by propolis levels.

***Keywords*:** Cholesterol, egg quality, haematological, performance, propolis.

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INTRODUCTION

Prohibition of the use of growth-promoting antibiotics in the poultry industry has started the search for alternative additives to antibiotics. Furthermore, concerns about the possible negative effects of synthetic feed additives in many regions of the world have prompt producers to look for alternative natural feed additives. For these reasons, poultry nutritionists strive to find natural feed additives with beneficial effects on performance, egg quality, meat yield and immune system (Salah Eldin et al., 2015; Raheema, 2016).

It is known that the raw materials, extracts and purified active compounds of bee products commonly used in traditional medicine have antioxidant, antimicrobial and anti-inflammatory properties (Vynograd et al., 2000; De Castro, 2001; Silici and Kutluca, 2005; Aygun et al., 2012; Premratanachai and Chanpen, 2014). Propolis, commonly called bee glue, is a bee product that is collected by worker honey bees (*Apis Mellifera L.*) from tree buds and plant leaks. Honey bees produce propolis by chewing it with enzymes and then mixing it with beeswax and other ingredients (Seven et al., 2010; Banskota et al., 2001; Aygun et al., 2012). Generally, propolis contains resin-polyphenolic fraction (55%), beewax (7%), bee pollen (5%) and aromatic essential oils (30%). In addition to them, it also contains other minor compounds (3%) like vitamins (A, C, D, E, B_1, B_2, B_3, B_6 and B_9) and some minerals such as iron, calcium, copper, nickel, zinc, magnesium, manganese, vanadium, strontium and cobalt (Castaldo and Capasso, 2002; Bankova, 2005). It is rich in vitamins, minerals, enzymes and fatty acids (Lotfy, 2006; Wagh, 2013; Kurek-Górecka et al., 2014). The main ingredients it contains are phenolic compounds, flavonoids and their terpenes and esters (Nolkemper et al., 2010). Besides propolis is used in traditional medicine, it is also used as a dietary supplement in poultry diets. There are research results indicating that propolis when added into broiler and laying hens diets can enhance the performance (Shalmany and Shivazad, 2006; Seven et al., 2010; Seven et al., 2012), improve some serum parameters (Galal et al., 2008; Attia et al., 2014) and decrease oxidative stress (Mannaa et al., 2011; Hosseini et al., 2015). According to Galal et al. (2008) propolis use in laying hens nutrition has positive effects on performance and egg quality, and these effects are due to its high palatability. It also contains compounds that increase the digestion and absorption of calcium, and it was stated to improve eggshell quality (Seven, 2008; Seven et al., 2011). It is also reported that it prevents anemia by increasing red blood cells in poultry (Khan, 2017). Considering the aforementioned beneficial effects of propolis in broilers and laying hens we hypothesized that similar effects could also be seen in quails

This research was carried out to investigate the effect of supplementing diets with different levels of propolis on performance, egg quality, and the haematological or biochemical properties of blood in Japanese quails.

MATERIALS AND METHODS

Ethical Approval

The animal care practices were used in the experiment in consistency with animal welfare rules stated in Article 9 in government law in Turkey (No. 5996)

Animals and Diets

This study was carried out with a total of 120 female Japanese quails at the age of 22 weeks and lasted 12 weeks. Quails were randomly distributed to six experimental groups. Each trial group was divided into four subgroups, each containing five quails. Treatment diets were prepared with six increasing levels of propolis (0, 2, 4, 6, 8 and 10 g/kg). Chemical analysis values of propolis used in the research are as follows: 39.95% mechanical mass, 2.54% beewax, 46.63% soluble solids, 3.31% flavonoid expressed in quercetin 3.75% minerals, 7.57% humidity. Propolis supplied from Konya, Turkey were cut into small pieces and frozen at -20 °C. Then, the frozen propolis was pulverized in the blender by adding double its weight of corn starch. The basal diet was prepared according to NRC (1994). The basal diet and its calculated nutrient content are shown in Table 1. Throughout the experiment, feed and water were given to quails *ad-libitum*.

Determination of Performance Parameters

Body weight change was determined by group weighing at the beginning and end of the experiment. Feed intake was calculated as g/d/quail. Egg production of birds was recorded daily and calculated as %. Egg weight was obtained by weighing the eggs collected in the last three days of the experiment. Egg mass was calculated from formula: *(egg production (%) x egg weight)/100*.The feed conversion ratio was calculated using the following formula: *feed intake (g feed/quail)/egg mass (g/egg/quail)*.

Table 1. Basal diet and its calculated nutrient content

Ingredients	g/kg	Nutrient contents	g/kg
Corn	542	Metabolisable energy, kcal ME/kg	2902
Soybean meal	270	Crude protein	200.9
Sunflower meal	70	Calcium	25.1
Sunflower oil	43	Available phosphorus	3.5
Limestone	56	Lysine	10.0
Dicalcium phosphate	11.5	Methionine	4.5
Salt	3.5	Cystine	3.7
Premix[1]	2.5	Methionine+cystine	8.2
DL methionine	1.5		
Total	1000.0		

[1]Premix is supplied that per kg of diet; Manganese: 80 mg, Iron: 60 mg, Copper: 5 mg, Iodine: 1 mg, Selenium: 0.15 mg, Vitamin A: 8.800 IU, Vitamin D_3: 2.200 IU, Vitamin E: 11 mg, Nicotine acid: 44 mg, Cal-D-Pan: 8.8 mg, Riboflavin: 4.4 mg, Thiamine: 2.5 mg, Vitamin B_{12}: 6.6 mg, Folic acid: 1 mg, Biotin: 0.11 mg, Choline: 220 mg.

Determination of Egg Quality Parameters

The egg quality parameters were determined by analysis of eggs collected in the last three days of the experiment. Egg length and egg diameter of each egg was measured by digital calliper. Egg shape index was calculated with (egg diameter / egg length) x 100 formula. Eggshell breaking strength was measured by applying supported systematic pressure to blunt of the eggs (Egg Force Reader, OrkaFood Technology, Israel). Afterwards, the yolk diameter was measured with a digital calliper and the yolk height was measured with a digital height gauge. The yolk index was obtained by using these parameters with *(yolk height / yolk diameter) x 100* formula. Similarly, length and diameter of albumen were determined with digital calliper and albumen height was determined with digital height gauge. Using these parameters, the albumen index was found from [*albumen height / (albumen length + albumen diameter) / 2)] x 100*. Additionally, Haugh unit was calculated as follows: Haugh unit = $100 \times \log (\text{albumin height} + 7.57 - 1.7 \times \text{egg weight}^{0.37})$ (Haugh, 1937). The egg quality analyses were completed within 24 hours after eggs were collected. The ratio of eggshell weight including the membrane to egg weight was determined by using *eggshell weight (g) /egg weight (g) x 100* formula. The thickness of eggshell including the membrane was calculated from the values obtained with digital calliper from three sections of the eggs.

Determination of Haematological and Biochemical Parameters of Blood

At the end of the study, two quails from each subgroup (eight quails per treatment group) were randomly selected and blood samples (2 ml per quail) were taken either into heparinised tubes for haematological analyses or in centrifuge tubes for biochemical constituents analyses. The analyses of blood and serum samples were made by an auto-analyser.

Statistical Analysis

Data were analysed in the SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA) with model of one-way ANOVA, using the group mean as an experimental unit. A probability value of $P<0.05$ was considered statistically significant. Orthogonal polynomial contrasts were used to evaluate the significance of linear and quadratic models to determine the response of the dependent variable to an increasing propolis level.

RESULTS

Performance

The body weight change, egg production, egg weight, egg mass, feed intake and feed conversion ratio are shown in Table 2. The supplementation of different levels of propolis to diets did not significantly affect body weight change, egg production, egg weight and feed intake ($P>0.05$). Egg mass increased linearly ($P<0.01$) when propolis was added to quail diets. Feed conversion ratio was affected linearly ($P<0.05$) with the supplementation of increasing levels propolis to diets in quails.

Egg Quality Parameters

The damaged eggs, egg shape index, eggshell breaking strength, eggshell weight and thickness or albumen and yolk indexes and Haugh unit results are presented in the Table 3.Some of the egg internal and

external parameters such as damaged eggs, egg shape index, eggshell weight and yolk index were not significantly affected by treatment diets (P>0.05). The addition of propolis to the diets linearly increased the eggshell breaking strength which was maximized at the dose level of 8 g/kg (P<0.05). Similarly, eggshell thickness was considerably enhanced by increasing levels of propolis, especially at the level of 8 g/kg (P<0.01). The albumen index decreased by supplementation with propolis and was minimized at the dose of 6 g/kg (P<0.05). Haugh unit also decreased at the dose of 6 g/kg level, but it was surprisingly maximized at the level of 10 g/kg (P<0.05).

Table 2. Effect of dietary supplementation of propolis on the performance parameters in layer quails

Parameters	Propolis (g/kg)						SEM*	*P*- value of contrast	
	0	2	4	6	8	10		Linear	Quadratic
Body weight change, g	26.42	19.50	21.58	18.33	24.58	12.25	4.906	0.203	0.833
Egg production, %	86.94	92.59	90.59	91.14	91.89	93.83	1.893	0.062	0.681
Egg weight, g	12.36	12.40	12.44	12.16	12.76	13.37	0.327	0.061	0.130
Egg mass, g/d/quail	10.75	11.48	11.28	11.08	11.70	12.56	0.343	0.010	0.292
Feed intake, g/d/quail	32.84	33.61	34.15	32.71	33.32	33.86	1.025	0.752	0.928
Feed conversion ratio, g feed/g egg	3.06	2.93	3.05	2.95	2.85	2.71	0.096	0.020	0.304

*Standard error of mean

Table 3. Effect of dietary supplementation propolis on the egg quality in layer quails

Parameters	Propolis (g/kg)						SEM*	P value of contrast	
	0	2	4	6	8	10		Linear	Quadratic
Damaged eggs, %	0.58	0.15	0.43	0.15	2.08	0.74	0.619	0.407	0.802
Egg shape index	76.37	79.62	77.30	78.72	77.22	77.92	1.253	0.878	0.503
Eggshell breaking strength, kg	1.29	1.44	1.42	1.33	1.51	1.47	0.045	0.021	0.763
Eggshell weight, % of egg weight	7.22	7.66	8.03	7.44	7.65	7.84	0.168	0.121	0.289
Eggshell thickness, µm	169	186	201	197	220	212	4.3	<0.001	0.045
Albumen index	6.04	5.09	5.52	4.89	5.46	5.93	0.284	0.984	0.018
Yolk index	52.16	51.08	51.38	47.92	50.81	50.71	1.427	0.357	0.269
Haugh unit	71.69	67.44	69.56	65.07	70.03	73.53	2.152	0.502	0.030

*Standard error of mean

Haematological Parameters of Blood

The haematological parameters of blood are demonstrated in Table 4. In the current study, white blood cell, lymphocyte, red blood cell, haemoglobin, haematocrit, erythrocyte volume, mean corpuscular haemoglobin, corpuscular haemoglobin concentration, red blood cell distribution width and haemoglobin/lymphocyte ratio were not affected by experimental diets (P>0.05). Among the haematological parameters, only the neutrophil concentration was quadratically affected by supplementation of quail diets with propolis, reaching a maximum at the level of 6 g/kg (P<0.05). However, it was diminished significantly by further supplementation of propolis.

Biochemical Analysis of Serum

The serum biochemical parameters are shown in Table 5. No treatment effect was observed except for the cholesterol and calcium concentrations of (P>0.05). The cholesterol and calcium levels were linearly affected (P<0.01 and P<0.05, respectively) by the addition of propolis to diets. The cholesterol concentration was decreased by dietary addition of propolis while its lowest concentration was obtained from the group of quails that received 8 g/kg. Besides, the calcium level reached a maximum at the dose of 2 g/kg while it decreased at higher levels.

Table 4. Effect of dietary supplementation with propolis on the haematological parameters in layer quails

Parameters	Propolis (g/kg)							*P*- value of contrast	
	0	2	4	6	8	10	SEM*	Linear	Quadratic
WBC, 10^3/µl	9.04	14.65	13.87	13.40	14.15	11.94	2.108	0.518	0.129
NEU, 10^3/µl	0.65	0.75	1.13	2.18	0.33	0.61	0.237	0.858	0.050
LYM, 10^3/µl	9.08	13.33	11.76	7.70	11.27	8.65	1.691	0.411	0.403
RBC, 10^6/µl	3.13	4.31	3.25	3.00	3.24	3.18	0.205	0.142	0.655
HGB, g/dL	17.60	19.97	17.67	16.30	18.00	17.15	0.687	0.140	0.986
HCT, %	46.20	55.10	45.65	42.13	44.25	44.70	2.230	0.061	0.868
MCV, µm^3	148	146	141	141	142	141	2.7	0.088	0.341
MCH, pg	56.33	53.77	54.35	54.57	56.20	53.92	1.565	0.761	0.729
MCHC, g/dL	38.13	36.50	38.70	38.90	40.77	38.37	0.846	0.081	0.550
RDW, %	10.33	10.72	9.62	10.63	11.25	10.22	0.449	0.707	0.971
HGB/LYM	2.10	1.65	1.73	2.86	1.66	2.07	0.425	0.808	0.852

WBC: White Blood Cell, NEU: Neutrophil, LYM: Lymphocyte, RBC: Red Blood Cell, HGB: Haemoglobin, HCT: Haematocrit, MCV: Erythrocyte Volume, MCH: Mean Corpuscular Haemoglobin, MCHC: Corpuscular Haemoglobin Concentration, RDW: Red Blood Cell Distribution Width, PLT: Thrombocyte, HGB/LYM: Haemoglobin/Lymphocyte Ratio.
*Standard error of mean

Table 5. Effect of dietary supplementation with propolis on the biochemical parameters of serum in layer quails

Parameters	Propolis (g/kg)							*P*- value of contrast	
	0	2	4	6	8	10	SEM*	Linear	Quadratic
Total protein, g/dL	4.47	5.42	4.17	4.35	4.30	4.00	0.310	0.052	0.627
Albumin, g/dL	1.50	1.75	1.42	1.45	1.55	1.32	0.098	0.110	0.484
Globulin, g/dL	2.97	3.67	2.62	2.87	3.22	2.65	0.219	0.168	0.714
Creatinine, mg/dL	0.35	0.36	0.37	0.34	0.34	0.35	0.010	0.657	0.738
Glucose, mg/dL	319	344	332	335	333	357	13.05	0.167	0.752
Cholesterol, mg/dL	250	212	183	166	138	149	16.9	<0.001	0.139
ALT, U/L	3.25	2.75	2.50	2.75	2.50	3.00	0.387	0.618	0.203
AST, U/L	219	219	220	242	258	240	17.16	0.118	0.866
Ca, g/dL	26.82	30.46	24.07	25.60	26.42	22.25	1.560	0.034	0.531
P, g/dL	8.02	8.52	6.22	7.40	7.52	6.80	0.896	0.319	0.679

ALT: Alanine aminotransferase AST: Aspartate aminotransferase.
*Standard error of mean

DISCUSSION

Performance

The results obtained from this study are in agreement with published studies claiming that the supplementation of different levels of propolis to the diets of birds positively influenced egg mass (Galal et al., 2008; Tatli Seven, 2008; Belloni et al., 2015; Mehaisen et al., 2019). The egg mass was enhanced by increasing dietary propolis and the highest value was observed at 10 g/kg level of propolis. Also, Mehaisen et al. (2019) stated that the egg mass increased by addition of 1 g/kg propolis in quails. Similar results have been reported by Belloni et al. (2015) and Soltani et al. (2019).In the present study, feed conversion ratio of laying quails improved by the supplementation of propolis in the diet, especially at the dose levels of 8 and 10 g/kg. This result agree with Zeweil et al. (2016) and Mehaisen et al. (2019), who showed that the addition of propolis (250-1000 mg/kg) to the diet improved feed conversion ratio in laying quails. At the same time, Galal et al. (2008) demonstrated that the feed conversion ratio of hens improved by dietary addition of propolis (100-150 mg/kg). Similarly, Tatli Seven (2008) reported that the supplementation of 2 or 5 g/kg propolis to the diet enhanced the feed conversion ratio in laying hens. In parallel, Belloni et al. (2015) noted that the feed conversion ratio of laying hens improved by the supplementation of 3% propolis. The positive effect of propolis on performance parameters such as egg mass and feed conversion ratio may be due to its potential to increase the digestibility and absorption of nutrients by promoting certain biological activities (Marieke et al. 2005; Kročko et al.2012). ShreifEffat and El-Saadany (2016) also reported that the high flavonoids and phenolic acids contained in propolis may increase the absorption of

crude protein and other nutrients by improving the gut microflora. Additionally, it may be propolis content of flavonoids that exerts antimicrobial and immunomodulatory effects and reduces the level of detrimental oestrogen (Burdock, 1998; Shaddel-Tili et al., 2017; Duarte et al., 1993; Hanasaki et al., 1994; Middleton and Kandaswami, 1994).

Egg Quality

In current study, the eggshell breaking strength and eggshell thickness increased by supplementation of propolis to the diets. The present results agree with those of previous studies (Galal et al., 2008; Tatli Seven et al., 2008; ShreifEffat and El-Saadany, 2016; Mehaisen et al., 2019; Soltani et al., 2019). Mehaisen et al. (2019) noted that the eggshell thickness increased by dietary addition of 1 g/kg level propolis in quails. Galal et al. (2008) reported that the eggshell thickness of laying hens was increased by supplementation of 100-150 mg/kg propolis to diets. For all that, Tatli Seven (2008) showed that the 2 or 5 g/kg of propolis positively affected the eggshell thickness in hens. Similar results have been reported by ShreifEffat and El-Saadany (2016) and Soltani et al. (2019). The enhanced eggshell thickness and eggshell breaking strength may be associated with the increased digestibility and absorption of calcium and phosphorus as a result of acid derivatives such as benzoic, 4-hydroxy-benzoic, etc., which are found in propolis (Haro et al., 2000). The albumen index diminished with the propolis supplementation to diets, especially 6 g/kg level. These results disagree with previous reports that the addition of propolis to the diet does not affect the albumin index (Tatli Seven, 2008; ShreifEffat and El-Saadany, 2016; Zeweil et al., 2016; Mehaisen et al., 2019). The Haugh unit is accepted as an index to predict the egg quality (Monira et al., 2003). At the present study, Haugh unit quadratically diminished with the addition of propolis to the diets, except for the 8 and 10 g/kg level. The highest Haugh unit was observed at the 10 g/kg level of propolis. Similarly, ShreifEffat and El-Saadany (2016) stated that dietary addition of 150, 300 or 450 mg/kg levels of propolis to the diet improved Haugh unit in laying hens. Additionally, Galal et al. (2008) and Abdel-Kareem and El-Sheikh (2017) reported that the Haugh unit was positively affected by the addition of propolis to the diet.

Blood Parameters

The propolis treatment did not affect haematological parameters linearly or quadratically, except for neutrophil concentration that quadratically increased for doses up to 6 g/kg of diet. Neutrophils represent the vast majority of white blood cells that consist part of the warrior cells of the defence system that protects the organism from various microbes and harmful toxins. Although there are some results about white blood cells and their sub-types such as lymphocyte, monocyte, eosinophil and basophil, among the studies carried out, there is no finding related to neutrophil count (Abdel-Rahman and Mosaad, 2013; Omar et al., 2003; Shaddel-Tili et al., 2017; Hassan et al., 2018). In the present study, cholesterol and calcium concentrations in serum were significantly affected by supplementation of propolis. The cholesterol level was linearly decreased and this result is in agreement with previous reports by Galal et al. (2008), Zeweil et al. (2016) and Abdel-Kareem and El-Sheikh (2017) who tested dietary inclusion of propolis at doses ranging from 100 to 1000 mg/kg of diet. However, there were also results indicating that serum cholesterol concentration was not affected by the addition of propolis to the diet (Denli et al., 2005; Soltani et al., 2019). Tatli Seven et al. (2008) and Arpasova et al. (2016) noted that these different results may be partially originated from variations in compositions of propolis as a result of collection area and time and bee flora. The calcium concentration was the highest at the dose of 2 g/kg and it decreased at higher levels of propolis. Contrary to the current study, Mehaisen et al. (2019) reported that the serum calcium level increased with the addition of propolis. Additionally, the calcium concentration of serum significantly decreased with the supplementation of propolis to quail diets and the lowest concentration was observed at 10 g/kg propolis level. The reason for the decrease in the serum concentration of calcium, which is the main component of the eggshell, may be that propolis uses calcium more effectively for the purpose of improving eggshell resistance and thickness.

CONCLUSION

In conclusion, it can be said that propolis, which is a natural feed additive, positively affected performance, eggshell quality, and cholesterol concentration of serum in layer quails but negatively affected the Ca metabolism at levels equal or higher than 4 g/kg.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Pharmacokinetics and plasma concentration of thiopental in normal and stressed chickens with hydrogen peroxide

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ABSTRACT: No previous study deals with hydrogen peroxide (H_2O_2)-induced oxidative stress (OS) and its influence on thiopental anesthesia, its plasma concentration and pharmacokinetic profile in 7-14 day old chickens. OS induction was made by the daily supply of H_2O_2 from day one to the completion of the experiments on day 14th of chickens' age. Median Effective Doses (ED_{50}s) of hypnosis and analgesia were revealed an increase in thiopental efficacy by 7 and 18%, respectively in the stressed chickens in comparison to the normal one. Thiopental plasma concentration was analyzed at time 0.5, 1, 2, 4 and 24 hours after its injection at 35 mg/kg, IP to be 91.42, 54.35, 38.27, 22.30 and 7.51 µg/ml in the normal chickens and increased significantly by 74, 84, 48, 85 and 82% to be 159.01, 100.06, 56.71, 41.30 and 13.63 µg/ml in the stressed chickens, respectively. Thiopental pharmacokinetics parameters, which included Area Under the plasma Concentration ($AUC_{0-\infty}$), Area Under the Moment Curve ($AUMC_{0-\infty}$), Mean Residence Time (MRT) and elimination half-life ($t_{1/2\beta}$) were increased in the stressed chickens by 82, 94, 6 and 8%, as well as, Clearance (Cl), elimination rate constant (K_{el}), and Steady-State Volume of Distribution (V_{ss}) were decreased in the same group by 33, 14 and 41%, respectively. The results of this trial concluded that OS status intensifies thiopental anesthetic action mainly by increasing its plasma concentration and altering the pharmacokinetics profile, suggesting veterinarians to bear in mind when preparing the dose of thiopental to be given to stressed animals.

Keywords: Chickens, plasma concentration, pharmacokinetics, stress, thiopental

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INTRODUCTION

Thiopental belongs to the barbiturates anesthetics that used for the induction of short duration of the general anesthesia by its mode of action resulting from the potentiation of the inhibitory neurotransmitter effect, Gamma-Aminobutyric Acid (GABA), on its $GABA_A$ receptor subtype, leading to an increase in the outflow of chloride ions inside the post-synaptic neurons that depresses the Central Nervous System (CNS) (Garcia et al., 2010; Clark et al., 2012; Naseri et al., 2017). Thiopental possesses excellent and reliable narcotic action with less efficient analgesia and muscle relaxation than other anesthetic agents like ketamine (Clark et al., 2012; Flecknell, 2015; Katzung and Trevor, 2015). H_2O_2 was used experimentally for the induction of the OS because it is well-known. A powerful oxidizing agent besides, its concentration (0.5%) and the length of treatment required to produce OS are well studied in the previous literature through its formation of free radicals that break down the vital cell components, stimulates lipid peroxidation and breaks down of proteins (including protein receptors) (Patockova et al., 2003; Sayre et al., 2008). The CNS considered more vulnerable to OS status, because of the high free radical formation, consuming a lot of oxygen, high amount of unsaturated fatty acids with less antioxidant activity (Pastore et al., 2003). The experimentally induced OS with H_2O_2 (0.5%) was found to modify the pharmacological response of some drugs that have a neuro-active mechanism like ketamine anesthesia (Mousa, 2014), diazepam and xylazine sedation (Mousa and Mohammad, 2012a;b) by pharmacodynamics interaction on their receptors and relevant pharmacokinetic interaction at a level of absorption, distribution, metabolism and elimination of the drugs which have an impact elevating drug efficacy and subsequent toxicity. The pharmacokinetics criteria of the drug are an important factor ensuring the drug delivery to the site of action on its target receptor to achieve efficacy and/or improve the preferred therapeutic effect with minimizing its adverse effect (Wen et al., 2015).

The purpose of this study was to use thiopental in normal chickens for the induction of the general anesthesia and to determine the possible efficacy of OS-induced by H_2O_2 at thiopental anesthesia, its plasma concentration and the pharmacokinetics profile.

MATERIALS AND METHODS

Animals

7-14-days old of broiler chickens (76 overall chickens used in all the experiments) for both genders were used in all the study experiments with a regular bodyweight of 80–125 g. They were kept in cages at an ideal condition of 32–35°C and the floor of the cages comprising woody shreds. Chickens have admitted to the water and food ration freely. Thiopental [Thiopentone (Thiopental sodium), 2.5%, Egyptian International Pharmaceutical Industries Co, Egypt] diluted using normal saline solution (0.9% NaCl) to be injected by a dosage volume 5 ml/kg. The route of administration of thiopental was intraperitoneally (IP) as mentioned by a previous study in the chicks (Church, 1957).

Ethical considerations

This research and the use of experimental chickens have been valid, qualified and authenticated by the professional scientific board of the Veterinary Medicine College / Mosul University, according to international standards.

Induction of OS in the chickens

Chickens gotten from a native hatchery at one day old, divided randomly to control normal group that provided with tap water (H_2O group) whereas the other remaining chickens had treated with 0.5% H_2O_2 (50%, Scharlab, Spain) in drinking water (stressed or H_2O_2 group). The earlier studies authenticated that a continuous daily fresh supply of 0.5% H_2O_2 in tap water can prompt OS whenever assumed to chickens from 1st day and continued for 14 days. Then, H_2O_2 can prompt OS on the 7th, 10th and 14th days of chickens' lifetime through glutathione reduction and malondialdehyde raising in their concentrations at the chickens' brain and plasma (OS biomarkers) (Mousa and Mohammad, 2012a; Mousa, 2014; Kabirian et al., 2018; Aksoy and Alper, 2019). So that, 7-14-days of chickens' lifetime, were used in the current study.

Assessment of thiopental ED_{50}s in normal and stressed chickens

A. Thiopental hypnotic ED_{50} in chickens

The hypnotic ED_{50} value of thiopental was estimated in the normal and stress chickens, implement to the up-down technique mentioned earlier (Dixon, 1980). The first dosage for thiopental at 35 mg/kg, IP depends on a preliminary study. Chickens observed individually for 4 hours waiting for the hypnotic occurrence of thiopental showed by losing the righting reflex. The dosages of thiopental then would be di-

minished or augmented 10 mg reliant on the presence or lack of the hypnotic effect, respectively (Mousa and Al-Zubaidy, 2019; Mousa et al., 2019).

B. Thiopental analgesic ED_{50} in chickens

For the normal and stressed chickens, the thiopental's analgesic ED_{50} is estimated according to the previously described routine (Dixon, 1980). The first thiopental dosage be 35 mg/kg, IP depends on an introductory study. The chickens were measured before and after 15 minutes of thiopental injection by using the electro-stimulator device (Harvard apparatus, USA), which indicated by pain perception (distress call) in the chickens (Mousa, 2019; 2020; Mousa and Al-Zubaidy, 2019), the doses of thiopental then should be reduced or raised 10 mg as look or lack of the analgesia occur (the decrease or increase in doses was chosen whereby not higher than 30% of the first dose used of thiopental for accurate results).

Thiopental plasma concentration of normal and stress chickens

A single dosage of thiopental at 35 mg/kg, IP (which resembles the ED_{100} of thiopental estimated in the previous experiment) was injected to normal and stressed chickens. Blood samples combined from the jugular vein of 5 chickens per measure time at 0.5, 1, 2, 4 and 24 hours for both the normal and stressed chicks. Then, adding the heparin (B. Braun Medical Inc, USA) (1:10) to the blood samples and undergoing centrifugation (Chalice, UK) at 3000 round per minute along with 15 minutes to get plasma samples (Chauhan et al., 2020) which then freezes at -18 °C pending spectrophotometric analysis for 72 hours.

Spectrophotometric analysis

Preparation and calibration of the thiopental standards

Thiopental standards were made of 2.5, 5, 10, 20, 40, 80 and 160 µg/ml (Gustafsson et al., 1996) by dilution with 0.5 *N* of Sodium hydroxide (NaOH) and filtration then, the absorbance by Ultra Violet (UV) spectrophotometry (Lovibond, Germany) at 330 nanometers (nm) was taken by the procedure described by Goldbaun (1948) to reveal the standard curve of the simple linear regression to calculate the thiopental concentration in the unknown plasma samples. By using the equation of the simple linear regression of thiopental standards with a coefficient of determination (R^2 = 0.8958) (Fig. 1), thiopental concentration in plasma samples can be calibrated and calculated in normal and stressed chickens (Barwick, 2003) (Figure 1) were:

y = a + bx (y = 0.0974 + 0.0017x)(Equation of the simple linear regression of thiopental standards)

y = absorbance of thiopental in the plasma samples at 330 nm

a = intercept

b = slope

x = thiopental concentration in the plasma samples (unknown)

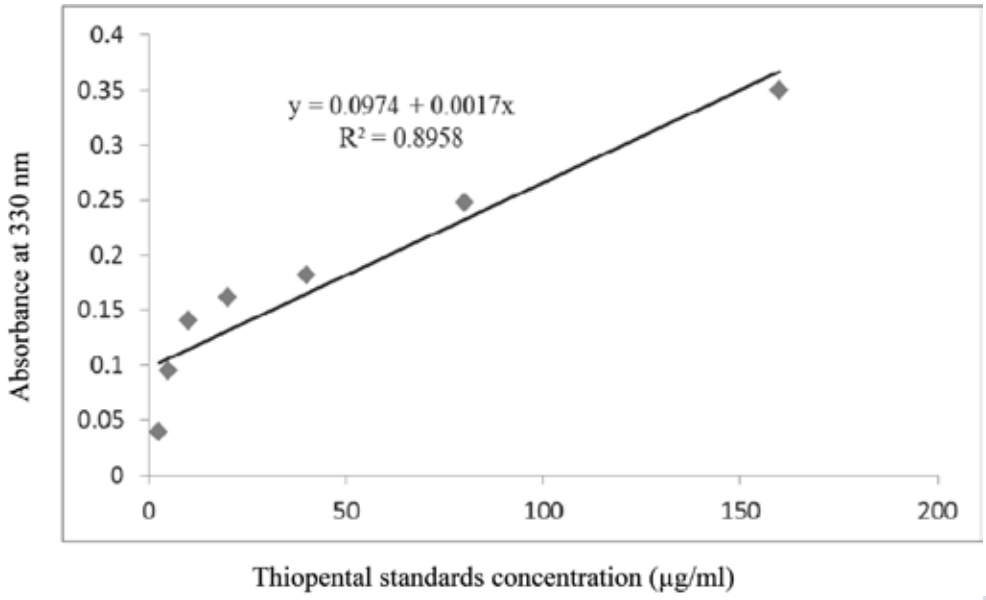

Figure 1. Equation of the simple linear regression for thiopental standards (2.5, 5, 10, 20, 40, 80 and 160 µg/ml), and their absorbance at 330 nm

Extraction and determination of thiopental in the plasma samples

A simple, validated and accurate method was used for determining thiopental concentration in the plasma (Goldbaum, 1948; Broughton, 1956) for both normal and stressed chickens in different measured times at 0.5, 1, 2, 4 and 24 hours after thiopental treatment. The procedure summarized by adding 10 ml of redistilled chloroform to 1 ml of the plasma and shake by tube shaker (Dragonlab, China) for 3 minutes. By using the filter paper, the aliquot will be obtained by using the funnel. Thereafter, 1 ml of NaOH (0.5 *N*) was added to the aliquot and the lower layer, then discarded and the remaining were centrifuged at 3000 rpm for 15 minutes to get the clear solution, which is the last consequent extraction result of the plasma sample which lastly analyzed by the UV spectrophotometry at 330 nm wavelength by using the quartz cuvette to record the absorbance for each unknown sample.

Determination of the thiopental pharmacokinetics parameters in the normal and stressed chickens

The non-compartmental method was used to find out the thiopental pharmacokinetics parameters in normal and stressed chickens by using a PKSolver program (Zhang et al., 2010) which included $AUC_{0-\infty}$ (µg.h/ml), $AUMC_{0-\infty}$ (µg.h^2/ml), MRT (AUMC/AUC)(h), Cl (dose/AUC)(L/h/kg), Tmax (h), Cmax (µg), $t_{1/2\beta}$ (h), K_{el} (0.693/ $t_{1/2\beta}$)(h^{-1}) and V_{ss} [dose. AUMC/(AUC)^2](L/kg). The increase or decrease in the percentages of the thiopental pharmacokinetics parameters was calculated in the normal chickens and correlate to determine the changes in the pharmacokinetics parameters in the stressed chickens.

Statistics

The unpaired student T-test was implemented to relate the means of two groups of parametric statistical examinations by using the SPSS program (Katz, 2011; Petrie and Watson, 2013). The data would be considered significant when $p < 0.05$.

RESULTS

Thiopental ED_{50} values the in normal and stressed chickens

A. The hypnotic ED_{50} value of thiopental

The ED_{50} values of thiopental that produce narcosis (lack of the righting reflex) in 50% of the chickens were 17.63 and 16.44 mg/kg, IP in the normal and stressed chickens, respectively, which have decreased by 7% that resembles the OS prompt by H_2O_2 to ameliorate the thiopental hypnotic ED_{50} value as determined by the up-down technique (Table 1-A) which is stated here for the first time. The signs elicited by thiopental hypnosis in the chicks were ataxia, recumbency, locked eyelids, defecation, loss of the righting reflex, paddling movements of the legs and quit sleeping with normal breathing. These signs, as it observed, it became exaggerated at the stressed chickens.

Table 1-A. Thiopental's hypnotic ED_{50} of normal and stress chickens

Parameter	Groups	
	Normal (H_2O)	Stress (H_2O_2)
Hypnotic ED_{50} value = XF + K × D	17.63 mg/kg, IP	16.44 mg/kg, IP
The extent of the doses	15-35 mg/kg	5-35 mg/kg
First dose used	35 mg/kg	35 mg/kg
Last dose used (XF)	25 mg/kg	15 mg/kg
Table Value (K) (Dixon, 1980)	-0.737	0.144
± in the dose (D)	10 mg	10 mg
Chickens used	6 (XXOXOX)*	7 (XXXOOXO)*
OS influence on thiopental hypnotic ED_{50} = H_2O- H_2O_2/ H_2O×100= 7%		

*X: effect (i.e. hypnosis), O: no effect (i.e. no hypnosis)
Tab water was given to the normal chickens while 0.5% H_2O_2 was given to the stress group from 1st to the termination of the experiments at day 14th

B. Thiopental's analgesic ED_{50} value

Thiopental antinociceptive influence in the stressed chickens with H_2O_2 was increased by 18% in association with normal chickens that have been fined by calculation of the analgesic ED_{50} value of thiopental essential to cause in 50% of the chickens to be 13.61 and 11.20 mg/kg, IP in the normal and stressed chickens, respectively that resemble the ameliorative influence of OS on the thiopental analgesic ED_{50} which is reported here for the first time in chicken and Table 1-B exemplify the various results gained from this trial.

Table 1-B. Thiopental's analgesic ED_{50} of normal and stress chickens

Parameter	Groups	
	Normal (H_2O)	Stress (H_2O_2)
Analgesic ED_{50} = XF + K × D	13.61 mg/kg, IP	11.20 mg/kg, IP
The extent of the doses used	5-35 mg/kg	5-35 mg/kg
First dose used	35 mg/kg	35 mg/kg
Last dose used (XF)	5 mg/kg	15 mg/kg
Table Value (K) (Dixon, 1980)	0.861	-0.380
± in the dose (D)	10 mg	10 mg
Chickens used	6 (XXOXXO)*	7 (XXXOOXX)*
OS influence on thiopental analgesic ED_{50} = Effect of OS= H_2O- H_2O_2/ H_2O×100= 18%		

*X: result (analgesia), O: no result (no analgesia)
Tab water was given to the normal chickens while 0.5% H_2O_2 was given to the stress group from 1st to the termination of the experiments at day 14th
Nociceptive effect recorded pre and post 15 min as thiopental treatment by using an electro-stimulator device

Thiopental plasma concentration in the normal and stressed chickens at different times

Table 2 and Figure 2 show a significant elevation in the thiopental plasma concentration of the stressed chickens compared to the normal one. The plasma concentration of thiopental measured at time 0.5, 1, 2, 4 and 24 hours after its injection at 35 mg/kg, IP were 91.42, 54.35, 38.27, 22.30 and 7.51µg/ml in the normal chickens while the plasma concentration was increased by 74, 84, 48, 85 and 82% to be 159.01, 100.06, 56.71, 41.30 and 13.63 µg/ml in the stressed chickens, respectively.

Table 2. Plasma concentration of thiopental in the normal and stressed chickens at different times

Groups	Times (Hour)				
	0.5	1	2	4	24
Normal (H_2O) (µg/ml)	91.42 ± 8.60	54.35 ± 4.95	38.27 ± 1.83	22.30 ± 2.12	7.51 ± 0.66
Stressed (H_2O_2) (µg/ml)	159.01 ± 10.19*	100.06 ± 5.54*	56.71 ± 2.85*	41.30 ± 3.90*	13.63 ± 0.75*
OS influence on plasma concentration of thiopental = Effect of OS= H_2O_2- H_2O/ H_2O×100= (%)	+74	+84	+48	+85	+82

Data represent Mean ± Standard Error for 5 chickens/measured time
Tab water was given to the normal chickens while 0.5% H_2O_2 was given to the stress group from 1st to the termination of the experiments on day 14th
Thiopental therapy 35 mg/kg, IP for both the normal and stress chickens
*: significantly different from normal group ($p < 0.05$)

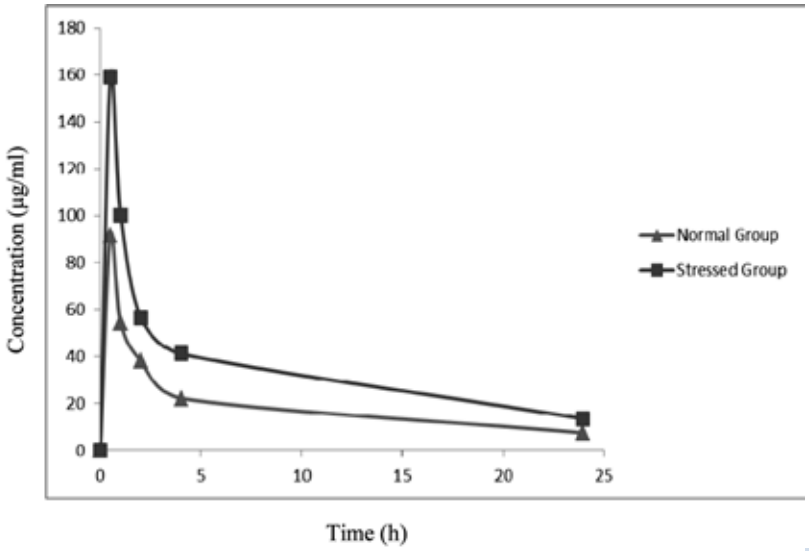

Figure 2. Thiopental's plasma concentration in the normal and stressed chickens at different times

Thiopental pharmacokinetics profiles in the normal and stressed chickens

Thiopental pharmacokinetics profiles in the normal chickens, which include $AUC_{0-\infty}$ (548.93 µg.h/ml), $AUMC_{0-\infty}$ (8046.58 µg.h^2/ml), MRT (14.66 h), $t_{1/2\beta}$ (10.49 h), Cl (0.06 L/h/kg), K_{el} (0.07 h^{-1}), V_{ss} (0.97 L/h/kg), T_{max} (0.5 h) and C_{max} (91.42 µg), while these values changed in the stressed chickens to be (999.55 µg.h/ml), (15573.44 µg.h^2/ml), (15.58 h), (11.36 h), (0.04 L/h/kg), (0.06 h^{-1}), (0.57 L/h/kg), (0.5 h) and (159.01 µg), respectively.

Table 3 shows the pharmacokinetic parameters of thiopental ($AUC_{0-\infty}$, $AUMC_{0-\infty}$, MRT and $t_{1/2\beta}$) were increased in the stressed chickens by 82, 94, 6 and 8%, respectively. On another hand, the Cl, K_{el}, and V_{ss} were decreased in the same group by 33, 14 and 41%, respectively in comparison to the normal group.

Table 3. Pharmacokinetics profiles of thiopental in normal and stressed chickens

Pharmacokinetics parameters	Groups		Effect of OS= H_2O_2-H_2O/ H_2O×100= (%)
	Normal (H_2O) group	Stressed (H_2O_2) group	
$AUC_{0-\infty}$ (µg.h/ml)	548.93	999.55	+82
$AUMC_{0-\infty}$(µg.h^2/ml)	8046.58	15573.44	+94
MRT (h)=AUMC/AUC	14.66	15.58	+6
$t_{1/2\beta}$ (h)	10.49	11.36	+8
Cl (L/h/kg)= dose/AUC	0.06	0.04	-33
K_{el} (h^{-1})= 0.693/ $t_{1/2\beta}$	0.07	0.06	-14
V_{ss} (L/kg)= dose.AUMC/$(AUC)^2$	0.97	0.57	-41
T_{max} (h)	0.50	0.50	0
C_{max} (µg)	91.42	159.01	-74

Tab water was given to the normal chickens while 0.5% H_2O_2 was given to the stress group from 1st to the termination of the experiments at day 14th

Thiopental treatment with dosage of 35 mg/kg, IP for both the normal and stressed chickens

DISCUSSION

The purpose of this study was to use thiopental in chickens for the induction of the general anesthesia and to find out the influence of OS status (induced with H_2O_2) on the thiopental anesthesia, its plasma concentration and pharmacokinetic profile. Thiopental is considered a general anesthetic that stimulates the rapid induction of anesthesia with its increasing the effect of GABA neurotransmitter on the $GABA_A$ receptor which causes depression of the CNS (Clark et al., 2012; Flecknell, 2015; Katzung and Trevor, 2015). Thiopental frequently used as an anesthetic for short surgical operation because it has a good hypnotic effect with occasionally less analgesic and muscle relaxant efficiency (Clark et al., 2012; Katzung and Trevor, 2015) and is considered a safe drug of choice for using in anesthesia because it possesses many benefits including a familiar mechanism of action, less myocardial and cerebral ischemia, decreasing histamine release with a uniquely stable hemodynamic status during anesthesia (Atasoy et al., 1993; Sumitraa et al., 2004; Ninu et al., 2015; Biswas et al., 2017) and was found to have efficient anesthesia than other barbiturates (Ferreira et al., 2013; Shaaban et al., 2018; Brohi et al., 2019). The result of this trial clears up the anesthetic profile of thiopental in the normal and stressed chickens through determining the analgesic and hypnotic ED_{50} values which were decreased in the stressed chickens with H_2O_2 that revealed an increase in thiopental efficacy and possibly increased its toxicity which was in agreement with earlier studies on diazepam sedation (Mousa and Mohammad, 2012a), xylazine analgesia (Mousa and Mohammad, 2012b) and ketamine anesthesia (Mousa, 2014). Thiopental interact and binds to the $GABA_A$/ chloride ionophore receptor complex, thereby enhancing the inhibitory actions of $GABA_A$ in the CNS and this will leads to synaptic inhibition, diminished neuronal excitability, induce anesthesia and lower the nociceptive threshold thus producing the analgesic effect (NCBI, 2020). Thiopental has a mode of action on the CNS (this attributed to reason for choosing it) because of the CNS is more vulnerable to OS status due to its high free radical formation, consuming a lot of oxygen, high amount of unsaturated fatty acids with less antioxidant activity (Pastore et al., 2003). The results show that thiopental plasma concentration was significantly increased with subsequent altering in its pharmacokinetic profile in the stressed chickens compared with the normal chickens. This modification in the thiopental pharmacological response in the stressed chickens attributed to H_2O_2-induced OS and thus causes phar-

macokinetics interaction (Mousa, 2014). H_2O_2-induce OS through its formation of free radicals that break down the vital cell components, especially the protein receptors (Patockova et al., 2003; Sayre et al., 2008), therefore, causes pharmacokinetic interaction by altering the biological constitutes important for absorption, distribution, metabolism and excretion of thiopental that determine the pharmacokinetic profile of thiopental. H_2O_2-induce OS can alter the pharmacokinetic profile of the drugs through its direct and indirect break down of the cell components (especially proteins which included plasma proteins and protein carriers) and thus the organs (liver, kidney, CNS and so on) which have a simultaneous modification at a level of the absorption, distribution, metabolism and excretion of thiopental and so, leading to pharmacokinetics interaction (Chauhan et al., 2020) which is stated here in this study through measuring the pharmacokinetics profile of thiopental indicated by increasing the half-life and decreasing the clearance, elimination rate constant and distribution volume of thiopental of the stress chickens compared to the normal one. The pharmacokinetics interaction has an impact to elevate drug efficacy and subsequent toxicity and this criterion of the drug are an important factor ensuring the drug delivery to the site of action on its target receptor to achieve efficacy and/or improve the preferred therapeutic effect (Wen et al., 2015).

CONCLUSIONS

The outcome of this study reveals the increase of the thiopental pharmacological response affected by H_2O_2-induced OS by increasing its concentration in the plasma and altering the pharmacokinetics profile. We recommended reducing the dose of thiopental to be given to the stressed animals.

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CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

The evaluation of dietary application of conjugated linoleic acid on performance, egg quality, blood parameters, antioxidant capacity and egg yolk cholesterol parameters in layer quails

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ABSTRACT: Conjugated linoleic acid (CLA) is a poly unsaturated fatty acid (FA) which is accepted as favorable for human health. The aim of this study is to ascertain the effects of CLA on performance, egg quality traits, egg yolk and albumin pH levels, blood serum biochemical parameters, egg yolk cholesterol content and blood antioxidant capacity in layer quails. 96 7-weeks-old female Japanese quails divided to 4 groups with 6 subgroups and the groups fed with basal diet supplied with 0 g/kg, 10 g/kg, 20 g/kg and 30 g/kg CLA respectively for 8 weeks. Our results indicated that CLA supplementation did not statistically affect performance, egg quality traits, egg pH levels, total antioxidant capacity in blood serum and yolk cholesterol content. Although dietary CLA did not significantly differ between groups for blood serum total protein, glycose, total cholesterol, triglyceride and high-density lipoprotein; lipoprotein lipase levels were significantly decreased in CLA supplemented groups (p<0.05). In summary, our results have shown dietary CLA supplementation might affect lipid metabolism and enzyme activity in female Japanese quails.

***Keywords*:** Conjugated linoleic acid, layer quail, performance, antioxidant, cholesterol

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INTRODUCTION

Conjugated linoleic acid (CLA) which is an aggregate term for a blend of geometric (*cis, cis*; *cis, trans*; *trans, cis*; and *trans, trans*) and positional isomers of linoleic acid (12:2n-6;LA) with conjugated double bound system which can be either *trans* or *cis* configuration (Hur et al., 2007; Shokryazdan et al., 2017). Since its discovery in 1980s, many studies have also been focused on to investigate the benefits of CLA to human health such as immune modulation, fat mobilization in body, anticarcinogenic and antiatherogenic effects (Suksombat et al., 2006; Liu et al., 2016). It's estimated that a human weight around 70 kg should consume minimum 3 g of CLA/d to benefit its health favorable effects (Ahn et al., 1999). CLA is found in many animal products as beef and dairy, pork, marine products, poultry products and vegetable oils besides synthetically produced ones (Hur et al., 2007). Although the main natural resource of CLA is ruminant meat and milk, several researchers stated meat and eggs of poultry could be enriched with CLA by dietary supplementation (Du and Ahn, 2002; Bölükbaşi, 2006). Since CLA readily integrated into the fat fraction, poultry eggs stand out in this regard due to the yolk's 30-35 % lipid content (Suksombat et al., 2006).

In animal nutrition one of the main goals is to provide sustainable healthy and good quality livestock products for human consumption. CLA might be consider as a potential feed additive to reach this target due to its beneficial effects on human health along with cholesterol reducing and antioxidant activity in livestock metabolism (Hur et al., 2007). Ha et al. (1990) mentioned that β-hydroxyl acrolein moiety in CLA structure might be the reason of its antioxidative effect. Several studies have showed CLA had improved oxidative stability in mice (Ha et al., 1990), broiler chickens (Ko et al., 2004; Zhang et al., 2008a) and pigs (Joo et al., 2002). Although several researches have shown that dietary CLA supplementation decrease blood lipid parameters in rats (Kloss et al., 2005), geese (Zhang et al., 2008b) and broilers (Bölükbaşi, 2006), the cholesterol reducing effect of CLA is unclear for egg yolk. Hur et al., (2003) demonstrated that 1, 2.5 and 5% of dietary CLA supplementation significantly decreased cholesterol content in egg yolk after 5 weeks of feeding trial in layers. On the other hand, Szymczyk and Pisulewski, (2003) stated no difference observed on cholesterol level of yolk in commercial layers that fed with diets comprised 0, 5, 10, 15 or 20 g pure CLA/kg. Different from those studies, Yin et al., (2008) observed CLA supplementation on White Leghorn and Brown Dwarf layer diets increased yolk cholesterol levels except for 2.5 % CLA in Brown Dwarf layers.

Quail eggs come to the forefront among the poultry eggs due to their great nutritional value, rich vitamin and mineral content (Tunsaringkarn et al., 2013). Studies on potential effect of CLA on layer and broiler quails are very limited. Aydin (2007) stated giving CLA alone with diet could create adverse effects on layer quails, and combination of CLA with 5% and 10% canola protect quail eggs against CLA-related pH changes in egg yolk by preserving the ratio of total unsaturated fatty acids and total saturated fatty acid. Aydin et al., (2006) reported that dietary 0.5% CLA supplementation significantly decreased body weight at the end of the study yet statistically improved breast muscle in male Japanese quail. Aydin and Cook (2004) observed 0.25 % dietary CLA supplementation increased egg size, yet 2 or 3% CLA had reduced egg proportions in Japanese quails. In the same study, all experiment groups except for the group fed with 0.25 % CLA had bigger liver sizes. No study has been conducted on effect of egg quality, antioxidant capacity and cholesterol content of egg yolk in layer quails.

In light of the above, the purpose of this study was to determine the outcomes of graded dietary levels of CLA (0, 10, 20 and 30 g/kg) on performance, egg quality, serum biochemical parameters, blood antioxidant capacity and egg cholesterol levels in layer Japanese quails.

MATERIALS AND METHODS

Birds, experimental diets and management

This research was approved by Selcuk University Faculty of Veterinary Ethics Committee of Experimental Animals Production and Research Center (2020/21). Furthermore, all procedures conducted in this study complied with the European directives for the use and care of animals in research (Directive 2010/63/EU).

A total of 96 7-weeks-old female Japanese quails (*Coturnix coturnix Japonica*) were split to 24 cages and divided into 4 experimental groups with 6 replicates non-specifically. The quails had *ad libitum* access to feed and water on 16 hours light and 8 hours dark program per a day. The animals fed with a commercial diet based on corn/soybean formulated to be isocaloric and isonitrogenous (Table 1) was manu-

factured in a local feed mill. CLA used in the study provided from a commercial company (Lutrell® Pure, BASF). The CLA supplement was a complex of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 18:2 isomers (11.8% of total FA composition was the *cis*-9, *trans*-11 isomer of CLA, 12.1% of total FA was the *trans*-10, *cis*-12 isomer of CLA, 16.5% of total FA was C16:0, 46.0% was C18:0 and 9.8% of total FA was C18:1 of the total fatty acid components) protected by the lipid encapsulation technique. The first group (Control) fed with a basal diet without supplementation. The experimental groups received 5 g/kg, 10 g/kg and 20 g/kg CLA supplementation in their diet respectively. During the trial, eggs were collected on a daily basis and weighed with 0.01 g scales to record average egg weight of each subgroup. In every two weeks, the quails weighed one at a time and feed intake of all birds in the cage were measured.

Table 1. Ingredients and calculated composition of the basal diets

Ingredient	%
Corn	50.60
Soybean Meal	25.62
Sunflowers Meal	10.50
Vegetable Oil	4.90
Limestone	6.00
Dicalcium Phosphate	1.56
Salt	0.40
Premix[1]	0.35
L-Lysine	0.25
DL-Methionine	0.22
Total	**100.00**
Chemical Composition	
Metabolizable Energy (kcal/kg)	2913
Crude Protein (%)	20.31
Ca %	2.72
Available P %	0.40
Lysine %	1.19
Methionine %	0.51

[1]Premix contains: Manganese: 60 mg; Zinc: 50 mg; Iron: 30 mg; Copper:5 mg; Selenium: 0.1 mg; Vitamin A: 8.800 IU; Vitamin D3: 2.200 IU; Vitamin E: 11 mg; Nicotine acid: 44 mg; Cal-D-Pan: 8.8 mg; Riboflavin: 4.4 mg; Thiamine: 2.5 mg; Vitamin B12: 6.6 mg; Folic acid: 1 mg; D-Biotin: 0.11 mg; Coline: 220 mg.

Egg quality characteristics, pH levels and yolk cholesterol determination

6 eggs were picked up from each subgroup for 3 days at the last week of the experiment. Each egg was weighed and an egg force reader (Egg Tester, Orka Technology, UT, USA) was used to determine egg shell strength. All collected eggs were broken on a flat glass plate a digital caliper was used to gauge the length and width of albumen and diameter of yolk. The obtained values applied to calculate albumen index [(albumen height/average of albumen length and albumen width) x 100], yolk index [(yolk height/yolk diameter) x 100], and Haugh unit [(100 x Log(H + 7.57–1.7W$^{.37}$), in the formula H means albumen height, W means egg weight, were calculated.

36 eggs from each group were gathered during the 8th week of the study to determine egg yolk and albumen pH levels. The collected eggs were stored in a refrigerator for 14 days at +4°C. Albumens and yolks of the eggs were separated and stirred at 1st, 7th and 14th day of the storage. A digital pH meter (Hanna Instruments, USA) was used to determine yolk pH and albumin pH of the eggs.

The cholesterol level of quail egg yolk was measured by using the methods descripted by Hammad et al. (1996) and Kaya et al. (2003). 3 eggs were randomly picked up from each subgroup at the end of the study to determined yolk cholesterol. The collected eggs were hard boiled for 5 minutes. 0,1 g egg yolk samples were separated and blended with 4 ml isopropyl alcohol. The samples were centrifuged at 3000 rpm for 10 min. cholesterol level in the quail egg yolk was measured with using of a commercial cholesterol kit (Siemens Healthcare Diagnostics, Marburg, Germany).

Serum biochemical and total antioxidant capacity examination

One quail was slaughtered at the end of 8 weeks' trial. Blood samples for the biochemical tests were collected from jugular vein and centrifuged at 3000 rpm for 10 min and stored at -24°C. Blood serum samples were measured to determine total cholesterol, total protein, glucose, lipase and high-density lipoprotein (HDL) with commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey). Total antioxidant capacity of blood serum was also measured with a commercial kit of same company.

Statistical analysis

Results are given as mean ± standard deviation. Performance, egg quality traits, egg pH levels, serum biochemical parameters, serum antioxidant capacity and egg yolk cholesterol data were analyzed by ANOVA in Minitab (2000). The significant differences between groups were determined by using Duncan post hoc test. Differences with values of $P < 0.05$ were considered to be statistically significant.

RESULTS

The effects of dietary CLA treatments on performance of layer quails are shown in Table 2. The changes in egg production, live weight gain, feed intake, egg weight and feed conversation rate between control and treatment groups were not statistically significant in the present experiment ($p>0.05$). On the other hand, the average egg weight was not significantly but numerically higher in the quails fed with 10 g/kg CLA supplementation than control and other treatment groups.

Table 3 illustrates the effects of different levels of CLA on eggshell breaking strength, Haugh unit, albumin index, yolk index and egg shape index of quail eggs. No significant differences between control group and treatment groups regarding the egg quality trait by feeding with CLA-enriched diets to Japanese quails ($p>0.05$).

Data illustrating the effect of dietary CLA supplementation in different levels on the egg pH values in 1, 15 and 30 days after the experiment are presented in Table 4. The CLA intake in Japanese quails did not differ in pH levels significantly in our study ($p>0.05$).

The results in Table 5 show the effect of dietary CLA on blood serum biochemistry status. Total protein, glycose, total cholesterol, triglyceride and HDL values didn't significantly differ between the groups because of CLA supplementation ($p>0.05$). On the other hand, Lipoprotein lipase decreased significantly in the groups fed with CLA enriched diets ($p<0.05$).

The effect of dietary CLA supplementation on blood serum total antioxidant capacity in Japanese quails are shown in Figure 1. CLA intake didn't differ total antioxidant capacity in blood serum between control and treatment groups ($p>0.05$). Data in Figure 2 demonstrates the effect of dietary CLA supplementation in different levels on egg yolk cholesterol content. Yolk cholesterol levels weren't affected by dietary CLA ($p>0.05$).

Table 2. The effect of dietary CLA supplementation on production performance in Japanese quails.

Parameters	CLA0	CLA5	CLA10	CLA20	*P*-Value
Egg production (%)	77.59±1.92	73.59±3.45	77.87±2.69	80.43±2.81	0.395
Body weight gain (g)	42.50±6.57	47.08±2.79	40.71±2.31	39.42±4.32	0.623
Feed Intake (g)	26.52±0.46	25.93±0.55	26.61±0.53	25.22±0.18	0.148
Egg weight (g)	11.44±0.32	11.72±0.18	14.22±1.81	11.73±0.10	0.150
Egg mass (g/bird per day)	9.63±0.41	9.00±0.34	10.10±0.55	9.45±0.20	0.295
Feed conversion ratio (g/g)	2.77±0.08	2.90±0.11	2.66±0.09	2.67±0.05	0.192

CLA0, the basal diet; CLA5, 5 g CLA/kg diet; CLA10, 10 g CLA/kg diet; CLA20, 20 CLA/kg diet. Values are expressed as mean ± SE

Table 3. The effect of dietary CLA supplementation on egg quality traits in Japanese quails.

Parameters	CLA0	CLA5	CLA10	CLA20	*P*-Value
Eggshell breaking strength (N)	1344.87±52.05	1373.57±55.95	1396.62±38.76	1317.53±57.40	0.757
Haugh unit	87.59±0.71	86.11±0.72	86.91±0.60	85.66±1.34	0.493
Albumen Index	4.72±0.15	4.43±0.16	4.79±0.15	4.63±0.13	0.384
Egg yolk index	45.08±0.60	44.83±0.54	45.67±0.66	45.69±0.53	0.744
Egg shape index	75.84±0.49	76.86±0.55	76.94±0.69	77.26±0.63	0.598

CLA0, the basal diet; CLA5, 5 g CLA/kg diet; CLA10, 10 g CLA/kg diet; CLA20, 20 CLA/kg diet. Values are expressed as mean ± SE

Table 4. The effect of dietary CLA supplementation on egg quality traits in Japanese quails at 1st, 15th and 30th days after the study

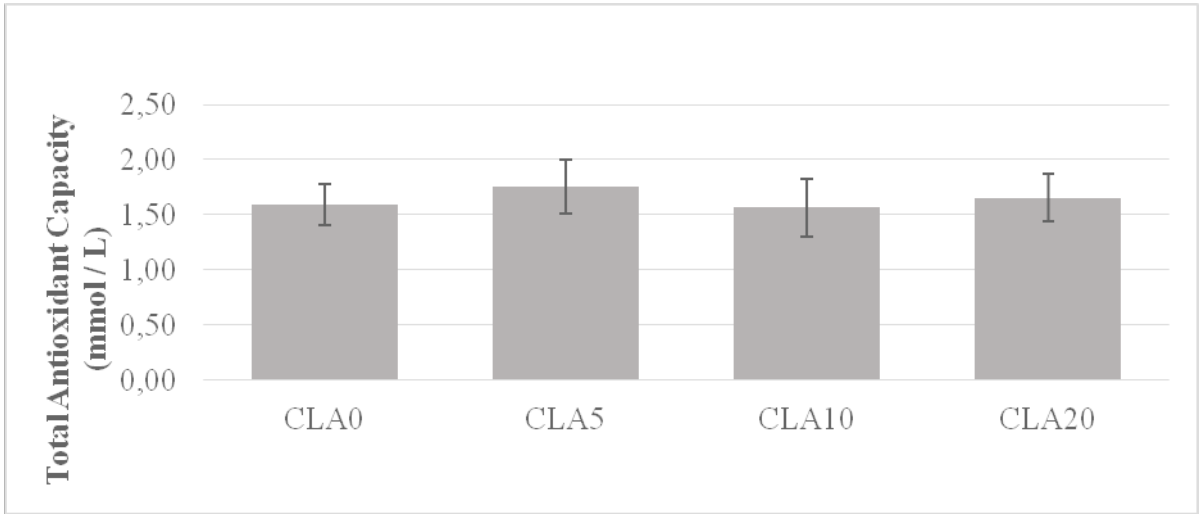
Days	Parameters	CLA0	CLA5	CLA10	CLA20	*P*-Value
1st Day	Yolk pH	5.95±0.22	5.88±0.06	5.78±0.56	5.88±0.10	0.548
	Albumen pH	8.84±0.15	8.84±0.16	8.86±0.20	8.88±0.10	0.884
15th Day	Yolk pH	6.27±6.27	6.26±0.17	6.23±0.23	6.35±0.24	0.547
	Albumin pH	9.16±0.05	9.11±0.12	9.15±0.04	9.12±0.13	0.549
30th Day	Yolk pH	5.89±0.36	5.26±0.25	5.61±0.32	5.83±0.24	0.532
	Albumin pH	8.27±0.30	8.69±0.16	8.19±0.26	8.65±0.10	0.386

CLA0, the basal diet; CLA5, 5 g CLA/kg diet; CLA10, 10 g CLA/kg diet; CLA20, 20 CLA/kg diet. Values are expressed as mean ± SE

Table 5. The effect of dietary CLA supplementation on blood serum biochemical parameters in Japanese quails.

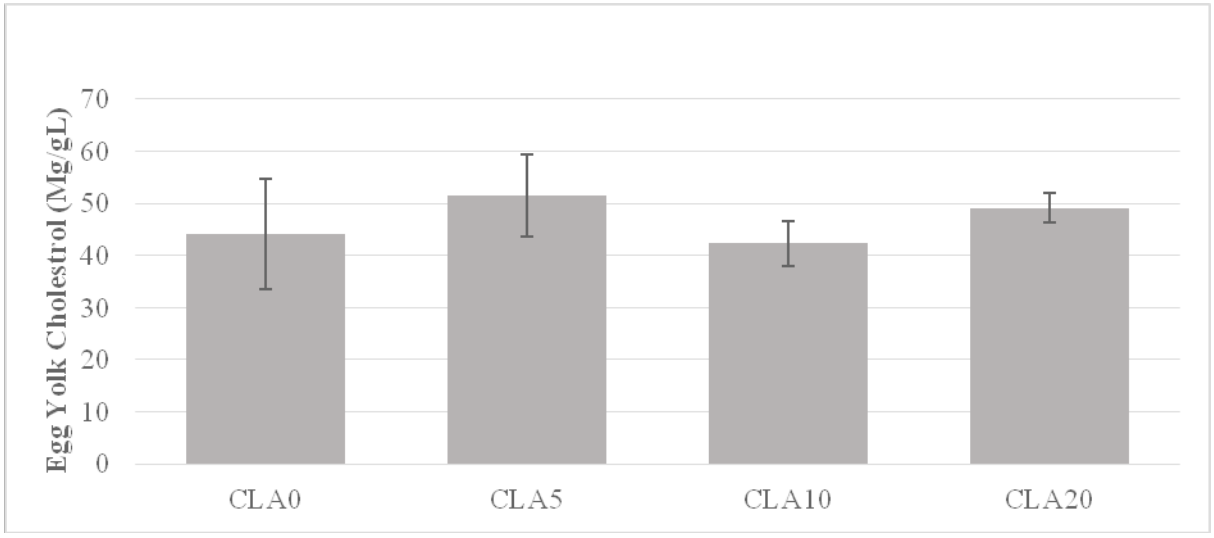
Parameters	CLA0	CLA5	CLA10	CLA20	*P*-Value
Total protein (g/dl)	3.40±0.18	3.70±0.20	3.26±0.26	3.35±0.16	0.471
Glycose (mg/dl)	345.92±10.54	344.67±11.69	325.33±10.36	348.33±6.75	0.351
Total cholesterol (mg/dl)	221.42±18.87	232.00±11.47	200.42±11.77	211.33±15.13	0.470
Triglyceride (mg/dl)	657.00±3.34	649.67±4.83	657.00±62.71	665.67±5.94	0.479
HDL (mg/dl)	166.25±42.46	230.56±44.34	207.30±43.69	197.40±43.45	0.765
Lipoprotein lipase(U/l)	15.08±0.89[b]	12.00±0.46[a]	11.50±0.38[a]	12.08±0.50[a]	0.0003

CLA0, the basal diet; CLA5, 5 g CLA/kg diet; CLA10, 10 g CLA/kg diet; CLA20, 20 CLA/kg diet. Values are expressed as mean ± SE. [a–b] Means within a row with unlike superscripts are different (p <0.05)



CLA0, the basal diet; CLA5, 5 g CLA/kg diet; CLA10, 10 g CLA/kg diet; CLA20, 20 CLA/kg diet.

Figure 1. The effect of dietary CLA supplementation on blood serum total antioxidant capacity in Japanese quails.



CLA0, the basal diet; CLA5, 5 g CLA/kg diet; CLA10, 10 g CLA/kg diet; CLA20, 20 CLA/kg diet.

Figure 2. The effect of dietary CLA supplementation on egg yolk cholesterol content in Japanese quails

DISCUSSION

CLA supplementation to the diets of layer Japanese quails didn't affect the animals' performance in the present experiment ($p>0.05$). Our results agree with previous reports that have reported no detrimental effect on performance of laying hens (Meluzzi et al., 2003; Shang et al., 2004; Alvarez et al., 2005; Kim et al., 2007; Koronowicz et al., 2016) and quails (Aydin et al., 2006) fed with CLA. Besides several researchers informed that layer hens fed with CLA enriched diets also had adverse effect on egg weight, egg production, feed consumption and feed conversion rate than the animals fed with basal diet (Ahn et al., 1999; Szymczyk and Pisulewski, 2003; Shang et al., 2004; Yin et al., 2008). It has been reported that certain amounts of CLA could be an intense inhibitor of body fat accumulation, therefore CLA might decrease feed intake and feed conversion rate in mice rats and chickens (Szymczyk et al., 2001). In our study, the average egg weight of the quails fed with 10 g/kg CLA supplementation was higher than the control and other treatments numerically. Dietary CLA applications have been reported to reduce egg weight and egg production in some research in poultry (Suksombat et al., 2006; Yin et al., 2008). Contrarily, Aydin et al., 2006 reported that dietary CLA at levels of 0.5 % improved average egg weights statistically at 3rd and 4th weeks of their experiment in Japanese quails. These might be due to promoting effect of certain amount of dietary CLA supplementation on thyroid hormone and estradiol which affect positively on egg weight and performance (Liu et al., 2016).

Egg quality traits were not affected with the treatments compared to the control group ($p>0.05$). Similar to our findings, Vashan et al. (2008) couldn't also report any significant differences between the layer hens fed with 0 %, %4 and %10 safflower oil, rich with linoleic acid. Furthermore, other researchers have mentioned dietary CLA treatments were ineffective on egg Haugh unit in hens (Suksombat et al., 2006; Liu et al., 2016). Whereas, Kim et al. (2007) recorded hens fed with 2 CLA-enriched diet had lower Haugh units but higher egg yolk index than control and other CLA treatments. (Alvarez et al., 2005) also noted dietary %2 CLA supplementation showed highest values in albumen height and Haugh unit in hens. The egg quality alterations in the hens fed with %2 CLA enriched diet might be related with the changes in yolk water content and fat composition in vitelline membrane by virtue of ion movement through the membrane (Alvarez et al., 2005; Kim et al., 2007).

pH of yolk and albumin are important measures for egg quality in poultry sector along with Haugh Unit, albumin and yolk index (Lee et al., 2016). No effect of dietary CLA concentration on pH levels of yolk and albumin were observed during storage at +4°C for 30 days ($p>0.05$). Correlatively, Alvarez et al. (2005) also informed CLA addition to hens' diet didn't statistically affect albumen and yolk pH. Whereas, it has been reported that discoloration of egg yolk and albumen or undesirable pH changes can be seen when the chicken were fed with CLA, supplemented with low fat diets. Probable cause of this result could be modification of the levels of mono-unsaturated fatty acids and saturated fatty acids in the eggs of animals fed with CLA-enriched diets (Aydin, 2007). Another study on hen eggs indicated that yolk pH was higher in the groups fed with CLA diets than control group (Ahn et al., 1999). In the related study, it is estimated that the ion movement from yolk and albumen through the yolk membrane in the eggs of hens fed with CLA was higher than control as a possible cause of elevated pH in yolk. This might be a result of *cis-trans* arrangements of CLA that increased permeability of yolk membrane.

From our observation, no significant differences were observed in blood serum biochemical parameters ($p>0.05$) except for Lipoprotein lipase ($p<0.05$). In addition, HDL levels in blood serum were not statistically significant, but numerical higher in the layer quails fed with CLA supplementation. Dietary CLA supplementation is accepted to have plasma and abdominal lipid lowering effects by inhibiting endogenous fatty acid production in the body (Lin et al., 2001). Several researches also demonstrate dietary CLA supplementation have increased blood HDL content in broiler (Szymczyk et al., 2001; Bölükbaşi, 2006) and layer chickens (Yin et al., 2008; Wang et al., 2019) and geese (Zhang et al., 2008b). Japanese quails submitted to CLA in our study had lower lipoprotein lipase activities in blood serum than the control group. Lipoprotein lipase is an extracellular enzyme that is synthesized mostly in skeletal muscle and adipose tissues. The enzyme mostly hydrolyze triglycerides in the structure of chylomicrons and lipoproteins to glycerols and free fatty acids (Zhang et al., 2007). The product used in the study contains *trans*-10 *cis*-12 isomers of CLA acknowledged to reduce lipid accumulation in adipocytes by inhibiting lipoprotein lipase activiy in metabolism (Kim et al., 2002). Similar to our findings, there are several studies noted lipoprotein lipase inhibiting effect of CLA

in broilers (Zhang et al., 2007), hamsters (Zabal*a* et al., 2006) and cultured 3T3-L1 adipocytes (Lin et al., 2001).

In the present study, CLA intake didn't statistically differ blood serum TAS concentrations between groups ($p>0.05$). The effect of dietary CLA to antioxidant defense system in the metabolism is still not clear (Zhang et al., 2008a). Several studies have also shown dietary CLA decreased lipid peroxidation in broiler meat (Zhang et al., 2008a; Liu et al., 2017) and hen eggs (Hur et al., 2003). Zhang et al., (2008a) indicated dietary CLA could show antioxidant effect both boosting the antioxidant enzyme activity and inhibiting the reactive oxygen species chain reactions. On the contrary, Ko et al. (2004) reported broiler chickens fed with the diet containing 1.5% CLA improved hepatic catalase activity significantly but other antioxidant enzymes were not affected . Leung and Liu (2000) reported that despite, *trans*-10, *cis*-12-CLA isomer indicated to have stronger free radical scavenging capacity, *cis*-9, *trans*-11-CLA could have prooxidant properties. The reason that we couldn't find any antioxidant effect of dietary CLA supplementation in the study might be due to the balance of the *trans*-10, *cis*-12-CLA (12.1% of total fatty acid components) and *cis*-9, *trans*-11-CLA (11.8% of total fatty acid components) in the product.

In our study, dietary CLA at the level of either 10, 20 and 30 mg/kg in diet didn't induce alterations in egg yolk cholesterol content ($p>0.05$). The effects of dietary CLA intake on egg yolk cholesterol levels are debatable. Similar to our results, Szymczyk and Pisulewski (2003) also reported CLA supplementation in layer diets didn't differ in egg yolk cholesterol level between groups. Wang et al. (2019) indicated that CLA addition didn't modify the levels of egg yolk cholesterol on day 28, yet caused a reduction in yolk cholesterol content on day 56. Besides, Hur et al. (2003) added the level of egg yolk cholesterol was decreased significantly in the groups fed with CLA supplemented diets for 5 weeks in layers. Yin et al., (2008) reported dietary CLA intake caused an elevation of yolk cholesterol amount in different breed of layer hens. The discrepancies between the outcomes of researches might be due to fatty acid composition CLA, experimental conditions, doses in diet or animal type.

CONCLUSION

The present study gave some evidence that dietary CLA supplementation might affect lipid metabolism and enzyme activity in female Japanese quails. More detailed studies are needed on correlation between metabolic lipid metabolism and egg lipid profile after dietary CLA supplementation in layer quails. Further studies should also aim to investigate the immunostimulant effects of CLA in poultry.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Research article
Ερευνητικό άρθρο

Comparative effects of addition of monensin, tannic acid and cinnamon essential oil on *in vitro* gas production parameters of sesame meal

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ABSTRACT: The aim of this experiment was to compare the effects of adding monensin, tannic acid and cinnamon essential oil on *in vitro* gas production parameters of sesame meal. Experimental treatments included sesame meal (control), sesame meal + 12 mg monensin/kg DM, sesame meal + 24 mg monensin/kg DM, sesame meal + 50 mg tannic acid/kg DM, sesame meal + 100 mg tannic acid/kg DM, sesame meal + 150 mg cinnamon essential oil/kg DM, sesame meal + 250 mg cinnamon essential oil/kg DM. The amount of gas produced by treatments fermentation was measured at 4, 6, 8, 12, 16, 24, 36, 48, 72, 96 and 120 hours after incubation. The results showed that gas production decreased significantly in 120 hours after incubation in the treatment containing monensin (at 12 and 24 mg/kg DM) and cinnamon essential oil (at 150 and 250 mg/kg DM) compared to the control treatment ($P<0.05$). The addition of monensin and cinnamon essential oil had a significant effect on increasing partitioning factor and fermentation efficiency compared to control treatment ($P<0.05$). Acid tannic at 100 mg/kg DM increased NEL, SCFA, OMDe and microbial protein compared to control ($P<0.05$). Ammonia nitrogen and total volatile fatty acids concentration at 120 h of incubation showed a significant increase in monensin and tannic acid supplementation compared to control ($P<0.05$), but cinnamon essential oil significantly decreased ammonia nitrogen concentration. In conclusion, cinnamon essential oil and monensin can be used in an environmentally conducive and acceptable way to diminish biogas emissions from ruminants; therewith ameliorate environmental conditions. However, the cinnamon essential oil can be easily used in livestock diets to improve fermentation and reduce biogas production.

***Keywords*:** Cinnamon essential oil, *in vitro* gas production, monensin, sesame meal, tannic acid

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INTRODUCTION

Sesame (*Sesame Indicum L*) belongs to the *Pedaliacea* family. Sesame has a straight stem of 150 to 180 cm in length and has single-branched and multi-branched varieties. The amount of oil from ranges 48 to 62 percent, in other sources, from 45 to 50 percent fat and some other sources mentioned a 50 to 55 percent range. Iranian sesame oil seeds have the highest amount of oil. Protein amounts of sesame arerange from 19 to 25 percent and carbohydrate content is from 10 to 15 percent (Jafari et al., 2019). This plant is cultivated to extract the oil (unsaturated containing several double bonds) present in its seeds.

By-products of agriculture industries such as sesame meal (SM) can have an important impact on diminishing production and ration costs. SM is a by-product of the sesame seed oil extraction. SM contains about 46% crude protein (CP) on dry matter basis, so it can be replacing soybean meal as a protein supplement in animal nutrition. Therefore, adding SM to livestock feeds in particular helps producers reduce the effect of a universal increase in feed costs. Numerable studies have appraised the efficacy of using SM on animal nutrition (Omer et al., 2019).SM is rich in methionine, arginine and leucine amino acids but has very little usable lysine (Rezaeipour et al., 2016).

In ruminants, feed efficiency is low due to rumen fermentation and gas production. Methane (CH_4) production has a direct relationship with feed consumption and about 7-9% of gross energy is consumed as CH_4. Ionophores such as monensin may be recognized to be effective in the rumen bio-hydrogenation process due to reducedCH_4 production by preventing the growth of H^+ bacteria. The utilization of ionophores as an oral antibiotic in livestock has been prohibited in some areas (such as the European Union). Therefore, secondary plant metabolites such as plant essential oils, saponins and tannins have been proposed as potential tools for manipulating the bacterial populations involved in gas production in rumen (Ishlak et al., 2015; Yao et al., 2020). Natural extracts from plants and tannic acid have been used to reduce methane production, reduce rumen degradation of protein and reduce ammonia production, increase rumen escaping protein for greater digestion in the gut and improve rumen fermentation efficiency (Al-Jumaili et al., 2017; Njidda et al., 2017).

Essential oils are used in animal nutrition for replacing growth promoting antibiotics (Besharati et al., 2020). In a study, researchers reported that among the chemical constituents of essential oils, oxygen monoterpens (especially alcohols) and monoterpene aldehydes strongly affect the growth and metabolism of rumen microbes, while hydrocarbons have lower monoterpene and inhibitory effects and sometimes arouse microbial activity (Taktak and Badawy, 2019). The chemical constituents of the essential oils have a great effect on the activity of rumen microorganisms and can improve energy and nitrogen consumption in the rumen. The researchers have found that adding cinnamon, thyme and clove essential oils (one µl per ml of culture medium) to the basal diet containing 80% forage and 20% concentrate reduced the rate of disappearance of dry matter (DM), crude protein (CP), and ammonia nitrogen concentrations. They as well as explained that the usage of cinnamon and thyme essential oils significantly reduced the rate of gas production (Jahani-Azizabadi et al., 2011).

Cinnamon is a dried and crushed cinnamomum tree bark. The original cinnamon species is scientifically named *Cinnamomum zeylanicum*. Cinnamon is a plant that extract of its stem, young shoots and leaves have therapeutic application. Cinnamaldehyde is one of the major active ingredients in cinnamon essential oil. Cinnamaldehyde is a compound of phenylpropanoid with strong antimicrobial activity that comprises about 75% of cinnamon essential oil (Sharma et al., 2016). In one study, researchers reported that, among other secondary metabolites (including thymol and carvacrol), cinnamaldehyde had no effect on cell membrane stability. The researchers (Nayanathara et al., 2018) suggested that the antimicrobial effects of cinnamaldehyde are exerted by the reaction between its carbonyl group with the proteins in the pre-plasm and inactivation of microbial enzymes.

The aim of this study was to compare the effects of adding monensin, tannic acid and cinnamon essential oil on gas production parameters of SM.

MATERIAL AND METHODS

Chemical composition of SM, comprisingDM, ether extract (EE), crude ash (CA), acid detergent insoluble fiber (ADF) and neutral detergent fiber (NDF) were determined according to the proposed AOAC (2002) methods (Table 1). Crude protein was measured using a Kjeldahl analyzer (Foss Tecator AB analyzer, Hoganas, Swede Kjeltec 2300 Auto analyzer) according to AOAC standard method (AOAC, 2002).

Experimental treatments included SM (200 mg

DM, control), SM+12 mg monensin/kg DM, SM+24 mg monensin/kg DM, SM+50 mg tannic acid/kg DM, SM+100 mg tannic acid/kg DM, SM+150 mg cinnamon essential oil/kg DM, SM+250 mg cinnamon essential oil/kg DM.

Table 1. Chemical composition of sesame meal (% of DM)

Chemical composition	Sesame meal
DM	93.21
CA	9.93
OM	83.28
CF	14.30
CP	40.95
NDF	41.50
ADF	18.40

DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; CA: crude ash; OM: organic matter; CF: crude fat

Extracting Cinnamon essential oil

Cinnamon essential oil extraction was performed according to standard method using water distillation using Clevenger apparatus (Jahani-Azizabadiet al. 2011). For this purpose, the researcher first crushed cinnamon and then 70 g of the crushed sample was placed into the balloon of the Clevenger apparatus and 750 ml of distilled water was added to each balloon. The samples were then boiled for 3.5 hours after boiling. After this period, the essential oil was collected at the appropriate location in sterile glass (Jahani-Azizabadiet al. 2011). The cinnamon essential oils were determined by GC-mas (Agilent Technologies 7890B). Composition of cinnamon essential oil is shown in Table 1. Cinnamaldehyde content of cinnamon was obtained 72.32%.

***In vitro* gas production (IVGP) test**

Fedorak and Hrudy (1983) method was used to measure gas production. A 200 mg milled SM was weighed and transferred into 50 ml sterile serum bottles.

Rumen fluid was collected in a slaughter house using a four-layer cheese cloth and transferred rapidly to the laboratory in a 39° C water flask. Prior to transferring the rumen fluid into the serum bottles, they were mixed with 1:2 buffer prepared by McDougall (1948) method (one portion of rumen fluid and two parts of buffer). In each glass containing the experimental treatment, 20 ml of rumen and buffer mixture was added and after the anaerobic injection into the glass, the glass lids were tightly closed by rubber cap and metal press.

All bottles were transferred to the incubator shakerset at 39° C to measure gas production, and record the amount of gas produced by food fermentation using the Fedorak and Hrudy (1983) method was recorded at the time of 4, 6, 8, 12, 16, 24, 36, 48, 72, 96 and 120 hours after incubation.

Gas production parameters were calculated using the following mathematical model in the SAS package program according to the model reported by Palangi et al. (2020).

$$P = a + b\ (1\text{-}e^{-ct})$$

where 'P' is the disappearance at time 't', 'a' quickly degradable fraction (or washing loss), 'b' denotes slowly degradable fraction and 'c' is constant rate of degradation of 'b' (Palangi and Macit, 2019). Metabolizable Energy (ME) amounts of gas production (GP) and organic matter digestibility (OMD) were calculated using the equality reported by Menke et al. (1979) as:

$$ME(MJ/kg\ DM) = 2.20+0.136GP+0.057CP+0.0029CP^2$$

$$OMD(\%)=14.88+0.889GP+0.45CP+0.0651XA$$

where, XA ash in g 100 g-1 DM and GP is the net gas production (mL) at 24h short chain fatty acid was calculated using blow equation as:

$$SCFA(mmoL)=-0.00425+0.0222GP$$

where,

Gas is 24 h net gas production (mL/g DM).

The Makkar method was used to determine the partitioning factor (representing the amount of microbial protein synthesis). The partitioning factor (PF) is the mg of degraded organic matter per ml of gas produced in according with the following equation (Makkar, 2010).

$$PF= \frac{c-(a-b)}{IVGP}$$

where,

c= OM (mg), a= undegraded OM (mg), b= ash of fraction a and IVGP= amount of produced gas at 24 h.

The undegraded organic matter was also calculated based on the following equation (Makkar, 2010):OMDe (mg) = *c - (a -b)*

After measuring the volume of gas produced

during 24 hours of incubation, the contents of the bottle were transferred to a container and washed for 1 hour with neutral detergent. The contents of the detergent solution were then filtered with ash-free filter paper and the residue was dried by oven at 100 ° C for 10 hours and calculated fraction b. With subtracting b from a, undegraded OM was calculated in mg (Makkar, 2010; Vercoe et al., 2010).

The values of produced microbial mass and the efficiency of microbial mass synthesis were also calculated using the following equation (Makkar, 2010; Vercoe et al., 2010).

$MM\ (mg) = [c- (a- b)]-[NG_{ml} \times 2.2]$

where;

MM, mg produced microbial mass, NG, ml of produced pure gas and 2.2, stoichiometric coefficient.

Measurement of ammonia

20 ml of the boric acid reagent was poured into a 100 to 150 ml Erlenmeyer flask. 0.8 g of magnesium oxide was placed in a 150 ml Erlenmeyer flask and it was fixed below the sample inlet. 20 ml of rumen fluid was poured from the sample inlet. Distillation continued until about 50 ml of distilled material was collected in the collection tank. Then the solution was titrated with 0.1N sulfuric acid (Markham, 1942).

Determination of volatile fatty acids

One ml of 25% metaphosphoric acid (v/w) was added to 5 ml of filtered extract to determine total volatile fatty acids. The prepared samples were stored at -20 ° C. Prior to analysis, the samples were incubated at room temperature overnight to melt the frozen samples (Markham, 1942). Then tVFA determined colorimetric method.

Statistical analysis

Data were analyzed withina completely randomized design with General Linear Model (GLM) using-SAS (2018), with Duncan's multiple range test used for the comparison of means.

RESULTS

Composition of cinnamon essential oilwas presented in Table 2. The effect of addition of monensin, tannic acid and cinnamon essence on gas production (GP) of SM was shown in Table 3. The GP characteristics, GP potential and rate of GP were presented in Table 4. The effects of adding monensin, tannic acid and cinnamon essential oil on the fermentation parameters estimated in GP, PF, and microbial biomass production efficiency of SM were shown in Table 5. Effect of treatments on VFA, N-NH_3 and pH after 120 h incubation were shown in Table 6. The graph of GC-mas of cinnamon essential oil was presented in Figure 1.

Table2. Composition of cinnamon essential oil in 100 ml.

Composition	Percentage
C_8H_8	0.55
C_7H_6O	1.31
C_9H_8O	72.32
$C_9H_{10}O$	0.1
$C_{10}H_8O$	0.13
$C_{10}H_{10}O$	3.2
$C_{10}H_{12}O$	0.69
$C_{10}H_{16}O$	3.17
$C_{10}H_{18}O$	0.16
$C_{13}H_{18}$	2.24
$C_{15}H_{20}$	0.75
$C_{15}H_{22}$	1.21
$C_{15}H_{24}$	14.17

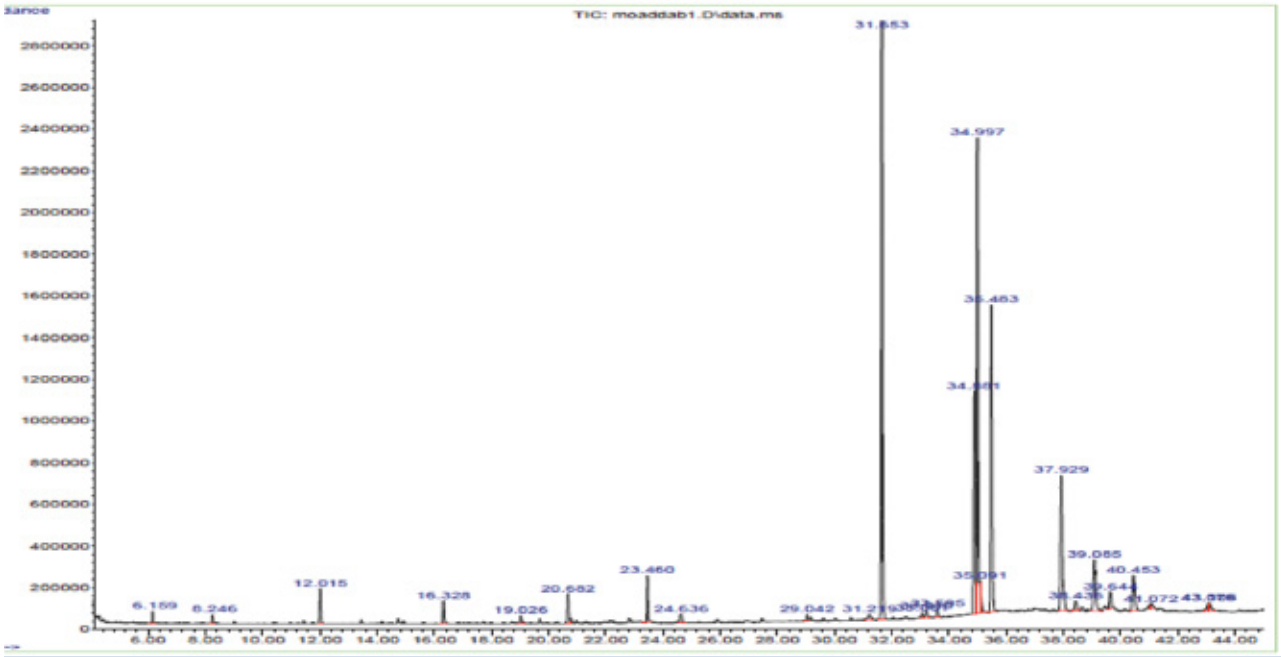

Figure 1. The graph of GC-mas of cinnamon essential oil

The use of agricultural oil seed meal in addition to the antioxidant approach from a nutritional point of view is also important. In the early hours of incubation (4 h and 6 h) the control had higher gas production than the other treatments (monensin, tannic acid and cinnamon essential oil) (P<0.05). Also, in the early hours of incubation (4 h and 6 h) SM treatments containing cinnamon essential oil (at 150 and 250 mg/kg DM) compared with monensin treatment (at levels 12 and 24 mg) and tannic acid (at 50 and 100 mg/kg DM) reduced gas production (P<0.05). After 24 hours of incubation, tannic acid treatment (at 100 mg/kg DM) showed the highest amount of GP, in the event that treating with monensin (at 12 and 24 mg/kg DM) and cinnamon essential oil (at level 150 and 250 mg/kg DM) reduced the amount of GP compared to the control group (P<0.05). After 120 hours of incubation, it was observed that treatment with cinnamon essential oil (at 150 and 250 mg/kg DM) showed the lowest GP and tannic acid treatment (at 100 mg/kg DM) had the highest amount of GP (P<0.05), whereasthere was significantly variation in GP of monensin (at 12 and 24 mg/kg DM) compared to the control group at all incubation times, that treating with monensin reduced the GP.

Table 3. Effects of adding monensin, tannic acid and cinnamon essential oil on gas productionof sesame meal (ml/ 0.2 g DM)

Treatments[1]	Incubation times (h)										
	4	6	8	12	16	24	36	48	72	96	120
SM	1.96^{a}	4.46^{a}	6.12^{a}b	8.66^{b}	11.32^{b}	15.79^{b}	18.17^{b}	20.72^{b}	21.98^{b}	23.25^{bc}	24.40^{bc}
SM+M12	1.68^{b}	4.17^{ab}	5.73^{b}	7.61^{d}	9.44^{c}	12.92^{c}	14.60^{c}	16.91^{c}	19.23^{c}	21.06^{cd}	22.24c^{d}
SM+M24	1.70^{b}	4.08^{ab}	5.76^{b}	7.86^{cd}	9.84^{c}	13.34^{c}	15.14^{c}	17.03^{c}	19.05^{c}	20.73^{d}	21.79^{d}
SM+TA50	1.56b	3.92^{b}	5.70^{b}	8.42^{bc}	11.03^{b}	15.55^{b}	18.21^{b}	20.60^{b}	22.30^{b}	23.87^{b}	24.86^{b}
SM+TA100	1.60^{b}	4.28^{ab}	6.50^{a}	9.88^{a}	13.41^{a}	18.61^{a}	21.19^{a}	23.68^{a}	25.56^{a}	27.03^{a}	28.12^{a}
SM+CEO150	1.50^{b}	3.25^{c}	4.90^{c}	7.10^{d}	9.60^{c}	11.88^{c}	14.88^{c}	16.93^{c}	18.68^{c}	19.68^{d}	20.18^{d}
SM+CEO250	1.50^{b}	3.52^{c}	5.17^{c}	7.37^{d}	9.87^{c}	12.09^{c}	15.09^{c}	17.08^{c}	18.83^{c}	19.83^{d}	20.33^{d}
SEM	0.071	0.139	0.169	0.252	0.336	0.514	0.653	0.771	0.762	0.787	0.784
P-value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Means within same column with different superscripts differ (P <0.05).

[1]SM: SM (control), SM+M12: SM+12 mg monensin/kg DM, SM+M24: SM+24 mg monensin/kg DM, SM+TA50: SM+50 mg acid tannic/kg DM, SM+TA100: SM+100 mg acid tannic/kg DM, SM+CEO150: SM+150 mg cinnamon essential oil/kg DM, SM+CEO250: SM+250 mg cinnamon essential oil/kg DM.

The GP characteristics, GP potential and rate of GP are presented in Table 4. Comparison of the GP process at different times and the GP parameters shows that the amount of gas obtained from the soluble fraction (a), (b) fraction, the gas production rate (c) and delay phase (L) of the treatments were significantly different (P<0.05). The protein content of the oilseeds was highly degradable, which is cause to the shortening of the delayed phase of SM containing monensin additive (at 12 and 24 mg/kg DM), cinnamon essential oil (at levels 150 and 250 mg/kg DM) and tannic acid (50 mg/kg DM) were significantly different from control and tannic acid treatment (at 100 mg/kg DM) (P<0.05). It is also observed that tannic acid (at 100 mg/kg DM) increased the lag time of SM.

Table 4. Effects of adding monensin, tannic acid and cinnamon essential oil on b, c and L parameters of sesame meal

Treatments[1]	Parameters[2]		
	b (ml/0.2 g DM)	c (ml/h)	L (h)
SM	23.70^{b}	0.045	1.67^{b}
SM+M12	21.90^{bc}	0.033	0.90^{cd}
SM+M24	21.21^{c}	0.038	0.75^{cd}
SM+TA50	23.87^{b}	0.091	1.51^{bc}
SM+TA100	27.24^{a}	0.048	2.50^{a}
SM+CEO150	20.91^{c}	0.037	1.22^{bc}
SM+CEO250	21.00^{c}	0.038	1.08^{bc}
SEM	0.775	0.018	0.266
P-value	<.0001	0.350	<.0001

Means within same column with different superscripts differ (P<0.05).
[1]SM: SM (control), SM+M12: SM+12 mg monensin/kg DM, SM+M24: SM+24 mg monensin/kg DM, SM+TA50: SM+50 mg acid tannic/kg DM, SM+TA100: SM+100 mg acid tannic/kg DM, SM+CEO150: SM+150 mg cinnamon essential oil/kg DM, SM+CEO250: SM+250 mg cinnamon essential oil/kg DM.
[2]b: Potential of gas production, c: rate of gas production, L: lag time.

The effects of adding monensin, tannic acid and cinnamon essential oil on the fermentation parameters estimated in GP, PF, and microbial biomass production efficiency of SM are shown in Table 5. The results show that by increasing the gas production (24h of incubation periods) of SM treated with tannic acid (100 mg/kg DM), increased ME and microbial protein (MP) (P<0.05). Treatments containing monensin and cinnamon essential oil had a significant difference with the control treatment by reducing the ME and the estimated MP content. The levels of short chain fatty acids (SCFAs), organic matter digestibility (DOM) and net energy of lactation (NE_L) were presented in Table 5. The highest amounts of SCFAs, DOM and NE_L were in the tannic acid treatment (100 mg/kg DM) and the lowest in the treatment containing cinnamon essential oil (150 mg/kg DM). There was no significant difference between treatment with monensin (12 and 24 mg/kg DM), cinnamon essential oil (150 and 250 mg/kg DM) and control. Significant differences were found in the content of tannic acid (50 and 100 mg/kg DM) (P<0.05), indicating that the levels of short-chain fatty acids, OMD, and NE_L were high. The decrease has been shown to be due to a decrease in GP within 24 hours after incubation. Maximum feedstuff fermentation efficiency (FFE) in monensin-containing treatment at levels 12 and 24 mg/kg DM was 10.82 and 10.94 mg/ml, respectively, and the lowest in control and treatment containing 100 mg tannic acid/kg DM were 7.95 and 7.77 mg/ml, respectively.

Table 5. Effects of adding monensin, tannic acid and cinnamon essential oil on estimated gas production parameters of sesame meal

Treatments[1]	Parameters										
	GP	SCFA	DOM	NE_L	FFE	ME	MP	OMDe	PF	MM	EMBS
SM	15.79^{b}	0.34^{b}	28.85^{b}	1.74^{b}	7.9^{5c}	4.35^{b}	34.80^{b}	79.61^{d}	5.07^{c}	44.87^{c}	32.05^{c}
SM + M12	12.97^{c}	0.28^{c}	26.01^{c}	1.42^{c}	10.82^{a}	3.89^{c}	31.37^{c}	90.69^{b}	7.02^{a}	62.27^{a}	44.48^{a}
SM + M24	13.34^{c}	0.29^{c}	26.43^{c}	1.47^{c}	10.94^{a}	3.96^{c}	31.87^{c}	92.31^{a}	7.05^{a}	62.96^{a}	44.97^{a}
SM + TA50	15.55^{b}	0.34^{b}	28.62^{b}	1.72^{b}	8.85b^{c}	4.31^{b}	34.51^{b}	87.47^{c}	5.67^{bc}	53.26^{b}	38.04^{b}
SM + TA100	18.61^{a}	0.40^{a}	31.64^{a}	2.07^{a}	7.77^{c}	4.79^{a}	38.17^{a}	93.51^{a}	5.03^{c}	52.57^{b}	37.55^{b}
SM + CEO150	12.09^{c}	0.26^{c}	24.98^{c}	1.30^{c}	9.91^{ab}	3.76^{c}	30.13^{c}	70.23^{e}	5.95^{b}	44.10^{c}	31.50^{c}
SM + CEO250	11.88^{c}	0.27^{c}	25.19^{c}	1.32^{c}	9.38^{b}	3.73^{c}	30.38^{c}	62.21^{f}	5.58b^{c}	40.60^{d}	30.01^{c}
SEM	0.560	0.012	0.555	0.064	0.398	0.088	0.669	0.494	0.249	1.161	0.829
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Means within same column with different superscripts differ (P<0.05).
[1]SM: SM (control), SM+M12: SM+12 mg monensin/kg DM, SM+M24: SM+24 mg monensin/kg DM, SM+TA50: SM+50 mg acid tannic/kg DM, SM+TA100: SM+100 mg acid tannic/kg DM, SM+CEO150: SM+150 mg cinnamon essential oil/kg DM, SM+CEO250: SM+250 mg cinnamon essential oil/kg DM.
GP: gas production (ml/0.2 g DM), SCFA: short chain fatty acid, DOM: digestible organic matter (%), NE_L: net energy for lactation (MJ/kg DM), FFE: feed fermentation efficiency, ME: metabolizable energy (MJ/kg DM), MP: microbial protein,OMDe: truly digestible OM (mg/0.2 DM), organic matter; PF: partitioning factor (mg DOM/ml GP), MM: microbial biomass production (mg). EMBS: microbial biomass production efficiency.

The effect of adding monensin, tannic acid and cinnamon essential oil, on the amount of total volatile fatty acid, ammonia nitrogen and ruminal pH using SM in the gastric test at 120 h after in vitro incubation was presented in Table 6. The pH of the control did not differ significantly (P>0.05). The highest pH reduction was observed in treatments containing tannic acid (100 mg/kg DM).

Table 6. Effects of treatments on VFA, N-NH3 and pH after 120 h incubation

Treatments[1]	Items[2]		
	tVFA (mmoL/l)	$N\text{-}NH_3$ (mmoL/l)	pH
SM[1]	226.33^{b}	368.67^{c}	6.76^{ab}
SM+M12	385.00^{a}	518.00^{b}	6.72^{b}
SM+M24	413.00^{a}	742.00^{a}	6.70^{b}
SM+TA50	434.00^{a}	679.00^{a}	6.71^{b}
SM+TA100	238.00^{b}	709.33^{a}	6.66^{b}
SM+CEO150	205.33^{b}	196.00^{d}	6.68^{b}
SM+CEO250	212.33^{b}	156.33^{d}	6.81^{a}
SEM	39.143	27.153	0.029
P-value	0.0006	<.0001	0.030

Means within same column with different superscripts differ (P<0.05).
[1]SM: SM (control), SM+M12: SM+12 mg monensin/kg DM, SM+M24: SM+24 mg monensin/kg DM, SM+TA50: SM+50 mg acid tannic/kg DM, SM+TA100: SM+100 mg acid tannic/kg DM, SM+CEO150: SM+150 mg cinnamon essential oil/kg DM, SM+CEO250: SM+250 mg cinnamon essential oil/kg DM.
[2]tVFA: total volatile fatty acids, $N\text{-}NH_3$: ammonium.

DISCUSSION

The results showed that SM treatment with tannic acid (100 mg/kg DM) produced more gas than control and cinnamon essential oils treatment. Moheghi et al. (2013) found that addition of tannic acid in the diet containing a mixture of forage (50%) and concentrate (50%) increased the amount of cumulative gas production at all incubation times compared to the control group. A possible reason for this increase in GP may be due to its anti-protozoa characteristic that results in more fermentation and *in-vitro* GP. Hence, in another study- on the ability of tannic acid to inhibit methanogenesis and bio-hydrogenation of unsaturated fatty acid, they found that the addition of 25 mg tannic acid at 250 mg DM was not different from control group, but addition of 50 mg tannic acid to 250 mg DM reduced GP and had significant difference with control (Al-Jumaili et al., 2017).

The results of *in vitro* and *in vivo* trials mostly show contradictory results from rumen hydrolyzed tannins. This is maybe due to diversity in the nature of tannins and the amount of inclusion in the diet (Buccioni et al., 2015). Besharati et al. (2013) also found that monensin containing cottonseed meal (24 mg/kg DM) reduced the production of gas compared to the other treatments, which was in agreement to the findings of present study. These results agreed to the findings of Callaway and Martin (1996) which proved the in vitro culture with monensin supplementation produced lower gas production than monensin-free additive group. Though some studies also reported a decrease in methane production by the addition of monensin (Jafari et al., 2019). Monensin may have reduced GP by ionophores lipophilic compounds that was toxic to many bacteria, protozoas, and fungi (Haque, 2018; Broucek, 2018). The toxicity of these compounds was due to their penetration into the cell membrane and changes in ionic charge inside. Ionophores penetrate into the cell membrane by binding to positive ions and transporting them (sodium, potassium, magnesium and calcium) and causing them to die by changes in ionic charge inside the cells (Holmes et al., 2018; Novilla, 2018).

The results of this experiment showed that the used cinnamon essential oil has the potential to affect ruminal fermentation of SM. The effects of natural plant essential oils and plant extracts on improving ruminal fermentation (e.g., reduced CH_4 production ruminal protein degradation, and-rumen ammonia nitrogen concentration) have been reported previously (Besharati et al., 2020). But in another study performed on a diet containing 80 % concentrate and 20 % forage, they found that adding cinnamon and thyme essential oil at all levels, reduced total GP and CH_4 production in comparison with control treatment ($P<0.05$). The results showed tannic acid at 50 and 100 mg/kg DM caused more gas production than those of treatments containing monensin and cinnamon essential oil. Reduction of GP in treatments containing cinnamon essential oils indicates that plant oils at low levels are useful for fermentation and but at high levels selectively restrict gram-positive and gram-negative bacteria (Oulkheir et al., 2017; Semeniuc et al., 2017).

The lag phase is required for the digestion of both parts that have the ability to degrade and the solve fractions. Moheghi et al. (2013) noted that the rate of gas production decreased with increasing levels of tannic acid in treatments. The highest potential of gas production (b) was obtained in the treatment with tannic acid (100 mg/kg DM) with 27.24 ml/0.2 g DM and the lowest in the treatment with cinnamon essential oil (at the level of 150 mg/kg DM) with 21.91 ml/0.2 g DM. The fraction b of SM significantly decreased with adding monensin (at 24 mg/kg DM) and essential oil (at 150 and 250 mg/kg DM) ($P<0.05$).

The rate of gas production (c) was not statistically affected by the additives ($P>0.05$). The study by El-Waziry et al. (2007) investigating the influence of different levels of tannic acid to soybean meal, statedthat there was no significant variation in the rate of GP. Tannins appear to inhibit the activity of microbial enzymes by forming protein complexes with enzymes in the bacterial cell wall, thereby reducing the digestibility of carbohydrates, especially structural carbohydrates, by cellulolytic bacteria, thus through this mechanism they exert their effect on the digestion of whole feedstuffs (Huang et al., 2018). The results of another study conducted by Besharati et al. (2013) on the effect of monensin supplementation (24 mg/kg DM) found that GP potential and GPrate were reduced, which was in agreement with the results of this study. Taghavi-Nezhad et al. (2011) reported that there is a negative relationship between peppermint essential oil at 250, 500, 750 and 1000 µg/ml and gas production parameters (gas production, gas production potential and rate, factor fractionation and microbial mass production), as the essential oil level increased, GP parameters decreased.

The results showed that monensin and cinnamon essential oil increased the fermentation efficiency and tannic acid (50 mg/kg DM) had not significant. Studies show that the addition of tannic acid to the experimental diet increases the digestibility of OM, the amount of short chain fatty acids and ME. There is a positive correlation between the amount of produced gas and the production of short-chain fatty acids (Menke, 1988) and GP can predict the amount of volatile fatty acids production that has a positive relationship with microbial mass production (Rabelo et al., 2017).

The partitioning factor (PF) index was the highest in monensin-containing treatments (12 and 24 mg/kg DM) with 7.02 and 7.05 mg of OM degraded per milliliter of gas, respectively, and the lowest value for control with 5.07 mg of degraded OM per ml gas ($P<0.05$), and there was a significant difference between treatments containing monensin with other

treatments (P<0.05). Treatments containing cinnamon essential oil (150 and 250 mg/kg DM) and tannic acid (50 mg/kg DM) also increased PF index, which showed a significant difference with control (P<0.05). Microbial mass index (MM) and microbial mass synthesis efficiency in treatment containing monensin (12 and 24 mg/kg DM) and tannic acid (50 and 100 mg/kg DM) were significantly different from others, but monensin-treated samples had a significant difference with tannic acid-treated ones. Control and cinnamon essential oil treatment (150 and 250 mg/kg DM) did not differ significantly (P>0.05). Makkar et al. (1995) reported that the effect of tannin on reducing feed degradation rate contributes to the simultaneous release of nutrients and this may be responsible for increased microbial efficiency. Research has shown that when consuming tannin-containing forages, the amount of non-ammonia nitrogen injected into the small intestine was higher than that consumed nitrogen, that part of which was attributed to increased MP production. Cinnamon essential oil and tannic acid reduced feed degradation rates, which can help coincide with the release of nutrients resulting in increased efficiency and in fact the amount of energy and ammonia available for MP synthesis has reached its equilibrium level, and the rumen microorganisms are able to grow better. In the Kiran and Kirishnamurti (2007) report, the range of PF content of feeds for protein sources ranged from 3.86 to 6.48 mg/ml and for energy sources between 3.28 and 4.53 mg/ml. In the present study, the PF parameter of SM was between 5.03 and 7.05 mg/ml, which was high and close to the reported by Kiran and Kirishnamurti (2007). The slight difference between treatments in this respect and the small variance of the results of the present experiment could be related to the low GP due to the protein degradation but not fermentation in the SM solution and increased the true digestible OM and consequently increased the PF parameter.

Ammonia nitrogen concentration (mmol/l) increased at both monensin and tannic acid levels compared to control, but cinnamon essential oil at both levels showed a decrease that resulted from decreased ammonia nitrogen concentration and increased MP production. It indicates that there is a linear relationship between these two indices and that nitrogen is probably used in the production of microbial protein. The researchers found that monensin supplement (24 mg/kg DM) had less inhibitory effect on rumen fermentation compared to thyme essential oil (100 mg/l culture medium). High partitioning factor in the feed indicates more conversion of the degraded material to microbial mass, greater efficiency of microbial mass production, less methane production and greater feed consumption, so PF can be used to predict microbial mass and methane production in ruminants. The increase in PF value indicates that the degradedOM is more likely to move towards microbial mass production and to decrease volatile fatty acid production (Kiran and Kirishnamurti 2007).

The results obtained in this study regarding pH decrease were consistent with the addition of tannic acid in the study of Moheghi et al. (2013). The decrease in pH by tannic acid and tannin has also been observed in other studies (Jafari et al., 2019). Probably one of the reasons for the decrease in pH could be the change in the patterns of rumen bacteria, especially cellulolytic bacteria (Jafari et al., 2019). On the other hand, protozoa depletion drives the pattern of volatile fatty acids production to produce more propionate and less acetate and butyrate (Silanikove et al., 2006). Silanikove et al. (2006) stated that under in vitro conditions, decreased absorption of volatile fatty acids from the rumen wall and increased ruminal fatty acid production were the main causes of pH decrease.

Ammonia nitrogen production in treatments tannic acid (50 and 100 mg/kg DM) and monensin (12 and 24 mg / kg DM) was higher than those of cinnamon and control (P<0.05). Cinnamon essential oil significantly reduced the amount of ammonia nitrogen in all treatments (P<0.05). Moheghi et al. (2013) who examined the in vitro experiment with a 50:50% forage to concentrate ration, observed that the addition of tannic acid reduced the amount of ammonia nitrogen in 24 h of incubation. One of the goals of using essential oils was to reduce the concentration of ammonia nitrogen, which indicates a reduction in the amino acid deamination by ammonia-producing bacteria, which is useful in feeding ruminants because it increases the amount of protein passing through rumen and ultimately increases the efficiency of protein utilization in ruminants (Van Nevel and Demeyer, 1988). The effect of essential oils in different experiments on the ammonia concentration was different. The cause of these differences may be related to the type and amount of essential oil and substrate used. The decrease in ammonia concentration can be linked to a decrease in the number of rumen protozoa (Talebzadeh et al., 2012), the inhibition of high-ammonia-producing bacteria, a decrease in amino acid deamination, and an increase in protein flow into the small intestine (Tager, 2010).

Most of the essential oils reduce rumen ammonia concentration due to antimicrobial activity on a specific group of gram-positive bacteria. Ammonia-producing bacteria make up only one percent of the population of rumen bacteria but have high de-amination activity and inhibition of these bacteria by secondary plant compounds by increasing protein utilization efficiency and reducing rumen ammonia production (Patra, 2011). Monensin reduces deamination and ammonia depletion by controlling the protein-degrading bacteria in the rumen (McGuffey et al., 2001).

Volatile fatty acid production was significantly increased in treatments containing tannic acid (50 mg/kg DM) and monensin (12 and 24 mg/kg DM) ($P<0.05$). The amount of volatile fatty acid production of cinnamon essential oil was not significantly different from the control ($P>0.05$). Moheghi et al.(2013) stated that tannic acid at 24 h incubation reduced pH and ammonia nitrogen compared to control. In most studies, the use of essential oils or their compounds did not reduce or alter the total concentration of volatile fatty acids. Since volatile fatty acids are a major source of energy for ruminant tissues, reducing their production is not beneficial, but no change in the concentration of volatile fatty acids is desirable if accompanied by a decrease in ammonia concentration, a decrease in methane production or a change in the ratio of volatile fatty acids.

The effect of the essential oil on the concentration of volatile fatty acids is dependent on the type of diet, the dosage and the pH of the rumen (Kholif et al., 2018;Besharati et al., 2020). Benchaar et al. (2007) reported that adding cinnamon (400 mg/l), clove (200 mg/l) and thyme (200 mg/l) essential oils to the medium had a significant effect on total volatile fatty acid concentration. Busquet et al. (2006) pointed out that the decrease in total volatile fatty acid production by the addition of dill essential oil and its main active ingredient showed a decrease in feed degradability. In the present study, cinnamon essential oil reduced the disappearance of DM, OM, and CP in addition to the reduction of ammonia nitrogen concentration, which was in accordance with the Jahani-Azizabadi et al. (2011) study.

CONCLUSION

Although the use of antibiotics increases the efficiency of feed, but for some reason they are not used, nowadays, other additives such as herbal essential oils are used for this purpose. Ionospheres such as monensin, may have been shown to be effective in the biodegradation process of rumen by inhibiting the growth of methane emissions by inhibiting the growth of gram-positive bacteria that produce hydrogen. The use of ionophores as an oral antibiotic in livestock has been banned in some areas. Therefore, secondary plant metabolites such as plant essential oils have been suggested as potential tools for manipulating bacterial populations involved in biogas production. The use of cinnamon essential oil as a biogas reducer for the first time is a novelty of this study. It is for first time that the effect of monensin and cinnamon essential oil on biogas production has been compared.

CONFLICT OF INTEREST

None declared.

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Case report
Κλινικό περιστατικό

Concurrent Occurrence of Avian Encephalomyelitis and Vitamin A Deficiency In a Commercial Broiler Chicken Flock

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ABSTRACT: Avian encephalomyelitis (AE) is accounted for a common infectious disease in poultry, which particularly targets the central nervous system and various parenchymatous organs of chickens.Vitamin A supplementation is considered as a viable option to strengthen the immune system in neonates. Vitamin A deficiency in broilers decreases the ability to synthesize specific antibodies and weakens the lymphocyte proliferation response leading to epithelial metaplasia. In the present study, the effect of vitamin A deficiency on the occurrence of AE in a day old commercial broiler chicken flock was evaluated.Twenty, Two-Day-old paralyzed chickens, had been referred to the clinic of Veterinary School.The chicks were selected from a broiler flock with 13,000 birds purchased from a broiler breeder flock, all vaccinated against the AE virus. After examination, ataxia, trembling, lateral recumbency, and incoordination were observed in the chickens. The chickens were euthanized, and the tissue samples from their brain, heart, and proventriculus were then collected for microscopic evaluation. According to microscopic studies, non-supportive encephalomyelitis was noted in the cerebellum and cerebrum. In the myocardium, fiber degeneration and lymphocytic aggregates between the muscle fibers were evidenced.The proventriculus in chickens revealed hyperplastic and thickened mucosa, and there was squamous metaplasia for some of the mucosal glands. Furthermore, some multifocal aggregations of lymphocytes were observed in the tunica muscularis layer of the proventriculus.Our findings showed that vitamin A deficiency in a broiler breeder farm might be had a significant effect on the occurrence of AE in their hatched chicks. This study, to the best of the authors' knowledge, is the first report of concurrent occurrence of avian encephalomyelitis and vitamin A deficiency in a Two-Day-old commercial broiler chicken flock in north of Iran.

***Keywords*:** Ataxia, brain, metaplasia, neuronal degeneration, proventriculus

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CASE HISTORY

Avian encephalomyelitis (AE), is recognized as a worldwide and economically important viral disease affecting mainly chickens, turkeys, quail, and pheasants caused by *Tremovirus A* of the *Picornaviridae* family (Sentíes-Cué et al., 2016).The outbreaks of the disease were first reported in the New England region of the USA in 1930. It is known as "epidemic tremor" since there are unusual tremors and vibrations of the head or neck as result of the disease (Koutoulis et al., 2015). Although the virus targets chickens of all ages, the nervous symptoms are only manifested in young chicks, between one to five weeks of age (Sentíes-Cué et al., 2016). In older chickens, infection is usually subclinical, resulting in a decline in egg production and hatchability (Sentíes-Cué et al., 2016). The virus may be spread by the horizontal (oral-faecal) and vertical routes. Vertically transmitted virus to the offspring of a breeder flock infected during egg production leading to an outbreak occurrence (Back, 2015).

Vitamin A is considered as an essential micronutrient throughout the life cycle of broilers. The deficiency of this vitamin decreases the ability to synthesize specific antibodies and weakens their lymphocyte proliferation response. The immune system of neonates can be strengthened by supplementing them with vitamin A and β-carotene (Fan et al., 2015). Vitamin A deficiency decreases the antibody titer and bile Immunoglobulins IgA concentration after vaccination (Çevİk, 2018). Furthermore, vitamin A and its derivatives contribute in the regulation of the growth and differentiation of gastrointestinal epithelial cells (Amit-Romach etal., 2009).

A clinico-pathological study was performed to understanding of the influence of the vitamin A deficiency on the occurrence of the AE in newly hatched chicks.

Twenty, Two-Day-old paralyzed chickens, had been referred to the clinic of Veterinary School. The chickens were taken from a broiler flock with 13,000 birds. Day-old chicks were purchased from a local hatchery from a broiler breeder flock vaccinated against avian encephalomyelitis virus (AEV). Clinical signs, including ataxia, trembling, lateral recumbency, and incoordination, were observed in the chickens. Although the majority of the affected chicks attempted to eat and drink, they were not able to compete for food and water due to the paralysis of their extremities and wings. According to the owner's answer, 5% of the birds were clinically affected on the first day, but the number ofthe affected cases increased by the second day.

According to the gross evaluations, haemorrhagicspots were observed under the occipital bone, andaround the cerebellum. Moreover, in some cases, the hearts were larger than normal size (Figure 1).

Microscopical evaluation in the brain revealed non-supportive encephalomyelitis characterized by multifocal foci of neuronal degeneration and necrosis, central chromatolysis of Nissl substance, multifocal lymphoid infiltration with perivascular cuffing (Figure 2 (A)), hyperemia, microglial proliferation, both in nodular and diffuse form and demyelination. In the myocardium, fiber degeneration and lymphocytic aggregates between the muscle fibers of the myocardium were observed. In the proventriculus, noticeable rete pegs and acanthosis were noted, indicating hyperplastic and thickened mucosa. Squamous metaplasia of glandular epithelium was also seen by mucosal glands. Furthermore, the accumulation of necrotic debris, admixed with inflammatory cells, and desquamated epithelial cells were found in the lumens of glands. On the other hand, in lamina propria and surrounding glands, the accumulation of granulocytes was demonstrated. Surprisingly, some multifocal aggregations of lymphocytes were observed in the layer of the tunica muscularis of the proventriculus (Figure 2).

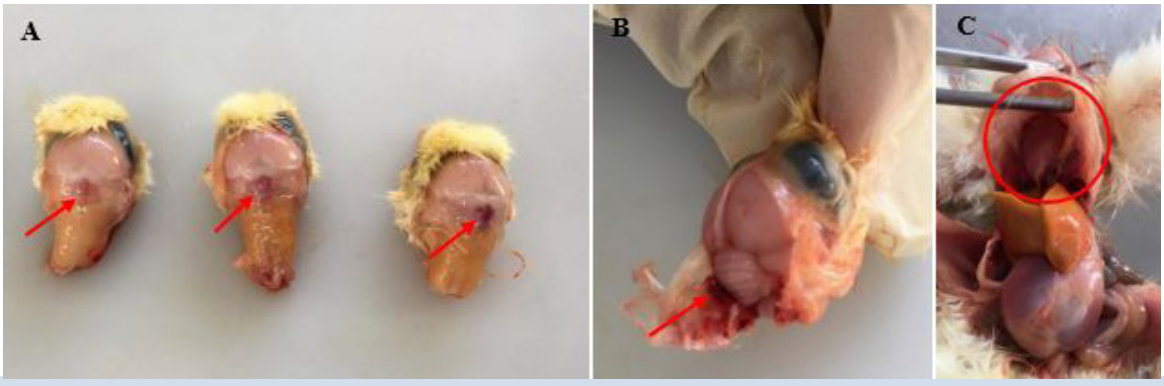

Figure 1. (A & B): The haemorrhagic spots under the occipital bone, around the cerebellum, **(C):** Cardiac enlargement in some cases

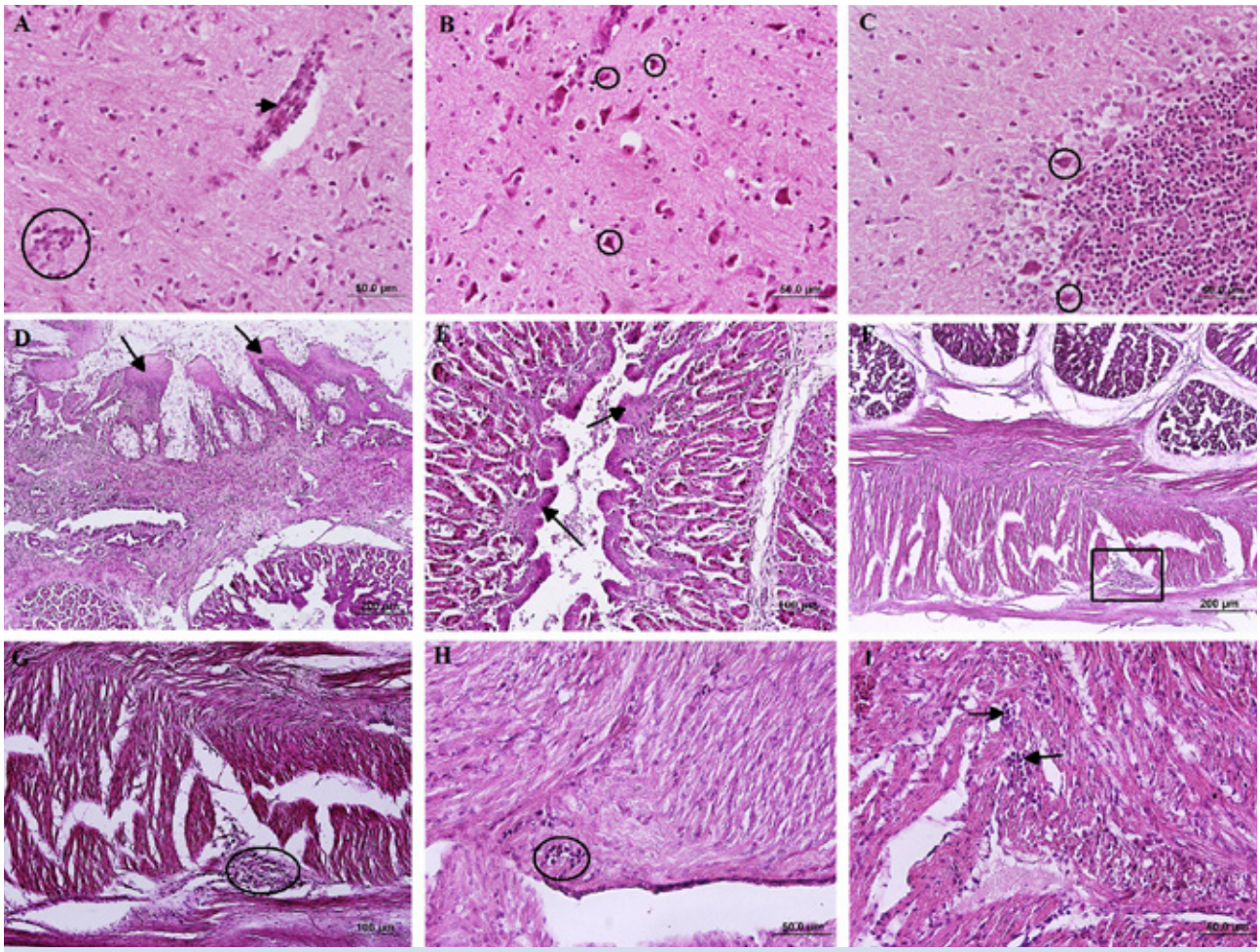

Figure 2. (A): Note tthe lymphocytic perivascular cuffing (arrow) and gliosis (circle), **(B):** Note the necrotic neurons (circle), **(C):** Necrotic and degenerative Purkinje cells (circle), **(D):** Note the squamous metaplasia and acanthosis in mucosal tunic of proventriculus (arrow), **(E):** Squamous metaplastic changes in glandular epithelium (arrow), **(F):** Note the aggregation of lymphocytes in muscularis tunic of proventriclous (rectangle), **(G):** Higher magnification of pervious slide, note thr lymphocytic aggregation (circle), **(H & I):** Foci of lymphocytic aggregates are seen in myocardium (circle and arrow respectively), (H & E, scale bar: A,B,C, G, H, and I: 50μm, D: 200 μm, E: 100 μm)

DISCUSSION

In the present study, the clinico-pathological features of a concurrent occurrence of AE and vitamin A deficiency in a two-day old commercial broiler chicken flock were described.

The major clinical symptoms of AE in adult chickens, are usually subclinical, detected by drop in egg production and the appearance of infected progeny. The clinical symptoms in young chickens are ataxia, and leg weakness from sitting on hocks to paresis that may lead to paralysis and recumbency (Marvil et al., 1999;Jana et al., 2005; Asasi et. al, 2008). In the initial stages of infection, lesions in chickens are frequently observed in the central nervous system than in other organs. Neuron degeneration may be a sign of initial changes of neural tissues, mainly in the medulla and the anterior horn cells of the spinal cord. The gradual disappearance of Nissl substance and the infiltration of perivascular by lymphoid cells were followed by progressing neuronal degeneration. This infiltration occurs mainly in the cortex, cerebellum, and medulla, while the grey matter of the spinal cord was usually unaffected. Although no significant gross changes were usually observed in other organs, there is a possibility of the histopathological lesions occurrence. Lesions in the parenchymous organs and body viscera are characterized by increased peri- and/or paravascular infiltration of lymphocytes and focal lymphoid hyperplasia. Eosinophilic swelling, necrosis, fragmentation, loss of striation in fibres, and heterophil infiltration were the fine changes in muscular structure (Tannock and Shafren, 1994).In the current study, in addition to clinical signs, some microscopic lesions including: multifocal foci of neuronal degeneration, necrosis in the cerebellum and cerebrum, fiber

degeneration and lymphocytic aggregates between the muscle fibers of the proventriculus were observed.

In addition, the squamous metaplasia of the glandular epithelium of mucosal glands was observed in the proventriculus. In a study conducted by Amit- Romach (2009), the role of vitamin A in regulating the growth and differentiation of gastrointestinal epithelial cells was described. Vitamin A deficiency leads to epithelial squamous metaplasia and loss, which affects its integrity and density, thereby allowing the pathogen to easily invade and infect the organism (Yuan et al., 2014; Fan et al., 2015).

In conclusion, since the symptoms of fat-soluble vitamin deficiency are exhibited with by delay,it is assumed that the studied case were selected from a breeder flock suffering from vitamin A deficiency leading to the formation of the eggs with metaplastic changes in the epithelium of the proventriculus. It is also assumed that the AEVin the one-day chickens has been transmitted by an AE infected breeder. The primary cause of the AE outbreak in the breeder flock is not clear. However, since the whole breeder flock was vaccinated against AEV, vitamin A deficiency may decrease the antibody titer concentration after vaccination leading to the occurrence of the disease in the progeny. This study, to the best of the authors' knowledge, is the first report on a broiler farm in north of Iran.

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CONFLICT OF INTEREST

The authors declare that there was no conflict of interest.

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Case report
Κλινικό περιστατικό

Pseudo-pericarditis in two cows with tropical theileriosis: clinical, haematological and biochemical findings

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ABSTRACT: Pseudo-pericarditis-shaped theileriosis is a rare phenomenon that only a handful of reports have been introduced. This case report aims to describe pseudo-pericarditis due to tropical theileriosis diagnosed in a 7-year old crossbreed Simmental cow and 3-year old crossbreed Holstein cow with a loss of appetite, weakness, and weight loss. We have detected high fever, paleness in mucous membranes (anaemia), petechial haemorrhages and icterus, an obvious enlargement in the left prescapular lymph node, distension in the jugular venous and positive venous pulse in the clinical examination. The factors of *Theileria annulata* were intensively present in the microscopic investigation of the peripheral blood smears of both animals. We determined considerably low levels of hematologic parameters. Finally, biochemical analyses revealed the elevated concentration of cTn-I, activities of CK-MB, LDH, AST, GGT, and the levels of TBIL. On the contrary, levels of TP, ALB, GLU, and Ca were significantly low. In this case report, we would like to touch upon some points that were not mentioned or merely investigated in the previous studies. In this context, we will first argue the increased risk of occurrence of pseudo-pericarditis in case of severe anaemia, petechial haemorrhages in conjunctival mucosa, greatly enlarged superficial lymph nodes and considerably low numbers of haematocrit. We also think that pseudo-pericarditis is a late-stage symptom of theileriosis. Then we further elaborate on the relationship between severe anaemia, pseudo-pericarditis and myocardial damage by referring to cardiac biomarkers (cTnI, CK-MB etc.) which was never done before. Later, we will finally elucidate on the biochemical parameters (AST, LDH, ALB, TBIL etc.) which revealed hepatic damage. Finally, we argue that in case of severe anaemia along with the expansion in vena jugularis and positive venous pulse, pseudo-pericarditis should be taken into consideration for the cows with tropical theileriosis.

***Keywords*:** Cow, theileriosis, positive venous pulse, pseudo-pericarditis.

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INTRODUCTION

Tropical theileriosis or Mediterranean coast fever is a common disease of cattle which can be seen worldwide especially southern Europe, North Africa, and Central Asia. Although it generally emerges in subtropical regions in the summer, it can be seen in tropical regions throughout the year (Cicek et al., 2009). A blood parasite, *Theileria annulata* (*T. annulata*), causes this progressive lymphoproliferative disease of cattle (Fartashvand et al., 2013). High fatality rate, diminished production, reproductive problems and an increased risk of secondary infection are the consequences of theileriosis which may result in economic losses (Keleş et al., 2001; Dumanli et al., 2005; Radostits et al., 2006; Cicek et al., 2009).

Theileria annulata is transmitted through the saliva of *Ixodid* tick of genus *Hyalomma* to the host (Mirzaei, 2007). This parasite is irregular or bacilliform shaped, ovoid, and small round with an apical complex and can be encountered with in both erythrocytes and lymphocytes of their host (Levine, 1985). Schizonts, also known as Koch's blue bodies, are formed in lymphocytes which produce merozoites in cooperation with host cell division. The merozoites escaping from the lymphocytes in host cells invade the erythrocytes. At this stage, the parasites become infective for the vector (Sayin et al., 2003) and are known as piroplasms (Mehlhorn and Schein, 1985).

The most important features of tropical theileriosis are hemolytic anaemia, secondary hypoxia, and vasculitis (Fartashvand et al., 2013). Also, the high body temperature, anorexia, conjunctival petechia, nasal discharge, lacrimation, and growth in superficial lymph nodes are characteristic features of tropical theileriosis (Hooshmand-Rad, 1976; Radostits et al., 2006). The occurrence of pseudo-pericarditis is quite rare in cattle with theileriosis (Sudhakara and Sivajothi, 2017; Satheesha et al., 2017). Jugular engorgement, oedema and anorexia form in cattle with pseudo-pericarditis due to the theileriosis (Keles et al., 2003; Radostits et al., 2006). Pseudo-pericarditis occurs with the symptoms that are likened to that of pericarditis without any heart abnormalities. The formation mechanism of pseudo-pericarditis is explained as pressure on the vena cava due to the swelling of the mediastinal lymph nodes around caudal vena cava and cranial vena cava which inhibits blood backflow leading to clinical manifestations such as oedema and jugular enlargement (Keles et al., 2003; Radostits et al., 2006).

Hematologic and biochemical alterations associated with anaemia occur in tropical theileriosis. These alterations are dependent on the virulence of the parasite, the infectious dose, the breed of the animal, the state of immunity, as well as climatic regional factors. (Singh et al., 2001; Mahmmod et al., 2011). In addition, the severity of anaemia, parasitemia and hypoxia has an effect on these alterations (Singh et al., 2001). This case report aims to notify the clinical, haematological and biochemical findings of two cows with pseudo-pericarditis due to tropical theileriosis.

CASE HISTORY

This study material consists of a 7-year old crossbreed Simmental cow and 3-year old crossbreed Holstein cow, which were brought to the Animal Hospital of Atatürk University with the complaints of loss of appetite, weakness, and weight loss. For the hematologic and biochemical analysis, we took blood samples from vena jugularis of the animals into the vacuumed anticoagulant and coagulant tubes. We performed hematologic analyses using a cell counter (Abacus Junior Vet5, Hungary) and obtained biochemical findings by an autoanalyzer (Beckman Coulter, USA). To verify if any *T. annulata* piroplasms are present, we used anticoagulant blood to prepare Giemsa stained blood smears which were then examined with an optical microscope at x1000 magnification with immersion oil.

RESULTS

In the physical examination of the cases; we detected high fever (39.8 °C and 40.2°C), tachypnoea (36/min and 44/min), tachycardia (100 bpm and 116 bpm), paleness in mucous membranes (anaemia), petechial haemorrhages and icterus in the conjunctiva, an obvious enlargement in the left prescapular lymph node, distension in the jugular venous and positive venous pulse (Figure 1 and 2). The auscultation of the heart did not reveal any pericardial rub, bubbling, splashing or tinkling. We have then checked the abdominal organs with a metal detector whether any metallic foreign bodies are present, no metallic foreign body was found.

Parasitological diagnosis revealed a high amount of *T. annulata* factors in the microscopic examination of the peripheral blood smears of both animals (Figure 3). Feces samples did not indicate fascioliasis or any other parasitic diseases under the parasitological examination.

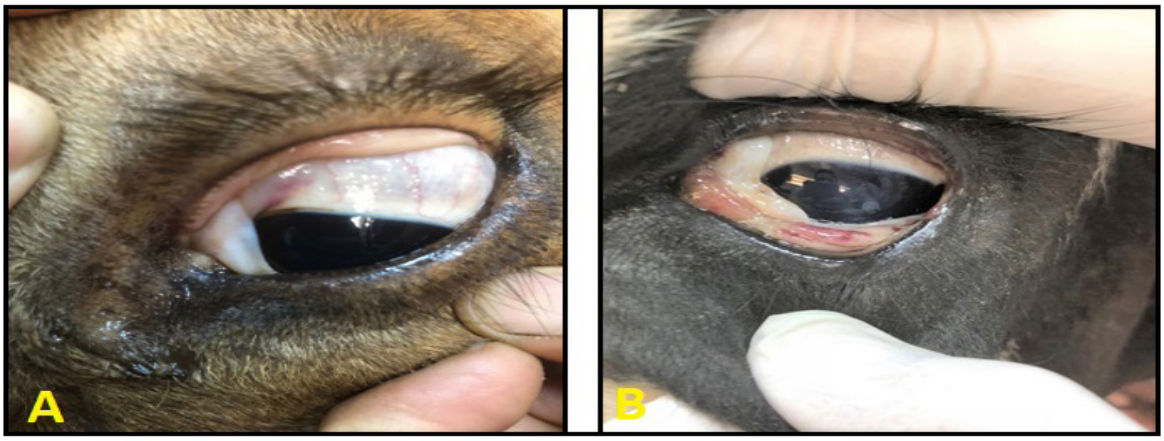

Figure 1. The paleness (A), petechial haemorrhages and icterus in conjunctival membranes (B).

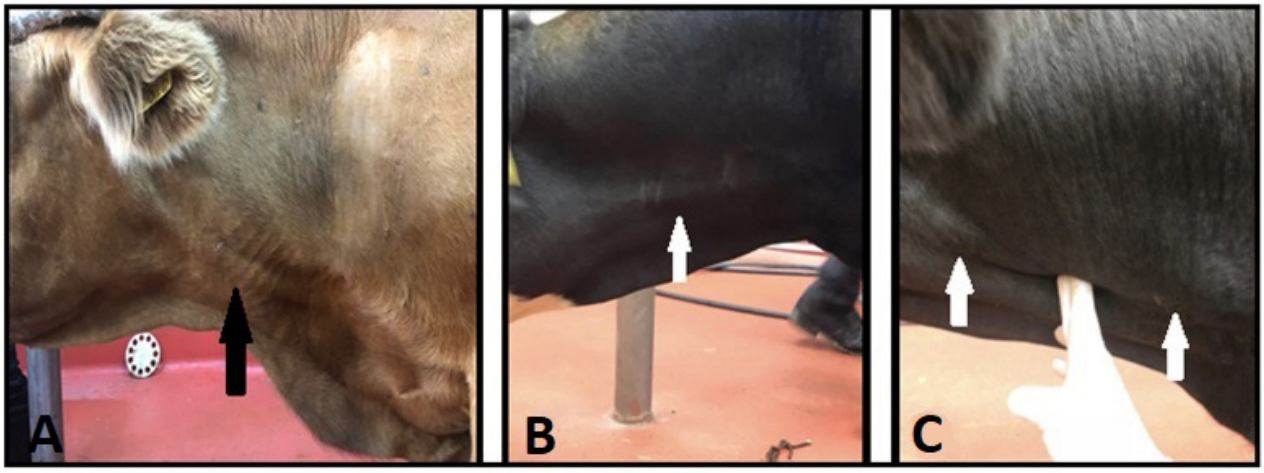

Figure 2. The fullness in the jugular vein (A and B) and positive vein pulse (C)

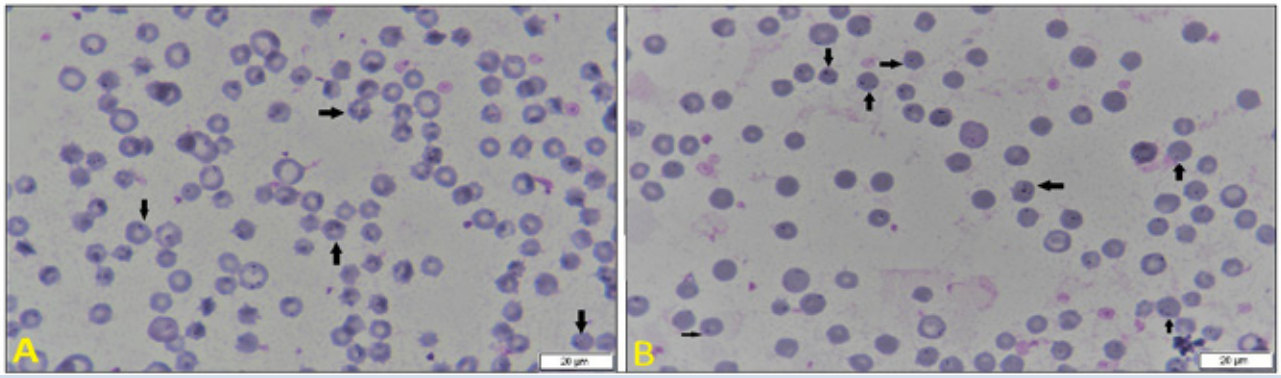

Figure 3. The ring forms of *Theileria annulata* piroplasms in red blood cells as shown with arrows in the figures (A and B)

In the hematologic examination, we observed much lower levels of lymphocyte, erythrocyte, haemoglobin, haematocrit, and platelets than the reference value (Roland, et al., 2014) (Table 1). Biochemical analyses demonstrated that levels of cardiac troponin-I (cTn-I), MB isoenzyme of creatine kinase (CK-MB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), blood urea nitrogen (BUN), and total bilirubin (TBIL) were higher than the reference value, on the contrary, the levels of total protein (TP), albumin (ALB), and glucose (GLU) were lower (Kilinc et al., 2018; Merck Veterinary Manual) (Table 2).

Table 1. Haematological findings of two cases

Parameter	Unit	Case-1	Case-2	Referance Range*
WBC	($x10^3/\mu L$)	5.65	8.00	4-12
LYM	($x10^3/\mu L$)	2.25	1.87	2.5-7.5
MON	($x10^3/\mu L$)	0.06	0.04	0-0.84
NEU	($x10^3/\mu L$)	3.30	5.95	0.6-6.7
EOS	($x10^3/\mu L$)	0.05	0.14	0.1-1
BAS	($x10^3/\mu L$)	0.00	0.00	0-0.5
RBC	($x10^6/\mu L$)	1.56	2.27	5-10
HGB	(g/dL)	2.9	3.6	8-15
HCT	(%)	9.26	11.32	24-46
PLT	($x10^3/\mu L$)	93	130	100-800

WBC: white blood cell; LYM: lymphocyte; MON: monocyte; NEU: neutrophil; EOS: eosinophil; BAS: basophil; RBC: red blood cell; HGB: haemoglobin; HCT: hematocrit; PLT: platelet
* Roland, et al. (2014)

Table 2. Biochemical findings of two cases

Parameter	Unit	Case-1	Case-2	Referance Range*
cTn-I	(ng/mL)	0.2	0.23	0.035-0.075 **
CK-MB	(U/L)	344	381	189-235 **
LDH	(U/L)	5427	7224	309-938
AST	(U/L)	364	642	60-125
GGT	(U/L)	107	22	6-17.4
BUN	(mg/dL)	26.64	38.79	10-25
TBIL	(mg/dL)	1.8	2.14	0-1.6
TP	(g/dL)	5.6	5.7	6.7-7.5
ALB	(g/dL)	1.97	2.15	2.5-3.8
GLU	(mg/dL)	30	26	40-100
Ca	(mg/dL)	5.4	7.9	8-11.4
P	(mg/dL)	6.7	6.8	5.6-8.0
Mg	(mg/dL)	1.68	1.5	1.5-2.9
Na	(mmol/L)	137	136	136-144
Cl	(mmol/L)	96	97	99-107
K	(mmol/L)	4.56	4.19	3.6-4.9

cTn-I: cardiac troponin-I; CK-MB: MB isoenzyme of creatine kinase; LDH: lactate dehydrogenase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transferase; BUN: blood urea nitrogen; TBIL: total bilirubin; TP: total protein; ALB: albumin; GLU: glucose; Ca: calcium; P: phosphorus; Mg: magnesium; Na: sodium; Cl: chloride; K: potassium
* Fielder SE (2015) **Kilinc et al. (2018)

DISCUSSION

There are many studies reporting the clinical findings of theileriosis, but only a few on pseudo-pericarditis-shaped theileriosis. In this context, Parashar et al. (2016) reported the most common clinical findings in 124 cattle infected with *T. annulata* as enlarged prescapular lymph node, pale mucous membranes, decreased appetite, high fever and tear discharge. Keleş et al. (2003) and Sudhakara and Sivajothi (2017) detected high fever, enlarged prescapular lymph node, oedema on the submandibular, neck and brisket region, enlarged jugular vein and positive vein pulse in cattle with pseudo-pericarditis-shaped tropical theileriosis. Also, they did not observe pericardial friction rub, gurgling, splashing or tinkling on auscultation of the heart. They described the mechanism of the development of pseudo-pericarditis as the result of the swelling of mediastinal lymph nodes around the caudal vena cava and cranial vena cava which causes pressure on the vena cava and inhibits the backflow of the blood. In this study, we obtained similar clinical findings with those reported in tropical theileriosis along with wilt and petechial haemorrhages in the mucous membranes. Besides, we verified the findings of the previous studies on the auscultation of the heart. Obtained findings rested on the pressure on the

vena cava which is due to the enlargement of the prescapular lymph node and possible mediastinal lymph node. Thus, we inferred that pseudo-pericarditis can be formed in tropical theileriosis.

As for the haematological findings, Kachhawa et al. (2016) and Ayadi et al. (2017) reported that the levels of total leukocyte, erythrocyte, haemoglobin and haematocrit were low in cattle with tropical theileriosis. Temiz et al. (2014) found that the number of leukocytes was high, but the haemoglobin concentration, hematocrit value and PLT numbers were significantly low in cattle with theileriosis compared to the control group. As can be seen, reports on the total number of leukocytes in these studies are controversial. The mechanism behind this controversy can be explained as follows: In theileriosis, the leukocytosis occurs mostly at the early stages of the disease, while leukopenia occurs at the late stages of the disease (Sayin, 1985; Gül, 1999). Lymphocytes proliferation forms in lymphoid organs as a defensive response to the entry and multiplication of *T. annulata* which gives rise to leucocytosis (Modi et al. 2015). On the other hand, leukopenia, which is mediated by TNF- α, is related to the destruction of lymphocytes in lymphoid organs and the infiltration of these cells to various organs (Sandhu et al., 1998; Forsyth et al., 1999; Omer et al., 2002). In this study, we discovered lymphopenia in two cattle with pseudo-pericarditis due to tropical theileriosis, so it suggests that the disease is at the late stage.

Further haematological findings indicate severe anaemia as the disease progresses. In this context, Issi et al. (2010) found a significant reduction in erythrocyte, haemoglobin and hematocrit values of 10 cattle with tropical theileriosis compared to healthy animals. Similarly, Keleş et al. (2003) determined that haemoglobin (9,7 g/dL) and HCT (29%) values approached the limit values in a cow with pseudo-pericarditis due to tropical theileriosis. Sudhakara and Sivajothi (2017) reported a reduction in erythrocyte (4,2 $x10^6/\mu L$), haemoglobin (7,2 g/dL) and HCT (22%) values in a bull with pseudo pericarditis due to tropical theileriosis. Temiz et al. (2014) detected pseudo-pericarditis in a severe anaemic cow with only 12.5% HCT from 28 tropical theileriosis cattle. Our numbers are compliant with these studies, however, they are significantly lower than those reported, such as erythrocyte (1,56 $x10^6/\mu L$ and 2,27 $x10^6/\mu L$), haemoglobin (2,9 g/dL and 3,6 g/dL), haematocrit (9,26% and 11,32%) and platelet (93 $x10^3/\mu L$ and 130 $x10^3/\mu L$). Anaemia occurs due to removal of the piroplasm-infected erythrocytes by macrophages in the organs of the reticuloendothelial system (Beniwal et al., 2000), pro-inflammatory cytokines, particularly TNF-α (Forsyth et al., 1999), increased levels of activated complement products and oxygen radicals (Clark et al., 1986). As the disease progresses in tropical theileriosis, marked anaemia with bilirubinaemia and bilirubinuria, thrombocytopenia, greatly enlarged superficial lymph nodes, icterus and petechial haemorrhages in the conjunctiva and diarrhoea occur (Priston, 2001). In addition, we have detected severe anaemia, leukopenia, thrombocytopenia, high TBIL levels, and petechial haemorrhages in conjunctival mucosa. Consequently, it can be easily inferred that the disease being at the late stage. Furthermore, we believe that animals with theileriosis are more likely to form pseudo-pericarditis if erythrocyte, haemoglobin and especially HCT values are very low, namely, severe anaemic.

As for the biochemical findings, we also checked the cTnI concentration and CK-MB levels, since some studies on animals with theileriosis reported myocardial damage. cTnI is a potent marker used for the diagnosis of myocardial damage (Gunes et al., 2008). CK-MB, LDH, AST, and alanine aminotransferase (ALT) serve the same purpose (Basbugan et al., 2010). In this context, Kilinc et al. (2018) reported that cTnI (0.14 ng/mL ± 0.02), creatine kinase (CK) (839.13 U/L ± 84.37), CK-MB (268.86 U/L ± 27.55) and AST (83.60 U/L ± 4.06) enzyme activity were quite high in 50 cattle with theileriosis compared to the control group. Razavi et al. (2015) noted that cTnI (0.06 ng/mL ± 0.004), CK (113.27 U/L ±2.59), AST (116 U/L ± 3.28) and LDH (647.5 U/L ± 18.83) levels in 50 cattle with theileriosis were significantly high. They attributed these high numbers with significant myocardial tissue damage due to *T. annulata* which can be severed by anaemia and hypoxia. Fartashvand et al. (2013) found that mean serum cTnI level (0.028 ng/mL; range: 0.005–0.21 ng/mL), CK-MB (301 ± 103 U/L) and AST (107 ± 46 U/L) enzyme activity were quite high in 90 cattle with theileriosis compared to the control group. In addition to the contributions of Razavi et al. (2015), they stated that the severity of parasitemia would also contribute to the pathophysiology of myocardial damage. To the best of our knowledge, so far there is no study investigating the cTnI, CK-MB and LDH levels (except for AST) in cattle with pseudo-pericarditis-shaped tropical theileriosis. In this case report, for the first time in the

world, we examined and noted very high numbers of cTnI, CK-MB, LDH and AST which revealed severe myocardial damage (Table 2). We think that these high numbers, even higher than the previous studies, could be the result of severe anaemia and intense parasitemia. Furthermore, the fullness in the jugular vein could have a negative effect on the heart. Therefore, we argue that by causing heart's dysfunction, both the myocardial damage due to theileriosis and the negative effect of fullness in the jugular vein give rise to the findings of pseudo-pericarditis to become evident. However, this claim should be supported by electrocardiography and echocardiography examination methods and studies with more animals.

Interestingly, the studies on this topic did not investigate the biochemical parameters, only with an exception of Sudhakara who preferred to check only AST, TP, ALB and Glucose. Here, we examined some of the further biochemical parameters to determine the hepatic tissue damage. It has been stated that high AST and total bilirubin levels are sufficient to determine liver damage in cattle (Gul and Grunder, 1988), but haemolytic anaemia may also cause increase total bilirubin in theileriosis (Omer et al., 2003). GGT is a sensitive indicator of liver disease (Murray et al., 1990). Many researchers have reported an increase in AST-GGT enzyme activities, total bilirubin and BUN levels along with hypoproteinemia and hypoalbuminemia in cattle with theileriosis (Sandhu et al., 1998; Omer et al., 2003; Ellah, 2015; Kachhawa et al., 2016). Razavi et al. (2015) reported that there was a marked increase in AST (116 U/L ± 3.28) and LDH (647.5 U/L ± 18.83) levels in cattle with theileriosis. However, a study examining the levels of LDH, GGT and BUN in cattle with pseudo-pericarditis-shaped theileriosis has not been found. In this study, we also found high levels of AST, GGT and LDH enzyme activities, BUN and total bilirubin levels along with hypoproteinemia and hypoalbuminemia (Table 2). We stipulate that our significantly higher numbers would allude to severe hepatic damage due to anaemia, strong heart damage, hypoxia and theileriosis (Sandhu et al., 1998). Moreover, we detected low levels of glucose which may be due to the consumption of glucose by the theileria agents and liver dysfunction in the disease (Col and Uslu, 2006). Finally, blood calcium levels were lower than the reference value which can be associated with hypoalbuminemia (Ellah, 2015).

In conclusion, this study touches upon three important points. Firstly, in case of a greatly enlarged superficial lymph nodes, severe anaemia and petechial haemorrhages in conjunctival mucosa along with the low count of haematological parameters, particularly HCT, the risk of occurrence of pseudo-pericarditis due to theileriosis significantly rises. This also implies that pseudo-pericarditis is a late-stage symptom of theileriosis. Secondly, the heart damage would be more severe in cattle with pseudo-pericarditis which is evidenced through high levels of cardiac biomarkers (cTnI, CK-MB, AST and LDH). So far, this case report would be the first investigating the cardiac biomarkers on cattle with pseudo-pericarditis-shaped theileriosis. Last but not least, there is a considerable risk of hepatic damage in such animals which is elucidated with the biochemical parameters (AST, GGT, LDH, TBIL, ALB etc.). Such biochemical parameters were not preferred in the previous studies on this matter, therefore we are happy to report a case based on such parameters. However, in order to fully comprehend the effect of pseudo-pericarditis on hemogram and biochemical parameters, more comparative studies with theileriosis and pseudo-pericarditis-shaped theileriosis should be made.

CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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