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Address: İstanbul University Faculty of Veterinary Medicine, 34320 Avcılar, İstanbul, Turkey
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Address: Büyükdere Avenue, 105/9 34394 Mecidiyeköy, Şişli, İstanbul, Turkey
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Address: İstanbul University Faculty of Veterinary Medicine, 34320 Avcılar, İstanbul, Turkey
Phone: +90 (212) 473 70 70/17260
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Contents

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Phylogenetic Grouping of Verotoxigenic *Escherichia coli* (VTEC) Obtained from Sheep and Broiler Chicken in Northwestern Iran

Habib DASTMALCHI SAEI[1], Mohammad ZAVARSHANI[2]

[1]Department of Microbiology, Faculty of Veterinary Medicine and Department of Cellular and Molecular Biotechnology, Institute of Biotechnology, Urmia University, Urmia, Iran

[2]Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

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ORCID IDs of the authors: H.D.S. 0000-0002-0628-3545; M.Z. 0000-0003-1672-2940

Abstract

Verotoxigenic *Escherichia coli* (VTEC) are major foodborne pathogens with an increasing public health concern. The purpose of this study was to investigate the occurrence and the phylogenetic groups of VTEC isolates from the feces of healthy sheep and broiler chickens at a slaughterhouse in Urmia region, Northwestern Iran. A total of 446 *E. coli* isolates (97 from sheep and 349 from broiler chickens) were assessed for the occurrence of the Vtx-encoding genes (vtx1 and vtx2) using polymerase chain reaction. Then, all the recovered VTEC isolates were phylogenetically grouped based on the Clermont phylotyping method using three genetic sequences, the so-called chuA, yjaA, and TSPE4.C2. The vtx gene-carrying *E. coli* was identified in 46.4% (45/97) of sheep-originated isolates and in 8.3% (29/349) of broiler chicken-originated isolates. In general, phylotyping revealed that 74 VTEC isolates segregated in the phylogenetic groups A (32.4%; designated as VTEC-A), B1 (44.6%; VTEC-B1), B2 (9.5%; VTEC-B2), and D (13.5%; VTEC-D). The results also showed that the dissemination of VTEC isolates of sheep and broiler chicken origin varied noticeably in their assignment to B1 and D phylogenetic groups ($p<0.01$). In addition, the virulent phylogenetic groups (B2 and D) were significantly more common in broiler chickens than in sheep ($p<0.01$). In conclusion, healthy sheep and broiler chickens could be a reservoir for VTEC belonging to virulent phylogenetic groups, thus representing a potential risk factor for public health. This study also demonstrated significant differences with respect to the phylogenetic group assignment of the VTEC strains between sheep and broiler chickens.

Keywords: Broiler chickens, phylogenetic groups, sheep, verotoxigenic *Escherichia coli* (VTEC)

Introduction

Escherichia coli is a bacterium generally found in the gut of warm-blooded animals (Kaper et al., 2004). Although most strains of this micro-organism are considered to be harmless symbionts of digestive tract, some strains cause human diseases. Verotoxigenic *Escherichia coli* (VTEC; also called Shiga toxin-producing *E. coli* or STEC) has emerged as an important zoonotic food-borne pathogen (Gyles, 2007) which can cause hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) in human (Girardeau et al., 2005). These strains are defined by making of one or more cytotoxins, called verocytotoxin 1 (VT1) and verocytotoxin 2 (VT2), usually encoded by bacteriophages. However, strains of this pathotype appear to circulate as a part of the gut flora with ruminants such as cattle, sheep and goats serving as the major animal reservoirs (Horcajo et al., 2010; Oporto et al., 2008). Healthy birds have also been reported to carry VTEC (Farooq et al., 2009).

Escherichia coli strains can be categorized into four main phylogenetic groups A, B1, B2 and D, by assessing the presence or absence of three genetic sequences called *chuA* (existing in B2 and D phylogroups, absent from B1 and A), *yjaA* (existing in B2, absent from D) and TSPE4.C2 (existing in B1, absent from group A) (Clermont et al., 2000). These phylogenetic groups apparently differ in their ecological niches, history of life, ten-

Address for Correspondence: Habib DASTMALCHI SAEI • **E-mail:** HDSaei561@gmail.com

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dency to cause disease (Gordon et al., 2008) and some characteristics such as their virulence genotype and genome size (Bergthorsson and Ochman, 1998; Girardeau et al., 2005). Commensal *E. coli* belongs generally to A and B1 phylogroups and rarely possess virulence genes (Dixit et al., 2004), whereas B2 and D strains are typically related to disease and carry a broad spectrum of virulence-factor genes (Nowrouzian et al., 2005). Phylotyping analyses have also revealed that the majority of the VTEC strains comprise phylogenetic group B1, representing that they most probably do not cause severe diseases in human (Girardeau et al., 2005; Ishii et al., 2007). In general, humans are infected with VTEC strains mostly through the ingestion of contaminated food or water or direct contact with animals, therefore identifying the sources of infection is an effective way towards decreasing the prevalence of this pathogen and thus reduce the risk of humans infection. Phylotyping of *E. coli* strains has previously been underscored as a valuable tool for bacterial source tracking (BST) and for surveillance programs in slaughterhouses (Carlos et al., 2010; Martins et al., 2013). Although calves have been considered to be a reservoir of VTEC in Urmia region, Iran (Saei and Ayremlou, 2012), there are limited information about the prevalence of VTEC in other food producing animals. The aims of the current study were to investigate the presence of VTEC and to determine their phylogenetic groups in feces from healthy sheep and broiler chickens at slaughter in Urmia, northwest of Iran.

Materials and Methods

Sample collection and *E. coli* isolates

A total of 446 fecal samples of apparently healthy sheep (n=97) and broiler chickens (n=349) were obtained during slaughter in Urmia region, Northwestern, Iran. All procedures in this study were in accordance with the ethical standards of the Animal Ethics Committee of Faculty of Veterinary Medicine, Urmia University (AECVU) and supervised by authority of Urmia University Research Council (UURC). The swab samples were placed directly in tubes containing Stuart transport medium (CM0111-Oxoid, Basingstoke, United Kingdom), and submitted to the laboratory for immediate processing. Each sample was streaked onto MacConkey agar (105465-Merck, Darmstadt, Germany) plates and incubated overnight at 37°C. Typical lactose-positive (pink *E. coli* colonies) colonies were further streaked on Eosin Methylene Blue (101347-EMB, Merck, Darmstadt, Germany) agar. From each plate, a single colony of typical morphology was selected and subcultured onto 5% sheep blood agar (110886-Merck, Darmstadt, Germany) for purity and biochemical tests. Furthermore, species-specific PCR was done as described previously (Riffon et al., 2001) using primers Eco 2083 (GCT TGA CAC TGA ACATTG AG) and Eco 2745 (GCA CTT ATC TCT TCC GCA TT). The confirmed *E. coli* isolates were kept in glycerol broth at -20°C for subsequent analysis.

Detection of *vtx* genes by PCR

The presence of the *vtx* genes in the *E. coli* isolates was examined by PCR using primers described earlier (Osek, 2003). The primer set vtx_1F (5′-CAG TTA ATG TCG TGG CGA AGG-3′) and vtx_1R (5′-CAC CAG ACA ATG TAA CCG CTG-3′) were used for the amplification of vtx_1, which yielded a PCR product of 384 bp in size. The primer set vtx_2F (5′-ATC CTA TTC CCG GGA GTT TAC G-3′) and vtx_2R (5′-GCG TCA TCG TAT ACA CAG GAG C-3′) were used for amplifying vtx_2, which allow the amplification of a DNA fragment at approximately 584-bp. *E. coli* ATCC43895 was used as positive control. Polymerase chain reaction and electrophoresis of products were performed as described previously (Saei and Ayremlou, 2012).

Phylogenetic group determination by triplex PCR

Three primer pairs used for the amplification of three genetic sequences called *chuA*, *yjaA* and TSPE4.C2 are presented in Table 1. Amplifications were done in a CORBETT thermocycler (Model CP2-003, Australia) with the following temperature profile: 1 cycle of 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and finally 1 cycle of 72°C for 7 min. The positive control used for the determination of three phylogenetic group markers (*chuA*$^+$, *yjaA*$^+$ and Tspe4C2$^+$) was *E. coli* reference strain ECOR62. Amplicons were electrophoresed on 1.5% (w/v) agarose gel containing ethidium bromide and visualized by the UV transilluminator. The sizes of PCR products were determined by comparing with GeneRuler 100 bp DNA ladder plus (Thermo Scientific, Germany). The main phylogenetic groups (A, B1, B2 and D) and subgroups (A_0, A_1, B_1, $B2_2$, $B2_3$, D_1, and D_2) of VTEC strains were determined according to the combinations of *chuA*, *yjaA*, and Tspe4.C2 markers as described earlier by Clermont et al. (2000) and Escobar-Paramo et al. (2004), respectively.

Statistical analysis

The Mann-Whitney U test was done using Statistical Package for the Social Sciences Software V22.0 (SPSS Statistics for Windows, IBM Corp.; Armonk, New York, USA) to compare the phylogenetic groups between the studied hosts. For each comparison, a p value less than 0.01 was considered significant.

Results

A total of 446 *E. coli* isolates comprising 97 from sheep and 349 from broiler chickens were obtained from same number of fecal samples by conventional culture as well as species-specific PCR techniques. Confirmed *E. coli* isolates were tested for the presence of vtx_1 and vtx_2 genes. VTEC isolates were recovered from 46.4% (45/97) in healthy sheep, and 8.3% (29/349) in broiler chickens.

The PCR results for phylotyping revealed that VTEC strains segregated mainly in phylogenetic group B1 (33 of 74 [44.6%]), designated as VTEC-B1. Of the remaining strains, 24 (32.4%), 10 (13.5%) and 7 (9.5%) segregated in main phylogenetic groups A (VTEC-A), D (VTEC-D), and B2 (VTEC-B2), respectively. The different banding patterns obtained by Clermont triplex PCR method for the phylogenetic groups are shown in Figure 1.

Table 1. Sequences of oligonucleotide primers used in this study

Gene	Sequence	Amplicon size	Reference
chuA	5'-GAC GAA CCA ACG GTC AGG AT-3'	279 bp	(Clermont et al., 2000)
	5'-TGC CGC CAG TAC CAA AGA CA-3'		
yjaA	5'-TGA AGT GTC AGG AGA CGC TG-3'	211 bp	(Clermont et al., 2000)
	5'-ATG GAG AAT GCG TTC CTC AAC-3'		
TSPE4.C2	5'-GAG TAA TGT CGG GGC ATT CA-3'	152 bp	(Clermont et al., 2000)
	5'-CGC GCC AAC AAA GTA TTA CG-3'		

Table 2. Phylogenetic groups and subgroups of VTEC strains isolated from sheep and broiler chickens

		Phylogenetic groups and subgroups of VTEC*						
		VTEC-A		VTEC-B1	VTEC-B2		VTEC-D	
Host	n	A_0	A_1	B_1	$B2_2$	$B2_3$	D_1	D_2
Sheep	45	5	8	29	2	-	1	-
Broiler chickens	29	2	9	4	-	5	5	4
Total	74	7	17	33	2	5	6	4

* Verotoxigenic *Escherichia coli*

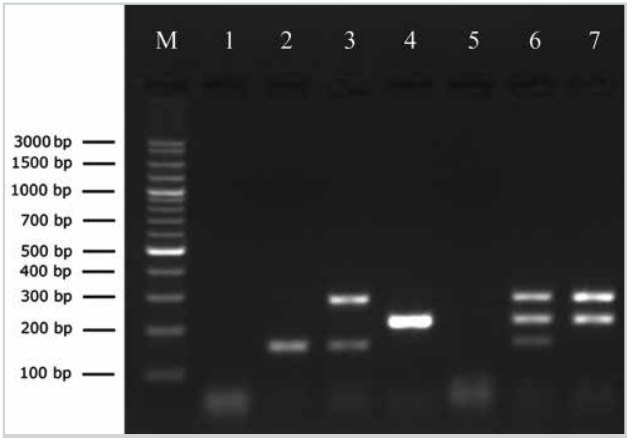

Figure 1. Triplex PCR patterns of representative *E. coli* phylogenetic groups. Lane M: GeneRuler™ 100 bp plus DNA ladder; Lane 1: negative control; Lane 2: group B1; Lane 3: group D; Lanes 4 and 5: group A; Lanes 6 and 7: group B2

Of 45 sheep VTEC isolates, 29 isolates (64.5%) were phylogenetic group B1, 13 (28.9%) phylogenetic group A, 2 (4.4%) phylogenetic group B2, and 1 (2.2%) phylogenetic group D. Among the isolates recovered from broiler chickens, 11 isolates (38%) were allocated into phylogenetic group A, 9 (31%) phylogenetic group D, 5 (17.2%) phylogenetic group B2, and 4 (13.8%) phylogenetic group B1. The analysis of phylotyping results among the studied hosts is detailed in Table 2. As shown, with the exception of B2 phylosubgroups ($B2_2$, $B2_3$), all other phylogenetic subgroups A (A_0, A_1), B1, D (D_1, D_2) were identified in VTEC isolates from both sheep and broiler chickens.

Table 3. The *vtx* genes in VTEC isolates from sheep and broiler chickens in relation to main phylogenetic groups

Phylogenetic group	Sheep				Broiler chickens			
***vtx** genes	A	B1	B2	D	A	B1	B2	D
vtx_1	8	17	1	1	4	1	3	3
vtx_2	3	8	1	-	6	3	2	5
vtx_1, vtx_2	2	4	-	-	1	-	-	1
Total	13	29	2	1	11	4	5	9

* Verocytotoxin

There was no significant difference in carriage of phylogenetic groups A and B2 between sheep and broiler chickens, whereas B1 significantly associated with sheep and D with broiler chickens ($p<0.01$). Concerning commensal (A and B1) and virulent (B2 and D) phylogenetic groups, statistical analysis also showed that commensal strains and virulent strains significantly associated with sheep and broiler chickens, respectively ($p<0.01$).

Out of 74 VTEC isolates, 38 isolates (51.4%) were positive for vtx_1, 28 (37.8%) for vtx_2, and 8 (10.8%) for both vtx_1 and vtx_2. Twenty-seven of sheep VTEC isolates were positive for vtx_1, 26 contained vtx_2 and 6 possessed both *vtx1* and *vtx2*. The corresponding counts in broiler isolates were 11, 16, and 2, respectively. The *vtx* genes in sheep and broilers VTEC isolates in relation to main phylogenetic groups are shown in Table 3.

Discussion

According to Wasteson (2001), Verotoxigenic *Escherichia coli* (VTEC) is the only *E. coli* pathogenicity group of major interest from zoonotic standpoint. In the current study, VTEC were isolated more frequently (45/97; 46.4%) in feces from sheep. This is in agreement with previous studies and confirms the importance of sheep as VTEC reservoir (Oporto et al., 2008). The frequency detected in the present study was, however, higher than the 29.9% and 7.9% reported in Switzerland and Brazil, respectively (Maluta et al., 2014; Zweifel et al., 2004). Another study on collection of *E. coli* isolates from healthy fat-tailed sheep in Iran showed that 13% of isolates belonged to VTEC pathotype (Ghanbarpour and Kiani, 2013). Differences in farm-level factors such as feed composition and sanitation

of drinking water may explain these discrepancies. A study of dairy cattle farms demonstrated that herd management factors related to cattle feeding practices were associated with fecal shedding of VTEC (Cho et al., 2013). In the current study, VTEC prevalence rate (29/349; 8.3%) in fecal samples of healthy broiler chickens was also higher than those reported in Kerman, southeastern of Iran (Ghanbarpour et al., 2011; Salehi, 2014). High incidence of VTEC observed in broilers may at least in part be due to geographical effects, hygienic measures and higher stocking density of birds in intensive chicken farming.

Despite the description of a new quadruplex PCR method to assign *E. coli* isolates to eight phylo-groups (A, B1, B2, C, D, E, F and clade I), Clermont genotyping triplex PCR is a cost effective and reasonably accurate method for detecting putative *E. coli* isolates from a variety of sample types (Higgins et al., 2007). Consistent with previous study (Girardeau et al., 2005), phylogenetic analysis revealed that VTEC isolates, irrespective of sheep or broilers origin, segregated mainly in phylogenetic groups A (24/74; 32.4%) and B1 (33/74; 44.6%). Selection through antibiotic pressure may explain this phenomenon, as most of antibiotic resistant *E. coli* strains have been shown to belong to the phylogenetic groups A and B1 (Obeng et al., 2012). Other speculation could be the ability of these phylogenetic groups to survive and persist in feces, manure, and soil in the environment. It is also hypothesized that bacteriophages carrying *vtx* genes probably could transduce with significant frequency to A and B1 phylogenetic group strains (Garcia-Aljaro et al., 2009).

Seven out of 74 (9.5%) VTEC strains analyzed in the study belonged to phylogenetic group B2, which is predominant among extraintestinal strains. In contrast to this result, none of the fecal isolates from domestic animals in South Korea and healthy fat-tailed sheep in southeastern of Iran belonged to B2 group (Ghanbarpour and Kiani, 2013; Unno et al., 2009). We supposed that they originated from food handlers or water contaminated with fecal material of humans. Carlos et al. (2010) stated that isolates belonging to the B2 group, particularly subgroup $B2_3$, represent an indicator for pollution by human feces.

According to statistical analysis, there were significant differences with respect to the phylogenetic group assignment of VTEC strains obtained from sheep and broilers. Carlos et al. (2010) also described a different dissemination of phylogenetic groups among *E. coli* strains isolated from humans, chickens, cows, goats, pigs and sheep, where high percentage of strains from the chicken samples were dominated by group A, whereas group B1 was predominant among *E. coli* strains from sheep. This non-random distribution of phylogenetic groups in the hosts may be due to ecological differences (e.g. in their behaviour, diet, antibiotic usage etc.) coupled with physiological differences (e.g. host genetic factors, gut characteristics, etc). A well-known example of the influence food ingestion may have is the prevalence of phylogenetic group A and B1 among omnivorous and herbivorous mammals, respectively (Carlos et al., 2010). Clermont et al. (2011) also concluded that gain (or loss) of few genes, e.g. adhesion-encoding genes, could contribute to the host specificity of non-B2 strains of different origin. Further studies of virulence factors which enable a phylogenetic group to colonize the gastrointestinal tract of different animal species are therefore needed to be evaluated.

Escherichia coli ST69, ST393, ST405 clones belonging to phylogenetic group D are increasingly reported as multidrug resistant strains causing extraintestinal infections (Novais et al., 2013). We found that the 10 VTEC strains studied belonged to the phylogenetic group D and significant differences on its association with hosts were also detected: only one VTEC isolate of phylogenetic groups D (2.2%) in sheep against 9 (31%) of broiler origin. Therefore, avian species appear to be a relevant reservoir of virulent phylogenetic group D. More expanded studies are needed to be undertaken in order to confirm this hypothesis.

In this study, we found that vtx_1 was the predominant gene over vtx_2 and vtx_1-vtx_2 in VTEC isolates in sheep. This distribution is in consistent with those previously described in two studies in sheep carried out in Spain (Blanco et al., 2003; Rey et al., 2003). However, in contrast with the results reported here, several studies have shown that most sheep VTEC carry vtx_1 and vtx_2 (Oporto et al., 2008; Vettorato et al., 2009). As results, vtx_2 was frequently found in broiler isolates. The same trend has been observed in chicken products in Argentina (Alonso et al., 2012). This is important because vtx_2-producing strains is more associated with severe disease in humans than vtx_1-producing strains (Paton and Paton, 2002).

Detection of *vtx* gene-carrying *E. coli* belonging to virulent phylogenetic groups (B2 and D), especially in broilers, represents a public health concern through fecal contamination of carcasses during slaughter operation at the processing facility. In Iran, studies have recently demonstrated that broiler and sheep carcasses could be considered as an important source of pathogenic *E. coli* (Bagheri et al., 2014; Tahamtan et al., 2010). On the other hand, high ratios of B2 and D isolates have been obtained from human clinical samples (Navidinia et al., 2013; Ramazanzadeh et al., 2013). However, further studies regarding phylogenetic background using other phylogenetic methods such as multilocus sequence typing (MLST), along with detection of serovars, *vtx* subtypes, and virulence genes are needed for predicting potential health hazards related to *E. coli* isolates from animals. In this regard, researches have pointed out the zoonotic potential of certain clonal groups such as avian pathogenic *E. coli* (APEC) O45:K1:H7-B2-ST95 (Mora et al., 2013) and O25b:K1:H4-B2-ST131 *ibeA* strains (Mora et al., 2010).

In conclusion, this study indicates that healthy sheep and broilers in Urmia region, Iran, could be considered as a source of VTEC strains. In addition, it demonstrates a different circulation

of the *E. coli* phylogenetic groups in the analyzed host. Regarding the presence of *stx* gene-carrying *E. coli* belonging to virulent phylogenetic groups in fecal samples of healthy animals, sufficient discrimination among VTEC strains to assess their public health significance is therefore recommended.

Ethics Committee Approval: Ethics committee approval was received for this study from the Animal Ethics Committee of Faculty of Veterinary Medicine, Urmia University (AECVU) and supervised by authority of Urmia University Research Council (UURC).

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SOD, CAT, TBARS, and TNF-α Concentrations in Uterine Tissues of Bitches with Pyometra and at Dioestrus

Tuğba Seval Fatma TOYDEMİR KARABULUT

Department of Gynaecology and Obstetrics, İstanbul University Faculty of Veterinary Medicine, İstanbul, Turkey

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ORCID ID of the author: T.S.F.T.K. 0000-0001-6416-8337

Abstract

Decreased antioxidant levels may be a cause of many diseases. Pus accumulation in the uterus literally called pyometra and generally occurs in median or late life span of bitches. The objective of the study was to evaluate the concentrations of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), oxidative stress parameter-thiobarbituric acid-reactive substances (TBARS) and inflammatory mediator, tumour necrosis factor alpha (TNF-α) in uterine tissues of bitches with and without pyometra. For this purpose, bitches with pyometra (n=27), and healthy bitches in dioestrus (n=8) included in the study and both of the groups went under ovariohysterectomy operation. SOD and CAT enzymes were found significantly higher in uterine tissues of dioestrus group ($p<0.01$, $p<0.05$, respectively). TNF-α did not differ in uterine tissues between the groups ($p>0.05$). Our data showing that decreased concentrations of antioxidant enzymes in the uterus occurs during the disease of pyometra in bitches. Due to antioxidants cannot deactivate free radicals occurred during the inflammatory process probably one of the main cause for uterine tissue damage in pyometra.

Keywords: Dioestrus, dog, oxidative stress, pyometra, TNF-α, uterus

Introduction

Free radicals are a reactive and unstable ion species, which become stable by acquiring electrons from wherever they can, which causes some adverse reactions resulting in cellular impairment. There are two types of free radicals: reactive oxygen species (ROS) and reactive nitrogen species (NOS) (Agarwall et al., 2005). Superoxide radical ($^{\cdot}O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) are referred as ROS and have became more recognizable in the last few decades. ROS are responsible for DNA damage, and thus are a cause of underlying diseases (Ercan et al., 2012, Todorova et al., 2005). More than 100 diseases including female reproductive disorders have been associated with ROS (Agarwall et al., 2005). Overabundance of ROS is termed "oxidative stress", and this stress is possibly an initiator of certain pregnancy-related disorders such as early pregnancy loss and embryopathies, in both humans and animals (Al-Gubory et al., 2010). Oxidative stress and antioxidants during pregnancy have been analysed in many studies on mammalian species (Gupta et al., 2007; Santos et al., 2004). It is clear that there is a link between oxidative stress and disorders of the female reproductive system, which is important in both human and animal reproduction (Al-Gubory et al., 2010).

The causes of infertility in bitches are difficult to identify and generally remain an unsolved problem in pet clinics. Infertility in bitches is presumed mainly caused by infectious agents (*Brucella canis, Campylobacter spp., Escherichia coli,* etc.), however, there are other causes including uterine lesions, hormonal imbalances, systemic diseases (Mir et al., 2013) as well as oxidative stress (Agarwall et al., 2005, Burton and Jauinaux, 2011). Diseases related to oxidative stress and inflammation has been

Address for Correspondence: Tuğba Seval Fatma TOYDEMİR KARABULUT • **E-mail:** sevaltoydemir@yahoo.com

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reported in numerous previous studies in dogs. When oxidative stress occurs, there may be no clinical signs, however, more energy is used for scavenging free radicals rather than for reproduction (Mohebbi-Fani et al., 2016) and this may lead to infertility in dogs.

Reactive oxygen species can be deactivated by antioxidants, which are 1) vitamins (A, C, and E) and 2) antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; and glutathione peroxidase, GPX). Antioxidants protect against peroxidation and DNA impairment (Al-Gubory et al., 2010, Todorova et al., 2005). If the balance between antioxidants and ROS fails, ROS is overproduced and binds to unsaturated fatty acids on cell membranes. This reaction is called "lipid peroxidation" and can be measured as thiobarbituric acid-reactive substances (TBARS) to show the degree of lipid peroxidation in an organism.

Superoxide dismutase is the first part of the defence mechanism during oxidative stress conditions and its main function is catalysing detoxification of superoxide radicals ($^{\cdot}O_2$) (Nakano et al., 1996, Todorova et al., 2005). Hydrogen peroxide (H_2O_2) is generated at the end of this reaction and together with GPX, CAT degrades H_2O_2 into water and oxygen. CAT is a more active antioxidant enzyme under high concentrations of H_2O_2 (Szczubial and Dabrowski, 2009).

Pyometra is sometimes a life-threatening disease, arising as a consequence of the systemic inflammatory response syndrome (SIRS). SIRS can be detected by determining the concentrations of cytokines in dogs, like in humans (Karlsson et al., 2012). Cytokines are mainly protein constructed elements that are released into the inter cellular space by leucocytes. SOD also neutralizes superoxide radicals formed by tumour necrosis factor alpha (TNF-α) cytokine (Agarwall et al., 2005).

In our study, oxidative stress biomarkers were chosen according to the results of some previous studies. In those studies, Cu SOD, Mn SOD, CAT and GPX (Sugino et al., 2000), malondialdehyde (MDA) and TBARS (Jozwik et al., 1999) were revealed to be present in the female reproductive tract.

The present study compared the level of oxidative stress by using TBARS, SOD and CAT, and the inflammatory mediator TNF-α during the dioestrus period in healthy bitches and bitches with pyometra. The hypothesis of the study was that, when the uterus gets infected, oxidative stress probably occurs in the uterine tissues, affecting the reproductive performance of bitches and also increasing the risk of pyometra disease.

Materials and Methods

Dogs

The studies were performed on 27 dogs with pyometra, with a mean body weight of 20.18±12.41 kg (pyometra group; PG), and 8 clinically healthy dogs in dioestrus with a mean body weight of 15.15±14.72 kg (dioestrus group; DG). There was only one young dog in each group while all others were geriatric in both groups. Dogs in both groups were free of common infectious diseases. Bitches were considered to be in dioestrus according to their vaginal cytology results (appearance of intermediate and parabasal cells, neutrophils, and large numbers of bacteria in vaginal smears) in DG. Five dogs in PG had closed-cervix pyometra while twenty-two had open-cervix pyometra. Physical examinations were performed on each dog to evaluate the general condition in both groups. Results of vaginal examination, vaginal cytology and ultrasonography were used to differentiate pyometra from other uterine pathologies such as mucometra and hydrometra. For this purpose, transabdominal ultrasonography was performed to visualise the uterus (Easote Piemedical MyLab Five Vet, Netherlands) and also vaginoscopy was used to see the cervix uteri and vagina. Visualisation of pus during vaginal examination and/or a hypoechoic fluid-filled uterus on ultrasonography was considered as pyometra.

Tissue collection

Both groups of bitches underwent ovariohysterectomy (OVH). OVHs were performed under general anaesthesia, induced with an intravenous 4-8 mg/kg propofol solution (Propofol 1% Fresenius; Fresenius Kabi, DE) and continued with 2-4% isofluorane (Forane Liquid; Aesica Queenborough, UK). Excised uterine tissues were promptly rinsed and cleaned of blood and pus with cold saline to minimize the effect of blood on free radicals. Three parts of the uterus at different levels were taken for homogenization and stored in Eppendorf tubes at −86°C until homogenization.

Homogenization of uterine tissues and ELISA assays

Uterine tissues were first weighed and then rinsed with phosphate-buffered saline (PBS, pH 7.4) and homogenized (MIC-CRA-D1, ART Prozess&Labortechnik GmbH&Co. KG., Germany) in 5-10 ml cold PBS (proportion: 1/10). Uterine homogenates were centrifuged at 4°C for 30 min at 3000×g and then supernatants were analysed for TBARS, SOD, CAT and TNF-α. These parameters were measured with commercially available canine sandwich ELISA kits (TNF-α; canine TNF-α, CK-E90814; SOD; canine SOD ELISA, CK-E91351; CAT; canine CAT ELISA, CK-E91349; TBARS; canine TBARS ELISA, CK-E91350; Hangzhou Eastbiopharm Co. Ltd., China) by the quantitative sandwich enzyme immunoassay technique (μQuant, Bio-Tech Instruments, High Point, NC, USA) according to the manufacturer's instructions. The detection range of TNF-α was 5-1000 ng/L, SOD was 0.5-200 ng/mL, CAT was 1-300 ng/mL, TBARS was 0.5-100 nmol/mL and intra- and inter-assay coefficients of variations for all parameters were <10% and <2%, respectively.

Statistical analysis

Groups (pyometra and dioestrus) were added to the statistical model as between-subject effects and an independent sample t-test was applied to compare each sampling time for uterine analyses. A value of $p<0.05$ was used to indicate statistical significance.

Table 1. Uterine concentrations of SOD, CAT, TBARS and TNF-α

Variable	Dioestrus group (Mean±SE) (n=8)	Pyometra group (Mean±SE) (n=27)	t-test significance
SOD (ng/mL)	67.93±18.78	31.11±2.32	**
CAT (ng/mL)	67.69±18.52	39.40±3.80	*
TBARS (nmol/mL)	89.49±21.32	97.90±10.14	NS
TNF-α (ng/L)	371.45±64.56	342.99±32.85	NS

SOD: super oxide dismutase; CAT: catalase; TBARS: thiobarbituric acid reactive substances; TNF-α: tumour necrosis factor alpha
*p<0.05; **p<0.01; NS: p>0.05

Results

Uterine concentrations of SOD, CAT, TBARS and TNF-α
The mean concentrations in the groups are shown in Table 1. SOD, CAT and TNF-α were higher in uterine tissues of DG compared with PG. Of these variables, SOD and CAT levels were significantly different between the two groups (p<0.01, p<0.05, respectively). Concentrations of SOD, CAT, TBARS and TNF-α did not differ between the open- and closed-cervix pyometra bitches.

Discussion

Reactive oxygen species production is correlated with inflammatory processes. The consumption of oxygen by activated macrophages and neutrophils occurs during inflammation. An overabundance of ROS is seen if ROS production cannot be controlled by antioxidative mechanisms and this is one of the causes of tissue damage (Agarwal et al., 2005).

In this study, comparisons were made between SOD, CAT, TBARS and TNF-α concentrations in uterine tissues of bitches in the dioestrus period and bitches with pyometra. The main aim of this study was to determine the effect of ROS occurring during pyometra on levels of antioxidant enzymes SOD and CAT and also on the lipid peroxidation product TBARS and their relationships with a cytokine, TNF-α.

In our study, SOD concentrations were higher in DG, which comprises animals under the influence of progesterone. In a previous study, changes in superoxide anion and SOD level were detected during the oestrus cycle of *Rattus norvegicus*. Low levels of superoxide radical and high levels of SOD were observed during the dioestrus period (Laloraya et al., 1990). In another study, the expression of SOD was investigated in corpora lutea collected from women. The Cu-Zn SOD expression in the corpora lutea paralleled levels of progesterone and these levels rose from the early to the mid luteal phase and decreased during regression of the corpus luteum. Enhanced expression of Cu-Zn SOD in luteal tissue from pregnant patients was found and it was correlated with high levels of hCG (Sugino et al., 2000). In addition to this, it was revealed that estrogen lowers mammalian uterine SOD levels (Jain et al., 1999). Our results are in accordance with these studies. Additionally, when compared with DG, SOD levels were significantly lower in PG (p<0.01). This is likely due to the increased levels of ROS during inflammation and infection occurring in the uterus such as in the case of pyometra.

Our findings showed that pyometra disease significantly decreases the activity of the antioxidant enzymes, SOD and CAT (p<0.01, p<0.05, respectively), however, unfortunately, the level of antioxidant activity in the uterus at the beginning of the infection in these dogs is not known. TBARS concentrations were increased as expected in PG but the difference between the groups was not significant (p>0.05). At the initiation of uterine infection, antioxidant enzymes probably increase because of cytokines released from the leucocytes. In another study in bitches, SOD and CAT levels were found to be similar between the uterine tissues of bitches with pyometra and healthy ones (Szczubial and Dabrowski, 2009). This result might have been obtained in that study due to the fact that those bitches were recognized promptly at the initiation of pyometra. The duration of infection is thought to affect the activity of SOD and CAT concentrations in uterine tissue.

In another study, in women with endometriosis, high TNF-α concentrations were detected in the peritoneal fluid (Bedaiwy and Falcone, 2003). Cytokines influence the redox status of the ectopic endometrium in patients with endometriosis. Also, the antioxidant MnSOD neutralizes superoxide anions generated by cytokine TNF-α (Agarwal et al., 2005). In our study, TNF-α concentrations did not differ in uterine tissues between DG and PG (p>0.05) and no relationship was found between concentrations of TNF-α and pyometra occurrence.

In conclusion, our data showed that pyometra disease negatively changes the oxidative status of the uterus. Additionally, significantly decreased concentrations of SOD and CAT found in infected uteri compared with dioestrus uterine tissue show the importance of these antioxidant enzymes in the uterus. Also, it was assumed that one of the main causes of uterine damage in pyometra is the generation of free radicals during the inflammatory process, which cannot be deactivated by decreased levels of antioxidants.

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Experimental Evaluation of the Wound-healing and Antioxidant Activities of Tamarind (*Tamarindus indica*) Pulp and Leaf Meal in the African Catfish (*Clarias gariepinus*)

Olarinke Victoria ADENIYI[1,2], Flora Eyibio OLAIFA[2], Benjamin Obukowho EMIKPE[3], Ademola Adetokunbo OYAGBEMI[4]

[1]Department of Animal Production, Fisheries and Aquaculture, Kwara State University, Malete, Nigeria
[2]Department of Aquaculture and Fisheries Management, University of Ibadan, Nigeria
[3]Department of Veterinary Pathology, University of Ibadan, Nigeria
[4]Department of Veterinary Physiology, Biochemistry and Pharmacology, University of Ibadan, Nigeria

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ORCID IDs of the authors: O.V.A. 0000-0002-0964-8300; F.E.O. 0000-0003-1263-4134; B.O.E. 0000-0003-2458-6504; A.A.O. 0000-0002-8996-8610

Abstract

The fruits and leaves of *Tamarindus indica* have been widely used in traditional medicine for treating wounds and other diseases in Africa. The aim of this study was to investigate the wound-healing property of *T. indica* pulp (TP) and leaf (TL) meal and the importance of antioxidant enzymes in the wound-healing process in the African catfish, *Clarias gariepinus*. Surgical incisions of 10 mm^2 were made aseptically on the lateral part of the catfish, above the pelvic fin and toward the caudal region. The fish were fed experimental diets composed of basal diet fortified with each of TP or TL at concentrations of 0.5%, 1.0%, 1.5%, 2.0%, and 0.0% (untreated) and 0.2% oxytetracycline (treated) as controls in triplicate groups. Changes occurring in the wound area (mm^2) were measured at 3-day-intervals for 15 days. The healing rates and the relative percentage of healing were calculated. Sera collected from the experimental fish were analyzed for oxidative stress biomarkers and antioxidant enzymes. The results showed that fish fed diets treated with TP or TL had significantly faster ($p<0.05$) daily healing rates at the lateral and caudal regions from the 6^{th} to the 12^{th} day compared with those in the control groups. Percentage wound-healing (PWH) at the lateral and caudal regions was significantly enhanced ($p<0.05$) from the 6^{th} day in the tamarind-treated groups. The PWH reached the peak (100%) at the lateral region on the 12^{th} day in fish fed 0.5–2.0% of TL and 1.5%–2.0% of TP diets. Dietary treatment with TP and TL resulted in a lower production of serum malondialdehyde and hydrogen peroxide levels, whereas the reduced glutathione, superoxide dismutase, and glutathione peroxidase levels increased. Fortifying diets of *C. gariepinus* with 1.0%–2.0% of TP and TL meal enhanced wound-healing significantly compared to that of natural healing and with oxytetracycline-fortified diet. The faster wound-healing rate might be a consequence of elevated levels of serum antioxidants in the fish fed tamarind-fortified diets.

Keywords: African catfish, antioxidant activity, experimental study, serum antioxidants, *Tamarindus indica*, wound-healing

Introduction

The skin forms the external covering of the body of fish, which protects the fish against mechanical injury and noxious agents. The skin consists of the epidermis and dermal layer. The epidermis of teleost fish consists of fusiform cells, which remain viable and retain the capacity for mitotic division significant for healing processes (Genten et al., 2009; Yang et al., 2015). Skin grows, differentiates and renews itself at all times. A wound is a loss or breaking of cellular and anatomic or functional continuity of living tissues (Ayello, 2005). The closing of an open wound initiates healing because of the responses triggered off by the damaged local cells. Wound-healing is a physiological response of animal to tissue injury which results in replacement of destroyed tissue and restoration of the tissue integrity.

Wound-healing is enhanced by circulation of oxygen and nutrients in wound sites (Abdulla et al., 2009). Although oxygen plays vital roles such as oxidative phagocytosis, synthesis of collagen, angiogenesis and epithelialization in wound-heal-

Address for Correspondence: Olarinke Victoria ADENIYI • **E-mail:** adeniyiovic@yahoo.com
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ing, it is also resulted to production of highly reactive oxygen species (ROS) such as free radicals and peroxide, which result to oxidative stress, decelerate these processes and impaired wound-healing. Excessive production of ROS is deleterious to wound-healing (Dunnill et al., 2017; Kanta, 2011), hence, balance between ROS and antioxidants is essential. Antioxidant enzymes play important roles in the detoxification of reactive oxygen metabolites during wound-healing process (Bryan et al., 2012; Keller et al., 2006; Kurahashi and Fujii, 2015).

Plants and their extracts are organic products with immense potential for the management and treatment of wounds. Wound-healing activity of herbal products has been associated with the antimicrobial and antioxidants properties of the phytobiotics (Abdulla et al., 2009, Mohammad et al., 2012; Vifayaraghavan et al., 2017). Plant phytochemical constituents such as tannins, alkaloids and flavonoids contribute to wound-healing activity in animals (Kim et al., 2011; Li et al., 2011; Pawar and Toppo, 2012). Herbal products from *Rafflesia hasseltii* flowers (Abdulla et al., 2009), *Tamarindus indica* (Linn 1753) seed (Mohammad et al., 2012), *Acorus calamus* root and rhizome (Shi et al., 2014) in rat or mice, *Allium cepa* bulb, *Tetracarpidium conophorum* leaf (Bello et al., 2013) and *Azadirachta indica* leaf and oil (Alam et al., 2014) in fish have been used to test the efficacy of herbal products in wound-healing with great potentials.

Tamarindus indica L, commonly called tamarind, is a large tree belonging to the family Leguminoseae (Fabaceae) and subfamily Ceasalpinioideae. Tamarind grows widely in most tropical and subtropical regions of the world (Bhadoriya et al., 2011; Dhamija and Parle, 2012). The bark or leaves of tamarind in the form of powder, decoction, and poultice are applied traditionally on cuts, wounds and abscesses as well as for cleansing wounds caused by guinea worm (Lockett et al., 2000). The ethnomedical use of tamarind in wound-healing in many African countries has been reported (Diallo et al., 2002; Fabiyi et al., 1993; Havinga et al., 2010; Inngjerdingen et al., 2004). Studies have demonstrated the *in vitro* antimicrobial (Adeniyi et al., 2017; Gumgumjee et al., 2012) and antioxidant (Khairunnuur et al., 2009; Lim et al., 2013) activities of tamarind extracts. The role of antioxidant property of tamarind in wound-healing has not been elucidated while scientific information on the wound-healing and *in vivo* antioxidant activities of *T. indica* in fish is limited. Intensive culture of *Clarias gariepinus* (Burchell 1822) is associated with wounds resulting from aggressive behaviours of fish and artificial breeding involving the cutting of testes. This study therefore investigated wound-healing and antioxidant activities of dietary *T. indica* pulp and leaf meal as feed additives in *C. gariepinus*.

Materials and Methods

Plant identification and diets preparation

Fresh tamarind leaves and dried fruits were obtained and authenticated as *Tamarindus indica* Linn with a Voucher Number: UIH-22550. Following the harvest of the plant materials, fresh leaves were removed from the stalk, washed with clean water, drained while the brittle fruit husks were carefully removed and the pulp scraped from the fruits. The leaves were air-dried under shade for 14 days and pulp for 21 days. Both the tamarind pulp (TP) and leaves (TL) were processed into meals. The meals, TP and TL, were included singly at 0.5. 1.0, 1.5 and 2.0% each to fortify the basal diets while 0.0% and 0.2% oxytetracycline (OXY 200 WSP; Kepro, Deventer, Holland) were untreated and treated controls, respectively to make 10 experimental diets.

Experimental fish samples and formation of the wounds

The experimental fish samples consisted of 150 healthy African catfish (*C. gariepinus*). The fish (33.97-45.69g) were randomly selected from each treatment of fish previously fed with the experimental diets in triplicate groups for 12 weeks. Five fish were selected from each replicate and distributed into 50 litre capacity rectangular tanks in triplicates, according to their treatment groups. Following cleaning the portion of the skin with 70% ethanol, surgical incisions wounds of 10 mm^2 were made on each of the fish to the dermis on the lateral part, above the pelvic fin and towards the caudal region (Bello et al., 2013). The fish were returned to the holding tanks (50 litre capacity) and fed the experimental diets at 3% body weight daily. The culture water was changed completely every 48 hours. The water temperature, pH and dissolved oxygen were 26.5±1.00°C, 7.23±0.02 and 5.20±0.50mg/L respectively.

Evaluation of the wound-healing rate

Progressive changes in the wound area were evaluated by measuring the wound area with transparent ruler. The percentage wound-healing (Ammar et al., 2015), daily healing rates (Bell, 2002) and relative percentage healing (Amend, 1981) were calculated using the initial wound area (on 0^{th} day) and areas determined on n^{th} day (n=3, 6, 9, 12, 15) as shown in Table 1.

Evaluation of antioxidant activity

After the wound-healing experiment earlier described, blood samples were collected from the caudal vein (Ejraei et al., 2015) of fish sampled from each replicate individually into plain tubes and allowed to clot. The clotted blood samples were centrifuged at 4000 rpm for 10 minutes. Clear sera were collected with micropipette into plain tubes and stored in the freezer until when analysed. The *in vivo* antioxidant properties of dietary tamarind pulp and leaves were evaluated by analysing some oxidative stress biomarkers and antioxidants in the serum of the experimental fish after fifteen days. Concentrations of total protein (Gornal et al., 1949), hydrogen peroxide (Wolff, 1994), malondialdehyde (Varshney and Kale, 1990) as described by Omobowale et al., 2015) Reduced glutathione (Jollow et al. (1974) and activities superoxide dismutase (Oyagbemi et al., 2014) glutathione peroxidase (Rotruck et al., 1973) glutathione-s-transferase (Habig et al., 1974) and myeloperoxidase (Xia and Zweier, 1997) were analyzed spectrophotometrically (Elx800, BioTek, Winooski, USA) using the standard procedures. The whole experimental protocols

were performed according to the International (2010/63/EU) and University of Ibadan Institutional rules of animal experiments, clinical studies and biodiversity rights.

Statistical analysis

One-way Analysis of Variance (ANOVA) was used to analyze the data. Duncan multiple range test was used to compare differences among means at 5% probability level using statistical Statistical Analysis System (SAS software, 2010; SAS Institute, Cary, USA).

Results

Wound-healing activity

Fish on diets treated with TP and TL had significantly faster ($p<0.05$) Daily Healing Rates (DHR) at the lateral and caudal regions from the 6th to 12th day compared to the control groups (Table 2). The DHR generally reduced progressively from the 3rd day to the 15th day. Similar to the pattern observed for DHR, Percentage Wound-Healing (PWH) was significantly enhanced ($p<0.05$) from the 6th day in tamarind-treated groups (Table 3). The PWH reached the peak (100%) at the lateral region on the 12th day for all TL and 1.5-2.0% TP groups. The healing pattern at the lateral and caudal regions was dose-dependent. The PWH significantly increased ($p<0.05$) as the levels of inclusion of TP and TL rose.

Treating the diets of *C. gariepinus* with OTC and tamarind enhanced Relative Percentage Wound-Healing (RPWH) at the lateral and caudal region compared to natural healing in untreated control group (Figure 1, 2). On the 9th-15th day (Figure 3-5), fish fed the tamarind-treated diets also demonstrated significantly higher ($p<0.05$) RPWH than those fed the control diets. On the 12th day healing was completed at the lateral region in all the experimental groups except in the control groups and 0.5-1.0% TP group. Healing seemed to be relatively higher in the fish fed TL-treated diets than those fed TP-treated diets.

Other biological indices

During the 15 days study, *C. gariepinus* fed diets fortified with 2.0% TP and 1.0% TL showed significantly lower FCR than those fed untreated control diet. The survival was 100% in all the experimental groups (Table 4).

Antioxidant activity

All tamarind-treated groups showed lower hydrogen peroxide (H_2O_2) than the control groups (Figure 6). Fish fed diets containing 1.5% TP and 2.0% TL had significantly lower ($p<0.05$) H_2O_2 compared to those fed untreated control diet. Treating the diets of *C. gariepinus* with 1.0% TP and 1.0-1.5% TL significantly reduced ($p<0.05$) the sera Malondialdehyde (MDA) compared to the untreated control diet (Figure 7). The concentration of reduced glutathione (GSH) rose with the increasing level of inclusion of TP and TL in the diets (Figure 8). The concentration of GSH in the sera of fish fed diets treat-

Table 1. Formulae used for the study

Parameters	Formulae
Percentage wound healing (%)	100 x (Healed area* / Initial wound area)
Daily healing rates (mm^2)	Healed area / Healing time (nth day)
Relative percentage healing (%)	1 – [% wound healing in treatment (on nth day) / % wound healing in untreated control (on nth day)] x 100
Feed Conversion Ratio (FCR)	Feed intake (g) / Weight gain (g)
Survival rate (%)	100 x (Initial fish number - mortality number / Initial fish number)

*Healed area = Initial wound area (on 0th day) – Wound area left (on nth day)

Table 2. Daily healing rate (mm^2/day) of surgically wounded *Clarias gariepinus* fed diet treated with tamarind pulp and at three days interval for 15 days

	3rd day		6th day		9th day		12th day		15th day	
Diets	**LA**	**CA**	**LA**	**CA**	**LA**	**CA**	**LA**	**CA**	**LA**	**CA**
0.0	0.98 ± 0.02^{g}	0.36 ± 0.03^{f}	0.83 ± 0.02^{e}	0.40 ± 0.02^{g}	0.83 ± 0.01^{e}	0.43 ± 0.01^{c}	0.69 ± 0.01^{c}	0.35 ± 0.02^{g}	0.66 ± 0.01^{b}	0.33 ± 0.01^{g}
0.2O	1.19 ± 0.02^{c}	0.53 ± 0.03^{e}	0.85 ± 0.02^{e}	0.49 ± 0.01^{f}	0.84 ± 0.01^{e}	0.44 ± 0.01^{c}	0.78 ± 0.02^{b}	0.37 ± 0.00^{f}	0.67 ± 0.00^{a}	0.33 ± 0.01^{g}
0.5P	1.04 ± 0.01^{f}	0.53 ± 0.02^{e}	0.86 ± 0.01^{e}	0.54 ± 0.02^{de}	0.92 ± 0.01^{d}	0.52 ± 0.02^{b}	0.81 ± 0.05^{ab}	0.44 ± 0.01^{e}	0.67 ± 0.00^{a}	0.37 ± 0.01^{f}
1.0P	1.16 ± 0.02^{de}	0.64 ± 0.03^{d}	0.91 ± 0.01^{cd}	0.53 ± 0.02^{e}	0.98 ± 0.02^{d}	0.54 ± 0.02^{b}	0.82 ± 0.02^{a}	0.47 ± 0.01^{d}	0.67 ± 0.00^{a}	0.42 ± 0.01^{e}
1.5P	1.19 ± 0.02^{c}	0.77 ± 0.02^{c}	0.95 ± 0.02^{b}	0.58 ± 0.01^{c}	1.00 ± 0.02^{b}	0.61 ± 0.02^{ab}	0.83 ± 0.00^{a}	0.54 ± 0.01^{ab}	0.67 ± 0.00^{a}	0.47 ± 0.01^{bc}
2.0P	1.22 ± 0.02^{b}	0.94 ± 0.01^{a}	1.03 ± 0.01^{a}	0.64 ± 0.02^{b}	1.06 ± 0.06^{a}	0.63 ± 0.02^{a}	0.83 ± 0.00^{a}	0.56 ± 0.01^{a}	0.67 ± 0.00^{a}	0.48 ± 0.01^{b}
0.5L	1.33 ± 0.03^{e}	0.52 ± 0.02^{e}	0.89 ± 0.01^{d}	0.56 ± 0.01^{cd}	1.00 ± 0.01^{d}	0.53 ± 0.03^{b}	0.83 ± 0.00^{a}	0.45 ± 0.01^{e}	0.67 ± 0.00^{a}	0.44 ± 0.01^{d}
1.0L	1.16 ± 0.0^{de}	0.56 ± 0.03^{e}	0.92 ± 0.02^{c}	0.63 ± 0.02^{b}	1.01 ± 0.02^{c}	0.59 ± 0.02^{ab}	0.83 ± 0.00^{a}	0.50 ± 0.02^{c}	0.67 ± 0.00^{a}	0.48 ± 0.01^{c}
1.5L	1.18 ± 0.02^{cd}	0.62 ± 0.04^{d}	0.92 ± 0.02^{c}	0.64 ± 0.01^{b}	1.05 ± 0.02^{bc}	0.61 ± 0.01^{ab}	0.83 ± 0.00^{a}	0.53 ± 0.01^{b}	0.67 ± 0.00^{a}	0.47 ± 0.01^{bc}
2.0L	1.27 ± 0.03^{a}	0.84 ± 0.04^{b}	0.95 ± 0.01^{b}	0.71 ± 0.01^{a}	1.06 ± 0.04^{b}	0.68 ± 0.02^{a}	0.83 ± 0.00^{a}	0.54 ± 0.02^{ab}	0.67 ± 0.00^{a}	0.51 ± 0.01^{a}

Means with similar superscripts (a-g) on the same column are not significantly different at $p<0.05$
LA: lateral; CA: caudal; O: oxytetracycline; P: pulp; L: leaves

Table 3. Percentage wound-healing of surgically wounded *Clarias gariepinus* fed diet treated with tamarind pulp and leaf meal at three days interval for 15 days

	3rd day		6th day		9th day		12th day		15th day	
Diets	**LA**	**CA**	**LA**	**CA**	**LA**	**CA**	**LA**	**CA**	**LA**	**CA**
0.0	29.3±0.5^{g}	10.7±0.7^{f}	50.0±1.0^{e}	24.0±1.0^{g}	74.3±0.6^{e}	38.7±1.1^{e}	83.3±1.1^{c}	42.0±2.0^{g}	98.3±2.1^{b}	49.3±1.1^{g}
0.2 O	35.7±0.7^{c}	16.0±1.0^{e}	50.2±0.7^{e}	27.5±0.5^{f}	75.5±1.8^{e}	39.3±0.6^{e}	93.3±2.9^{b}	44.7±0.6^{f}	99.8±0.4^{a}	49.2±1.4^{g}
0.5P	31.3±0. 3^{f}	16.0±0.5^{e}	51.5±0.5^{e}	32.0±1.0^{e}	82.7±1.1^{d}	46.7±1.5^{d}	96.7±5.8^{ab}	53.0±1.0^{e}	100±0.0^{a}	56.0±2.0^{f}
1.0P	34.7±0.6^{de}	19.3±1.0^{d}	54.7±0.6^{cd}	32.7±1.1^{de}	88.0±2.0^{e}	47.3±1.5^{d}	98.3±2.9^{a}	56.7±1.1^{d}	100±0.0^{a}	62.7±1.5^{e}
1.5P	35.7±0.6^{c}	23.0±0.8^{c}	57.0±1.0^{b}	34.7±0.58^{abc}	90.0±2.0^{bc}	55.3±1.1^{b}	100±0.0^{a}	64.7±1.1^{ab}	100±0.0^{a}	70.0±1.0^{bc}
2.0P	36.7±0.6^{b}	28.1±0.2^{a}	62.0±0.5^{a}	38.7±1.1^{ab}	95.3±5.0^{a}	58.3±1.5^{a}	100±0.0^{a}	67.3±1.1^{a}	100±0.0^{a}	72.5±2.6^{b}
0.5L	34.0±1.0^{e}	15.7±0.4^{e}	53.7±0.6^{d}	33.7±0.6^{a}	90.0±1.0^{bc}	47.3±2.3^{d}	100±0.0^{a}	53.7±1.5^{e}	100±0.0^{a}	66.0±3.5^{ab}
1.0L	34.7±0.3^{de}	16.8±0.7^{e}	55.3±1.5^{c}	38.0±1.0^{ab}	91.3±2.3^{abc}	52.7±1.5^{c}	100±0.0^{a}	60.0±2.0^{c}	100±0.0^{a}	68.5±1.0^{ab}
1.5L	35.3±0.6^{cd}	18.7±0.5^{d}	56.0±1.0^{bc}	38.7±0.6^{abc}	94.5±1.8^{ab}	55.0±1.0^{bc}	100±0.0^{a}	63.0±1.0^{b}	100±0.0^{a}	71.0±2.6^{bc}
2.0L	38.0±1.0^{a}	25.3±1.1^{b}	57.3±1.5^{b}	42.3±0.6^{ab}	96.0±3.61^{a}	57.3±1.5^{ab}	100±0.0^{a}	65.3±2.1^{ab}	100±0.0^{a}	76.00±1.0^{a}

Means with similar superscripts (a-g) on the same column are not significantly different at p<0.05
LA: lateral; CA: Caudal; OTC: oxytetracycline; P: pulp; L: leaves

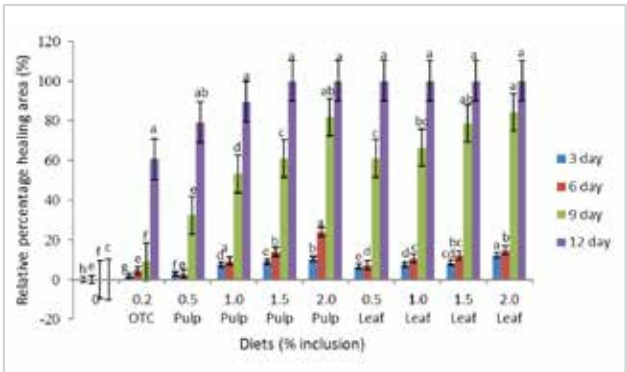

Figure 1. Relative percentage wound healing area at the lateral region of *Clarias gariepinus* fed diets treated with tamarind pulp and leaf meal. Bars with similar letters are not significantly different at p<0.05

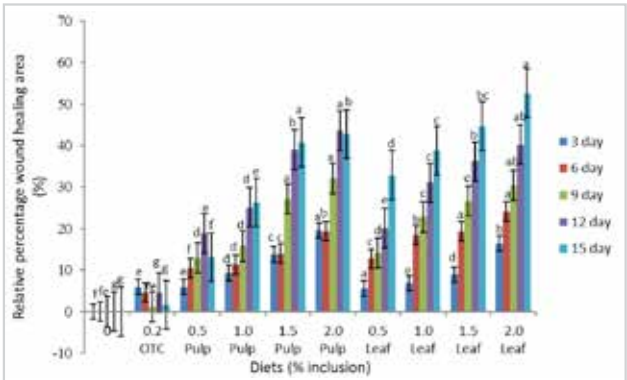

Figure 2. Relative percentage wound healing area at the caudal region of *Clarias gariepinus* fed diets treated with tamarind pulp and leaf meal. Bars with similar letters are not significantly different at p<0.05

ed with 1.0-2.0% TL were significantly higher (p<0.05) than the values obtained from the TP-treated and control groups. The activity of Glutathione Peroxidase (GPx) increased significantly (p<0.05) in the sera of the groups of fish fed diets containing 1.5-2.0% TP compared to the untreated control group. Groups of fish fed TP-treated diets exhibited higher activity of Superoxide Dismutase (SOD) than those fed control and TL-treated diets (Figure 9).

Although, TP-treated groups showed higher activity of Gluthathione-S-Transferase (GST) compared to OTC-treated group, the values did not differ significantly (p>0.05). Contrary to the observation with GST activity in TP groups, the activity of GST decreased with increasing level of TL in the diet (Figure 10). Figure 11 shows that fish on control diets had higher Myeloperoxidase (MPO) activity, than those on diets containing TP and TL. The activity of MPO did not differ significantly (p>0.05) among the tamarind-treated groups.

Discussion

This study investigated the wound-healing and antioxidants activities of *Tamarindus indica* pulp and leaf meal and related possible role of natural antioxidants in wound-healing. Faster wound-healing was observed at three-day intervals in groups of wounded *C. gariepinus* fed with tamarind-treated diets compared to the group on OTC-treated and untreated control diets.

The faster wound-healing observed in fish fed OTC-treated diets, compared to the untreated control, is similar to observation on faster healing of wounds / lesions treated with OTC earlier reported (Ajith et al., 2016; Chandler et al., 2010). The faster healing obtained from the fish fed tamarind-treated diets confirms the efficacy of this plant in traditional wound-healing. The

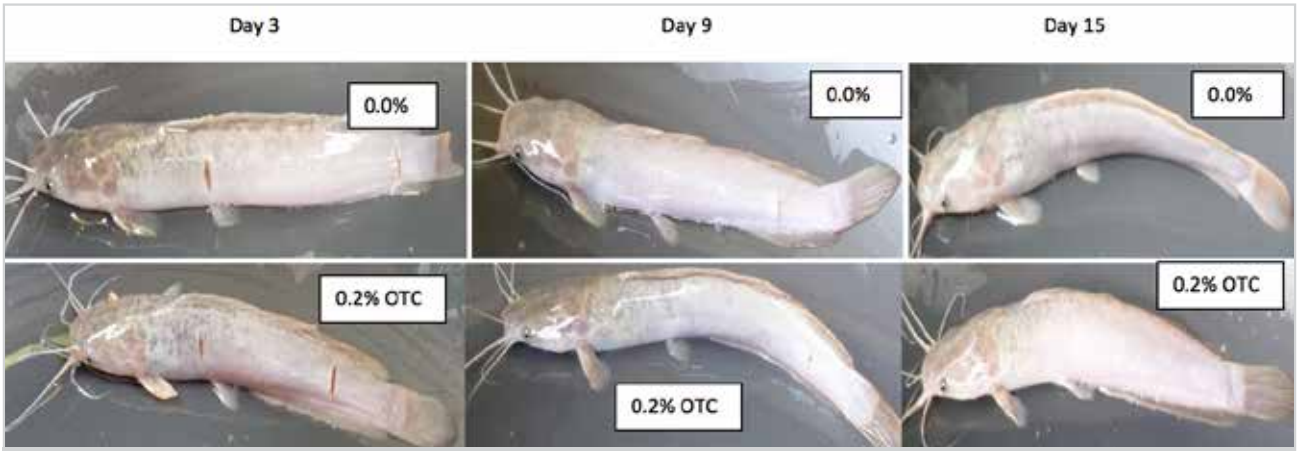

Figure 3. Wound-healing phases in African catfish fed diets fortified with 0.0% (untreated control) and 0.2% oxytetracycline (OTC-treated control)

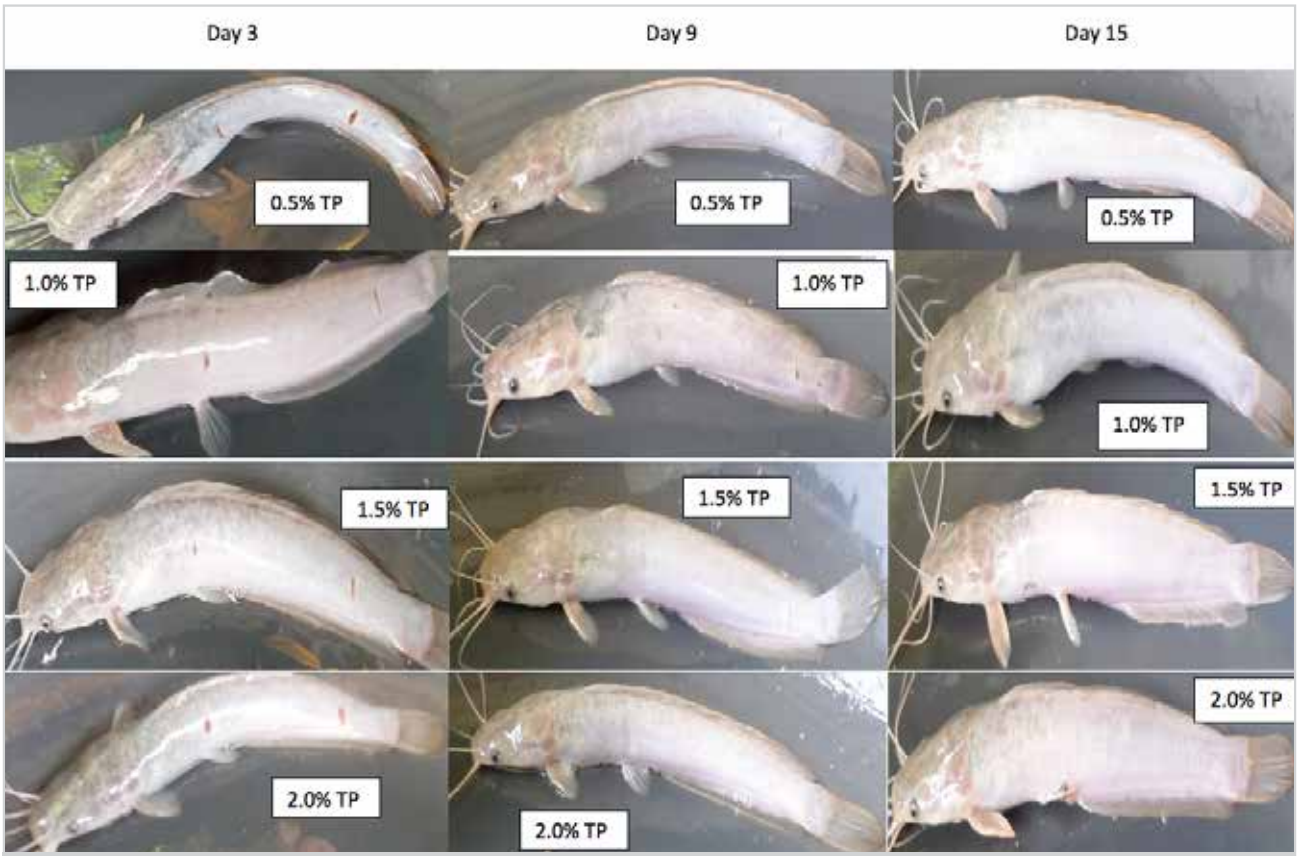

Figure 4. Wound-healing phases in African catfish fed diets fortified with 0.5% - 2.0% TP (tamarind pulp) meal

bark or leaves of tamarind have been reported to be used traditionally for healing wounds (Fabiyi et al., 1993; Havinga et al., 2010; Lockett et al., 2000). The healing activity of tamarind pulp and leaves in the diets of *C. gariepinus* might be due to the ability of the phytochemical in these herbal products to promote formation of collagen. Collagen is the principal component of connective tissue, which plays a key role in tissue regeneration (Abdulla et al., 2009; Cohen et al., 1992).

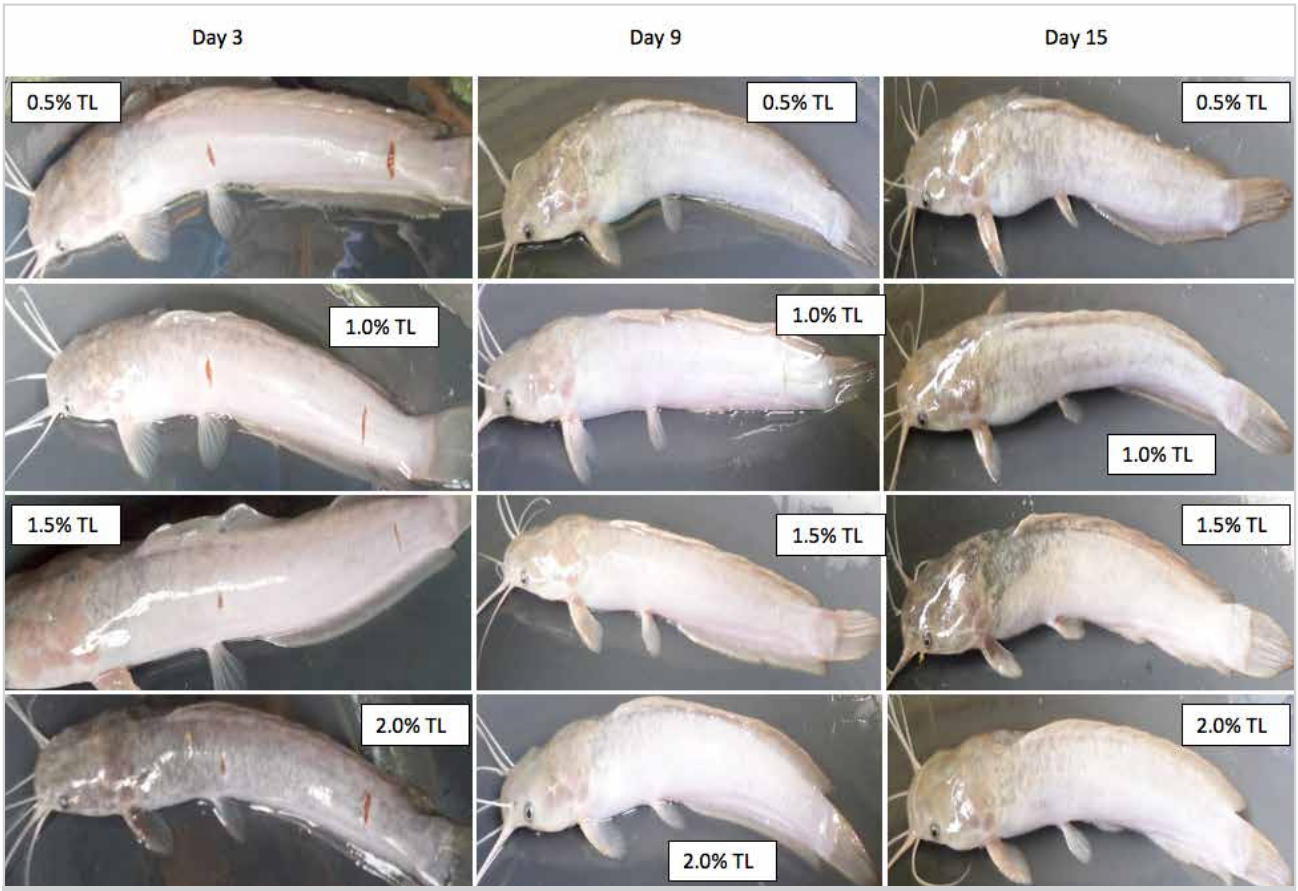

Figure 5. Wound-healing phases in African catfish fed diets fortified with 0.5% - 2.0% TL (tamarind leaf) meal

Table 4. Feed conversion ratio and survival of experimentally wounded *Clarias gariepinus* fed diets fortified with varying levels of *Tamarindus indica* pulp and leaves for 15 days

Treatments	Feed conversion ratio	Survival (%)
0.00%	1.64[a]	100
0.20% Oxytetracycline	1.48[ab]	100
0.50% Pulp	1.37[ab]	100
1.00% Pulp	1.36[ab]	100
1.50% Pulp	1.24[ab]	100
2.00% Pulp	1.13[b]	100
0.50% Leaves	1.40[ab]	100
1.00% Leaves	1.13[b]	100
1.50% Leaves	1.34[ab]	100
2.00% Leaves	1.36[ab]	100

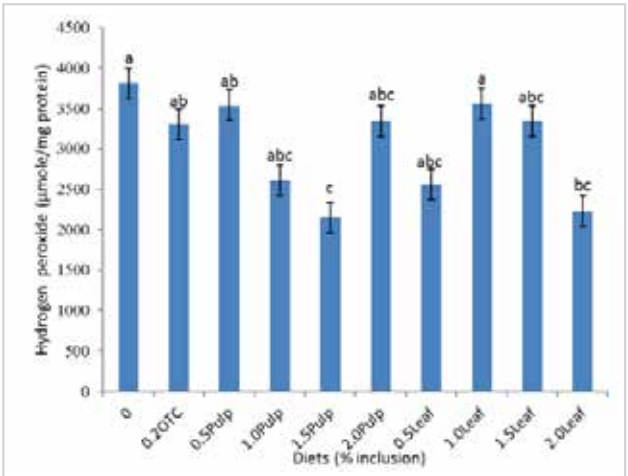

Figure 6. Sera hydrogen peroxide of *Clarias gariepinus* fed diets treated with tamarind pulp and leaf meal. Bars with similar letters are not significantly different at $p<0.05$

Better wound-healing rate was similarly observed in *C. gariepinus* fed diets containing walnut leaf and onion bulb (Bello et al., 2013). Alam et al. (2014) reported enhanced wound-healing in fish on kalojira seed oil, neem seed oil and leaves extract compared to control diets. Inclusion of extracts of *Rafflesia hasseltii* in the diets of Sprague Dawley rat has also been proved to enhance wound-healing (Abdulla et al., 2009). Shi et al. (2014) further re-

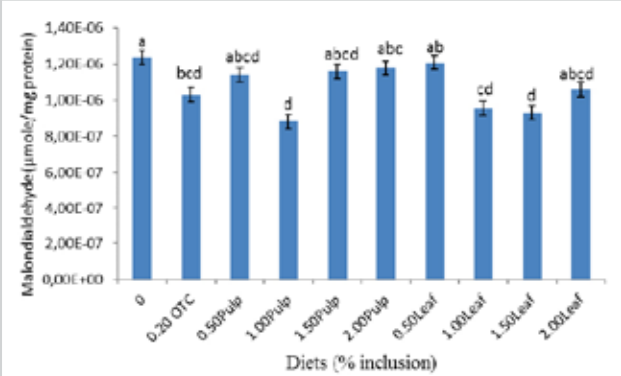

Figure 7. Sera Malondialdehyde of *Clarias gariepinus* fed diet treated with tamarind pulp and leaf meal. Bars with similar letters are not significantly different at p<0.05

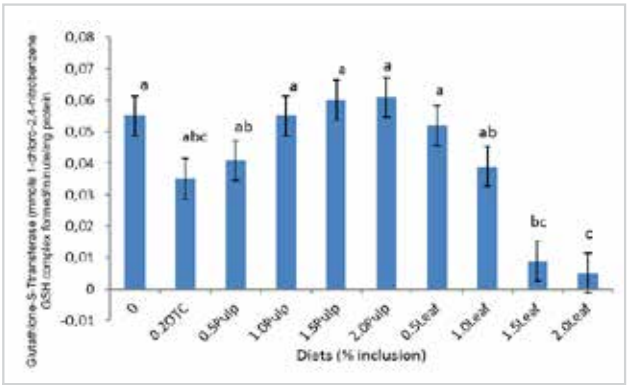

Figure 10. Activity of sera glutathione-S-transferase of *Clarias gariepinus* fed diets treated with tamarind pulp and leaf meal. Bars with similar letters are not significantly different at p<0.05

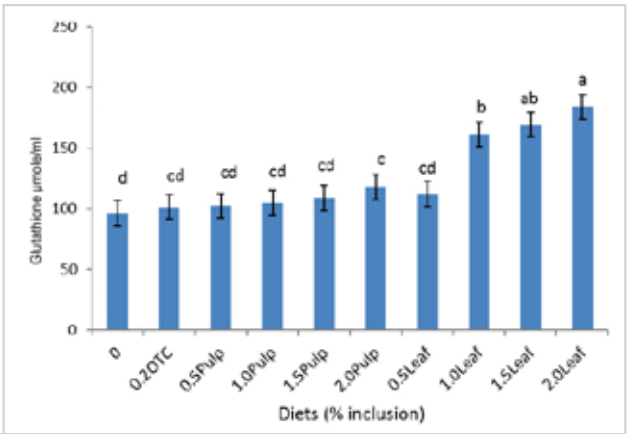

Figure 8. Sera reduced glutathione of *Clarias gariepinus* fed diets treated with tamarind pulp and leaf meal. Bars with similar letters are not significantly different at p<0.05

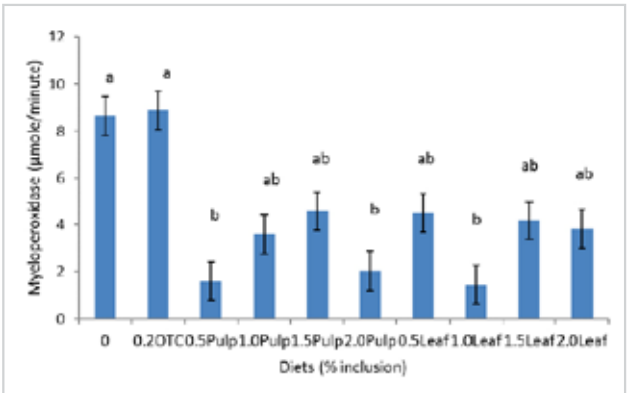

Figure 11. Activity of sera myeloperoxidase of *Clarias gariepinus* fed diets treated with tamarind pulp and leaf meal. Bars with similar letters are not significantly different at p<0.05

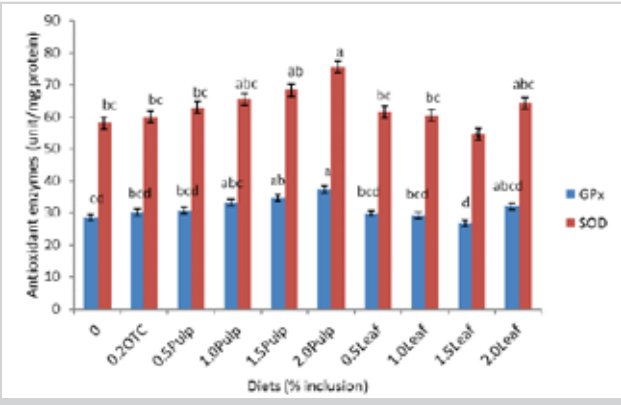

Figure 9. Activities of sera Glutathione Peroxidase (GPx) and Superoxide Dismutase (SOD) of *Clarias gariepinus* fed diets treated with tamarind pulp and leaf meal. Bars with similar letters are not significantly different at p<0.05

ported higher wound-healing rate in mice fed with diets treated with *Acorus calamus* extracts compared to the control diet.

Antioxidants in phytobiotics have been reported to promote wound-healing activity in animals (Abdulla et al., 2009; Mohammad et al., 2012). Reduction in the biomarkers of oxidative stress and complementary higher activities of sera GSH, GPx and SOD in *C. gariepinus* on dietary tamarind demonstrated antioxidant ability of TP and TL. Spontaneous dismutation of superoxide radicals to H_2O_2 and less reactive oxygen is enhanced by SOD while GPx remove it in the presence of GSH as substrate (Kohen and Nyska, 2002). The antioxidant activities demonstrated might be due to flavonoid in the tamarind pulp and leaves (Adeniyi et al., 2017) resulting to the enhanced wound-healing of the fish. Dietary flavonoid in animal has been recognized for antioxidants activities (Yao et al., 2004).

The activity of GST was not seriously affected except the significant reduction at 2.0% TL inclusion level. Cell inflammation and oxidative stress have been associated with increased MPO

activities, as high MPO may be released from neutrophil when reactive oxygen species is high (Akinrinde et al., 2015). Therefore, the reduction in MPO activity in the tamarind-treated *C. gariepinus* demonstrated the chemoprotective effects of the tamarind additives and this might have contributed to faster healing observed in fish fed diets fortified with tamarind pulp and leaf meal.

Similar antioxidant properties of some phytobiotics have been reported: Activity of SOD was similarly higher in common carp and prawn fed diets containing 0.1-0.2% *Rheum officinale* anthraquinone extract than those fed control diet (Liu et al., 2010; Xie et al., 2008). Giannenas et al. (2012) also observed decreased MDA and increased GSH-based enzymes in the fillet of rainbow trout fed diets containing thymol and carvacrol. Furthermore, SOD activity was increased in pacific red snapper fed microalgae (Reyes-Becerril et al., 2014) and in Nile tilapia on diets supplemented with *Astragalus* polysaccharides (Zahran et al., 2014). The later authors however reported insignificant effect on MDA.

The possible mechanism of the enhanced wound-healing of *Clarias gariepinus* fed with diets fortified with tamarind in this study might be reduction of oxidative stress. Utilization of antioxidants has been reported to enhance repair of tissues and wound-healing (Fitzmaurice et al., 2011; Shetty, 2013; Kurahashi and Fujii, 2015). Elevated levels of MDA and hydrogen peroxide accompanied with higher production of antioxidant levels seemed to enhance wound-healing in this study. Low levels of antioxidants and elevated levels of markers of oxidative stress have been reported to delay wound-healing due to damage to cellular membranes, proteins and lipids (Rasik and Shukla, 2000).

In conclusion, this study revealed the *in vivo* antioxidant activity of *Tamarindus indica* pulp and leaves and its utilization as proven wound-healing agent. These natural antioxidants might have been responsible for enhanced wound-healing observed in *Clarias gariepinus*. Utilization of 1.0-2.0% air-dried *Tamarindus indica* pulp and leaf meal as feed additives significantly enhanced wound-healing in *Clarias gariepinus* and it is therefore recommended for use.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of University of Ibadan, Nigeria.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – O.V.A.; Design – O.V.A., F.E.O., B.O.E.; Supervision – F.E.O., B.O.E.; Resources – O.V.A., A.A.O.; Materials – O.V.A., A.A.O.; Data Collection and/or Processing – O.V.A.; Analysis and/or Interpretation – O.V.A.; Literature Search – O.V.A.; Writing Manuscript – O.V.A.; Critical Review – O.V.A., F.E.O., B.O.E., A.A.O.

Conflict of Interest: No conflict of interest was declared by the authors.

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The Effect of Safflower on the *In Vitro* Digestion Parameters and Methane Production in Horse and Ruminant

Alper ÇAĞRI[1], Kanber KARA[2]

[1]Kırşehir Directorate of Provincial Food Agriculture and Livestock, Kırşehir, Turkey

[2]Department of Animal Nutrition and Nutritional Diseases, Erciyes University, Faculty of Veterinary Medicine, Kayseri, Turkey

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Abstract

The aim of this study was to determine the effects of using 5%, 10%, and 20% of safflower (*Carthamus tinctorius* L. Dinçer; without thorns) grain, and its hay and straw, on the *in vitro* fermentation parameters in the diets of horses and ruminants. The addition of up to 5% of crushed safflower grain to a horse's diet had no negative effect on the *in vitro* total gas production, true dry matter digestion (T-DMD), metabolic energy (ME), gas yield at 24 h (GY_{24}), partial factor (PF_{24}), microbial crude protein production (MCP) and short chain fatty acid composition (SCFA) of digestion fluid; however, increasing the grain content negatively affected certain parameters ($p<0.05$). The hay and straw at 5%–20% ratio in a horse's diet had a positive effect on *in vitro* gas production, ME, SCFA, and GY_{24}. We observed that 5% safflower grain in ruminants' diets did not negatively affect the *in vitro* cumulative gas production up to 96 h, T-DMD, true organic matter digestion (T-OMD), ME, net energy lactation (NEL), GY_{24}, PF_{24}, and MCP values and SCFA compositions; but 10% and 20% levels negatively affected the *in vitro* gas production, ME, NEL, and SCFA values ($p<0.05$). The use of up to 20% hay and straw had no negative effect on the parameters ($p>0.05$). Using safflower grain, hay and straw in horse and ruminants' diets did not affect the *in vitro* methane production ($p>0.05$). Consequently, using up to 5% safflower grain, and 20% hay and straw has the potential as a feed source in the diets of horses and ruminants.

Keywords: Digestion, horse, *in vitro* gas production, ruminant, safflower

Introduction

Safflower is *Carthamus tinctorious* L species in the *Compositae (*or *Asteraceae*) family of the *Campanulatae (Asterales*) order. The gene centre of safflower is known as Africa, the Middle- East and Asian continents; it can be planted in winter or summer, or as a crop rotation plant. This plant, which can be grown in different environmental and soil conditions, is one of the earliest crops used by humans. It is an annual and is stake rooted; and there are thorny and thorn-less species and it is an oil-seed plant which can include 70-80% linoleic acid or 80% oleic acid in oils (Baumler et al., 2006; Gilbert, 2008; Gumus and Kucukersan, 2016; Landau et al., 2004; Sahebi et al., 2011). This oil-seed plant can adapt more easily to different soil conditions than those of other oil-seed plants. Safflower, which has attracted attention as a food crop resistant to drought, is of extreme importance today due to the effects of global warm (Altin et al., 2012).

The grain of safflower is described as a hulled seed (*achene*) due to its covering with a hull layer. This seed is shaped like a sunflower seed, but is white-coloured and is smaller and harder than a sunflower seed. Previous studies have found the hull ratio in safflower grains to be 33-60% (Gumus and Kucukersan, 2016). The safflower grain contains 13-19% crude protein (CP), 24-28% ether extract (EE), 42% neutral detergent fibre (NDF), 32% acid detergent fibre (ADF) (Bozan and Temelli, 2008; Dschaak et al., 2011). Recently, safflower varieties have been developed with high oil levels (47% EE) and low fibre (25% NDF, 18% ADF) (Dschaak et al., 2011). The safflower herbage includes 9.5-13.8% CP, 37.2-42.1% NDF, 0.4-0.7% tannin and 0.2-0.4% non-protein nitrogen in total nitrogen (Asgharzadeh et al., 2013). Quality

Address for Correspondence: Kanber KARA • **E-mail:** karakanber@hotmail.com; kanberkara@erciyes.edu.tr

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herbage can be obtained from safflower and despite its thorny leaves can be consumed by sheep and goats. In previous study, the preference for and rejection of safflower herbage (especially stems) by sheep and dairy cattle was determined to be very close to that of wheat straw (Landau et al., 2005). The herbage or hay of safflower has been used as forage in the diets of cows in Australia and sheep in Italy (Landau et al., 2004 and 2005).

The drought in the Mediterranean Levant has reached its the highest level for the last 900 years, and semi-arid soils have turned to arid; and arid soils have turned to desert (Altin et al., 2012; Cook et al., 2016; IPCC. 2014). The importance of safflower, which is an oilseed plant cultivated on moorland and arid/semi-arid lands, has increased in arid and semi-arid countries. In countries which experience drought safflower stands out as an alternative culture plant in terms of oil for human nutrition and feed (grain and forage) for herbivorous nutrition. Recently, the plantation areas and amount of production in the harvest of the safflower plant in areas which have arid and semi-arid climatic conditions of Turkey, have also increased (Gumus and Kucukersan, 2016; TSI, 2016). In 2015, the safflower plant was cultivated on 0.43 million decares and a total of 70 thousand tonnes (162 kg/decare) was harvested in Turkey (TSI, 2016). The planting area of safflower in Turkey is increasing day by day. In the study, the use of up to 5%, 10% and 20% of safflower grain, safflower herbage and safflower straw in horse and ruminant total mix ration (TMR) aimed to determine the effect on *in vitro* digestion parameters.

Materials and Methods

The scientific procedures of the study were conducted according to research protocol approved (Date: January 14, 2015; Decision number: 15/10) by the Local Ethics Committee for Animal Experiments of Erciyes University.

The samples of safflower herbage, safflower straw and safflower grain

The safflower (*Carthamus tinctorius* L. Dinçer) samples used in the present study were collected from the province of Kırşehir, Turkey. Kırşehir is located (38°49″-39°48″ north latitudes, 33°25″ - 34°43″ east longitudes) in Turkey's Central Anatolia region. The Dinçer type safflower is without thorns and grows 985 m above sea level. Steppe and dry forests are the dominant vegetation in this location. Arid conditions and desert-like steppe vegetation are dominant in the Kırşehir province due to temperature and rainfall (Altin et al., 2012). Samples of safflower herbage obtained by cutting the green safflower plant in the pre-flowering stage (Figure 1) using scissors. The herbage samples were cut 1 cm above the soil, included the aerial parts (leaf, stem, preflowering bud) of the plants. Fresh-wet herbage was dried and then used in chemical and digestion analyses of the study. Safflower grain and straw were mature grains obtained after the safflower plant was harvested (Figure 2, 3).

Chemical analysis

The samples of safflower herbage were dried in an oven (Binder, Germany) for 24 hours at 55°C and then 8 hours at 105°C. The grain and straw samples of safflower which were ground to size to pass through a 1 mm sieve (IKA Werke, Staufen im Breisgau, Germany), were also dried in an oven for 24 hours at 105°C. The safflower herbage was ground to size to pass through a 1 mm sieve (IKA Werke, Staufen im Breisgau, Germany) and then dried for 24 hours at 105°C. After this procedure, the dry matter (DM) values of these samples were calculated. The ash, crude protein (CP) and ether extract (EE) contents were detected according to the Association of Official Analytical Chemists (AOAC 1995; method 920.39; method 942.05; method 942.01). The neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) contents, were analysed using a glass crucible on an FIWE3 fibre analyser (Velp, Italy) (Van-Soest et al., 1991). The NDF was detected using sodium sulphite and thermo-stable α-amylase (Megazyme, Ireland) (called as "aNDF"). The aNDF, ADF, and ADL contents were corrected for ash residue (called as "aNDFom, ADFom, and ADL", respectively). Analyses were carried out in triplicate.

The metabolic energy (ME) values were calculated using the following formula (MAFF, 1984) by the nutrient contents determined by analysis of the grain, herbage and straw of the safflower.

ME (kcal/kg DM): 3227 + 62.86 * EE% - 31.79 * ash% - 32.50 * ADFom% (MAFF, 1984).

In vitro digestion

Ruminant and horse rations used in the study and added rations, including 5%, 10% and 20% of the safflower grain, safflower herbage and safflower straw are given in Table 1 and 2.

In vitro digestion technique for ruminants

As inoculum, fresh rumen fluid was used. Rumen fluid (approximately 1.0 L) was obtained from two beef cattle (*Hereford*) fed with a diet containing an 80% concentrated mix feed and 20% forage in DM applied in intensive fattening. Rumen fluid was obtained via a stomach tube into two hours after the morning feeding and collected in a thermos including water at 39°C using CO_2 gas, and filtered with four layers of cheesecloth in the laboratory. The total mix ration (TMR) for beef cattle evaluated to determine *in vitro* digestion in ruminants is given in Table 1. This TMR was prepared for fattening cattle which are 12 months of age, with 400 kg of live weight and 1.4 kg of live weight gain.

The *in vitro* digestion technique performed in the current study is the Hohenheim *in vitro* gas production technique (Menke et al., 1988), which incubated filtered rumen fluid (10 mL), buffer mixture (20 mL) and substrate (milled feed sample, 200±10 mg). This buffer mixture includes 474 mL of bi-distilled water, 237.33 mL of macro-mineral solution, 237.33 mL of buffer solution, 0.12 mL of trace-mineral solution, 1.22 mL of resazurin solution and 50 mL of reducing solution in one litre. Dried samples were incubated in rumen fluid and buffer mixture in 100 mL glass syringes (Model For-

Figure 1. Safflower (*Carthamus tinctorius* L. Dinçer) herbage

Figure 2. Safflower (*Carthamus tinctorius* L. Dinçer) grain

Figure 3. Safflower (*Carthamus tinctorius* L. Dinçer) straw

tuna, Germany) (n=6) (triplicate; cumulative gas production plus triplicate; dry matter-organic matter loss). The three blank syringes (no template; rumen fluid plus buffer mixture) were incubated to calculate the total gas production. The syringes were incubated in a water bath with a thermostat (Special Waterbath, Yapar Stainless Steel Ltd., Kahramanmaraş, Turkey), which has a stainless reservoir, at 39°C for up to 96 h.

Table 1. The supplementation of safflower to beef cattle total mix ration

		Supplementation of safflower								
	Feed kg/day	Safflower grain			Safflower herbage			Safflower straw		
Feeds	**(as DM)**	**5%**	**10%**	**20%**	**5%**	**10%**	**20%**	**5%**	**10%**	**20%**
Safflower	-	0.50	1.00	2.00	0.5	1.00	2.00	0.50	1.00	2.00
Corn silage	1.20	1.20	1.20	1.20	0.70	0.20	-	1.20	1.20	1.00
Wheat straw	1.80	1.80	1.80	2.25	1.80	1.80	1.00	1.30	0.80	-
Barley grain	3.15	2.65	2.15	0.00	3.15	3.15	3.15	3.15	3.15	3.15
Concentrated feed mix*	3.60	3.60	3.10	2.80	3.60	3.60	3.60	3.60	3.60	3.60
Cotton seed meal	-	-	0.50	1.50	-	-	-	-	-	-
Total feed kg/day (as DM)	9.75	9.75	9.75	9.75	9.75	9.75	9.75	9.75	9.75	9.75
Crude protein and energy composition (Calculated)										
CP (% DM)	12.00	11.58	11.66	11.96	12.03	12.06	12.45	12.01	12.02	11.98
ME (kcal/kg DM)	2457.00	2498.00	2515.00	2488.00	2449.00	2441.00	2483.00	2465.00	2472.00	2473.00
Nutrient composition analysed										
Ash	7.25									
CP	11.50									
EE	2.64									
aNDFom	36.15									
ADFom	20.58									
ADL	3.29									

CP: crude protein as %; aNDFom: assayed with a heat stable amylase and expressed exclusive of residual ash as %; ADFom: ADF expressed exclusive of residual ash as %; ADL: acid detergent lignin as %; EE: diethyl ether extract as %.
*: Beef cattle concentrated feed mixture included 15%CP and 2700 kcal/kg ME.

***In vitro* digestion technique for horses**

The *in vitro* digestion technique in horses was carried out according to Sunvold et al. (1995) and Sweney (2012), which incubated feed sample in faeces inoculum and fermentation medium, which included solution A, solution B, trace mineral solution, water-soluble vitamins, folate:biotin solution, riboflavin solution, hemin solution, short-chain fatty acids, rezurine, yeast extract, trypticase, Na_2CO_3 and Cystein HCl*H_2O (Table 3). The faeces samples used as an inoculum in the current study were obtained from two thoroughbred horses (6-7 years of age, 480-500 kg in body weight) that were fed with a diet containing 70% forage and 30% concentrate feed, in DM basis. Faeces samples were collected soon after defecation and transferred into a thermos containing water at 39°C under CO_2 gas and transferred to the laboratory. Faeces samples were diluted at a 1:10 ratio with 0.9% sterile serum physiologic solution (Polifleks, Polifarma, Turkey) using a laboratory type blender (Waring Products Division, Torrington C.T., USA). Diluted faeces inoculum was filtered through four layers of cheesecloth under constant CO_2 gas (anaerobically) and used in the *in vitro* digestion technique.

The horse *in vitro* digestion technique was carried out in glass syringes with 100 ml volume (Model Fortuna, Haberle Labortechnik, Germany). The samples (500±10 mg as DM) were incubated with a medium mixture (30 mL) and faeces inoculum (5 mL) in glass syringes (n=6). The syringes were closed using clips and then the initial volume recorded and incubated in a water bath with a thermostat (Special Waterbath, Yapar Stainless Steel Ltd., Kahramanmaraş, Turkey), which has a stainless reservoir, at 39.0±0.2°C for up to 48h. In addition, six blank syringes (no template; medium mixture plus faeces inoculum) were used to calculate the total gas production.

Determination of cumulative gas production

In *in vitro* incubations, the total gas volume was recorded from the calibrated scale on the syringe at 3, 6, 12, 24, 48, 72, and 96 hours for ruminants and at 6, 12, 18, 24, 36, and 48 hours for horses.

Determination of methane production

After measuring the total gas volume at 24 h, the tubing of the plastic syringe outlet was inserted into the inlet of the methane analyser (Sensor, Europe GmbH, Erkrath, Germany) and the piston was pushed to insert the accumulated gas into the analyser.

Determination of *in vitro* true dry matter disappearance and *in vitro* true organic matter disappearance values

Three of the *in vitro* fermentation syringes for both ruminants

Table 2. The supplementation of safflower to horse total mix ration

		Supplementation of safflower								
	Feed kg/day	Safflower grain			Safflower herbage			Safflower straw		
Feeds	**(as DM)**	**5%**	**10%**	**20%**	**5%**	**10%**	**20%**	**5%**	**10%**	**20%**
Safflower		0.50	0.90	1.80	0.50	0.90	1.80	0.50	0.90	1.80
Wheat straw	3.00	3.00	3.00	3.50	3.00	2.65	2.30	2.50	2.30	2.20
Grass hay, mature	3.00	3.00	2.70	2.00	2.50	2.50	2.50	3.00	2.80	2.00
Alfalfa hay, mid maturity	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Barley grain	0.50	-	-	-	0.50	0.50	-	0.50	0.50	0.50
Vegetable oil	0.10	0.10	-	-	0.10	0.05	-	0.10	0.10	0.10
Oat grain	0.50	0.50	0.50	-	0.50	0.50	0.50	0.50	0.50	0.50
Cotton seed meal	0.50	0.50	0.50	0.30	0.50	0.50	0.50	0.50	0.50	0.50
Total feed kg/day (as DM)	9.10	9.10	9.10	9.10	9.10	9.10	9.10	9.10	9.10	9.10
Crude protein and energy composition (Calculated)										
CP (% DM)	12.31	12.30	12.48	11.57	12.16	12.36	12.34	12.30	12.15	12.00
DE (kcal/kg DM)	2043.00	2022.00	2048.00	2088.00	2069.00	2069.00	2012.00	2065.00	2074.00	2078.00
Nutrient composition analysed										
Ash	10.69									
CP	10.62									
EE	3.34									
aNDFom	30.26									
ADFom	23.81									
ADL	4.68									

CP: crude protein as %; aNDFom: assayed with a heat stable amylase and expressed exclusive of residual ash as %; ADFom: ADF expressed exclusive of residual ash as %; ADL: acid detergent lignin as %; EE: diethyl ether extract as %; DE: digestible energy; DE=ME/0.80.

and horses were stopped after 24 h. The *in vitro* true dry matter disappearance (T-DMd) and the *in vitro* true organic matter disappearance (T-OMd) values of substrates were calculated at 24 h of incubations.

The *in vitro* dry matter- and organic matter - disappearance was determined by filtering the fermentation residues using a vacuum unit (Velp Dietary Fibre Analyzer, Italy) on pre-weighed glass crucibles (Velp, porosity #2, Italy) the fermentation residues, which was dried at 105°C and burning the residual at 550°C. *In vitro* T-DMd was calculated as 1 - [(DM residue - DM blank)/initial DM)] x 100. *In vitro* T-OMd was calculated as 1 - [(OM residue - OM blank)/ initial OM)] x 100.

Determination of estimated digestion values and end-products

The ME and OMD contents of the samples were calculated using the equations of Menke and Steingass (1988).

The gas yields (GY_{24}), partial factor (PF_{24}), and microbial crude protein production levels (MCP) of the samples at 24 h were calculated using the equations:

$GY_{24} = [(GP_{24} \times 10^3) : T\text{-}DMd]$

$PF_{24} = T\text{-}DMd : GP_{24}$

MCP (mg/g DM) = mg T-DMd - (mL gas × 2.2 mg/mL)

T-DMd: *in vitro* dry matter disappearance (mg) for g DM at 24 h (mg/g DM)

GP_{24}: volume (mL) of total gas produced by g DM at 24 h (mL/g DM)

The molarities of estimated short chain fatty acid (SCFA) produced by substrate at 24 hours of *in vitro* fermentations were calculated using the following formula of Getachew et al. (2008):

SCFA (mmol/0.2 g DM) = 0.0222 GP - 0.00425

The GP is net gas production at 24 h (mL/0.2 g DM)

Statistical analysis

The experiment data were first subjected to Levene's test to detect the variance homogeneity. One-way variance analyses (ANOVA) were implemented for homogeneous variances by General Linear Model procedures to test treatment differences.

Table 3. Composition of *in vitro* fermentation medium

Component	mL/L	Amount
Solution A[a]		330.0
Solution B[b]		330.0
Trace mineral solution[c]		10.0
Water-soluble vitamins[d]		20.0
Folate: biotin solution[e]		5.0
Riboflavin solution[f]		5.0
Hemin solution[g]		2.5
Short chain fatty acids[h]		0.4
Resazurine[i]		1.0
Distilled H_2O		296.0
	g/L	
Yeast extract		0.5
Trypticase		0.5
Na_2CO_3		4.0
Cystein HCl*H_2O		0.5

[a]Composition (g/L): NaCl, 5.4; KH_2PO_4, 2.7; CaCl2*H_2O, 0.16; $MgCl_2$*$6H_2O$, 0.12; $MnCl_2$*$4H_2O$, 0.06; $CoCl_2$*$6H_2O$, 0.06; (NH4)2SO_4, 5.4.
[b]Composition: K_2HPO_4, 2.7 g/L.
[c]Composition (mg/L): ethylene diamine tetraacetic acid (disodium salt), 500; $FeSO_4$*$7H_2O$, 200; $ZnSO_4$*$7H_2O$, 10; $MnCl_2$*$4H_2O$, 3;H_3PO_4, 30; $CoCl_2$*$6H_2O$, 20; $CuCl_2$*$2H_2O$, 1; $NiCl_2$*$6H_2O$, 2; Na_2MoO_4*$2H_2O$, 3.
[d]Composition (mg/L): thiamin-HCl, 100; d-pantothenic acid, 100; niacin, 100; pyridoxine, 100; p- aminobenzoic acid, 5; vitamin B12, 0.25.
[e]Composition (mg/L): folic acid, 10; d-biotin, 2; NH_4HCO_3, 100.
[f]Composition: riboflavin, 10 mg/L in 5 mmol/L of Hepes.
[g]Hemin: Hemin 500 mg/L of 10 mmol/L NaOH
[h]Composition: n-valerate, isovalerate, isobutyrate and DL alpha- methylbutyrate, 250 mL/L
[i]Composition: 1 g resazurine/L distilled water

Data was analyzed based on the statistical model: Y*ij* = µ*ij* +S*i* + e*i*. Where, Y*ij* = the general mean common for each parameter under investigation. S_i = the *i*th effect of the safflower grain, safflower herbage or safflower straw on the observed parameters, and e*i* = the standard error term. The means were separated by Tukey's multiple range test at p<0.05. Analyses were performed using Statistical Package for the Social Sciences (SPSS) 17.0 software (IBM Corp.; Armonk, NY, USA).

Results

The nutrient compositions of safflower grain, safflower herbage and safflower straw are given in Table 4.

In vitro digestion parameters in horse

Supplementation of up to 20% crushed safflower grain in horse ration did not have a linear effect on cumulative total gas production for the first 12 hours (p>0.05). *In vitro* total gas production during the 12th and 18th hours of incubation was higher than that of 0% safflower grain (p<0.05; quadratic); but the increasing dose of safflower grain was negatively affected

Table 4. The nutrient matter and energy composition of safflower grain, safflower herbage and safflower straw used in study

	Safflower grain	Safflower herbage	Safflower straw
CP	12.30	8.10	3.74
Ash	2.02	8.82	7.71
EE	27.42	2.13	1.37
aNDFom	49.33	39.05	49.98
ADFom	40.53	31.99	44.29
ADL	13.24	4.75	6.67
ME	3569.18	2040.83	1628.59

CP: crude protein as % in dry matter; aNDFom: assayed with a heat stable amylase and expressed exclusive of residual ash as % in dry matter; ADFom: ADF expressed exclusive of residual ash as % in dry matter; ADL: acid detergent lignin as % in dry matter; EE: diethyl ether extract as % in dry matter; ME: metabolisable energy as kcal/kg in dry matter

(p<0.05; linear). Although *in vitro* total gas produced by 5% safflower grain supplementation to horse TMR was similar to the control ration (0% safflower grain) at 24 - 48 hours of incubation, those produced by 10% and 20% safflower grain were low linearly than that of control ration (p<0.05) (Table 5).

Safflower herbage supplementation to horse TMR positively affected *in vitro* cumulative total gas production during the all incubation (6, 12, 18, 24, 36 and 48 hours) (p<0.05).

In horses, at 24 hours of incubation, *in vitro* total gas production of TMR with safflower herbage reached 183-195 mL/g DM, and this production level reached 229-253 mL/g DM at 48 hours (Table 5).

Up to 20% safflower straw was used in horse TMR and increased linearly the *in vitro* cumulative gas production at 6, 12, 18 and 24 hours of incubation (p<0.05). At the 36th and 48th hours of incubation, the horse TMR with up to 20% safflower straw did not negatively affect *in vitro* cumulative gas production (p>0.05) (Table 5).

Supplementation of up to 20% of crushed safflower grain, safflower herbage and safflower straw to horse TMR did not have a significant effect on methane production at 24 hours of *in vitro* gas production (p>0.05). The *in vitro* methane production ranged from 0.22 to 0.42 mL/g DM (Table 5).

Up to 20% safflower in horse TMR decreased linearly the *in vitro* T-DMd, T-OMd, ME and SCFA values (p<0.05). On the other hand, the *in vitro* GY_{24}, PF_{24}, and MCP values of horse TMR did not change with the use of up to 20% safflower grain (p>0.05). It was determined that the use of 5%, 10% and 20% of safflower herbage in horse TMR decreased *in vitro* T-DMd, PF_{24} and MCP values (p<0.05). The *in vitro* T-OMd value of TMR were not affected by the use of safflower herbage in horses (p>0.05) (Table 6). The *in vitro* GY_{24}, ME and SCFA values of horse TMR showed

Table 5. Effect of safflower in horse TMR on *in vitro* cumulative gas production and methane production

			***In vitro* cumulative gas production (mL/g DM)**					
Supplementation to TMR		**Methane**	**6 h**	**12 h**	**18 h**	**24 h**	**36 h**	**48 h**
0% Safflower grain		0.29	3.94	43.37	73.94	183.11	221.39	229.29
5% Safflower grain		0.36	6.58	53.25	87.78	184.08	222.09	231.92
10% Safflower grain		0.27	5.27	43.49	72.16	161.45	198.10	200.08
20% Safflower grain		0.22	5.26	41.08	66.52	158.37	172.18	174.13
	SEM	0.06	0.51	1.70	2.71	3.86	6.17	7.12
p value	L	0.640	0.380	0.136	0.029	0.002	<0.001	<0.001
	Q	0.709	0.210	0.026	0.015	0.445	<0.001	<0.001
0% Safflower herbage		0.29	3.94	43.37	73.94	183.11	220.92	229.43
5% Safflower herbage		0.38	5.94	58.17	92.22	194.35	243.96	247.94
10% Safflower herbage		0.39	6.60	56.81	91.17	195.57	251.18	253.50
20% Safflower herbage		0.37	7.30	53.13	90.65	189.94	243.50	251.16
	SEM	0.07	0.54	1.91	2.45	2.17	3.80	4.47
p value	L	0.771	0.033	0.010	0.001	0.038	0.003	<0.001
	Q	0.799	0.507	0.001	0.002	0.007	0.004	0.004
0% Safflower straw		0.29	3.94	43.37	73.94	183.11	220.92	229.43
5% Safflower straw		0.33	4.64	59.18	93.74	195.51	233.34	232.03
10% Safflower straw		0.40	5.93	61.94	99.85	204.32	237.78	241.46
20% Safflower straw		0.42	7.84	66.71	108.89	214.52	240.37	245.98
	SEM	0.08	0.52	2.74	3.96	4.02	4.21	4.04
p value	L	0.604	0.002	<0.001	<0.001	<0.001	0.132	0.145
	Q	0.976	0.379	0.014	0.030	0.427	0.572	0.909

L: linear; Q: quadratic; Methane: *In vitro* methane production as mL/g DM at 24; SEM: standard error of means

an increase in all levels of safflower herbage ($p<0.05$). It was observed that this increase slightly decreased with the use of 20% (Table 6). It was determined that the use of safflower straw in horse TMR decreased linearly the *in vitro* T-DMd, PF_{24} and MCP values ($p<0.05$). The *in vitro* GY_{24}, ME and SCFA values of horse TMR were increased in linear contrast ($p<0.001$) depending on the increase in the level of safflower straw. In contrast, *in vitro* T-OMd were not affected by up to 20% safflower straw ($p>0.05$) (Table 6).

***In vitro* digestion parameters in ruminant**

Safflower grain supplementation of up to 20% to beef cattle TMR decreased linearly *in vitro* cumulative gas production at 3, 6, 12, 24, 48, 72, and 96 hours ($p<0.05$). The in vitro methane production (mL/0.2 g DM) of beef cattle TMR reduced linearly by safflower grain supplementation level ($p<0.05$) (Table 7).

The safflower herbage and safflower straw (5-20% in DM) used in beef cattle TMR did not change *in vitro* cumulative total gas production up to 96 hours and methane production at 24 hours of incubation ($p<0.05$). In the beef cattle TMR, the use of crushed safflower grain did not change *in vitro* T-DMd, T-OMd, GY_{24} and PF_{24} values ($p>0.05$). In relation to the increase of safflower grain in beef cattle TMR, the ME and NEL values of TMR and the SCFA concentration of digestion fluid decreased linearly ($p<0.001$) (Table 8). The *in vitro* estimated T-DMd, T-OMd, GY24, PF24, ME, NEL and SCFA values of beef cattle TMR were not affected by up to 20% safflower herbage and safflower straw ($p<0.05$) (Table 8).

Discussion

Nutrient composition in the grain, herbage and straw of safflower

Similar to our present study findings, Oğuz et al. (2014) reported that safflower grain grown in Turkey contained about 12% CP, 33% EE, 33% ADF and 44% NDF in DM. In another study, Ingale and Shrivastava (2011) stated that safflower grain (*C. tinctorius* PBNS-12 and PBNS-40) grown in India contained approximately 16% CP, 25-29% EE and 3.5% ash. Paya et al. (2014) (arid climate, Iran) found CP values (16%) of safflower grain was high than that of our findings, in line with the findings of Ingale and Shrivastava (2011). Stanford et al. (2001) reported that safflower straw, which containing seed-bound plant heads, contained about 13% CP, 13% EE, 40% ADF and 50% NDF in DM. The aNDFom and ADFom

Table 6. Effect of safflower in horse TMR on *in vitro* fermentation parameters

Supplementation to TMR		T-DMd	T-OMd	GY_{24}	PF_{24}	MCP	ME	SCFA
0% Safflower grain		494.46	560.36	371.18	2.69	90.70	8.12	0.81
5% Safflower grain		480.26	527.98	383.64	2.60	75.27	8.13	0.81
10% Safflower grain		439.79	449.37	367.15	2.72	84.60	7.52	0.71
20% Safflower grain		438.12	503.83	361.56	2.78	89.70	7.43	0.69
	SEM	7.79	15.46	5.12	0.03	4.96	0.10	0.01
p value	L	<0.001	0.037	0.365	0.330	0.899	0.001	0.001
	Q	0.274	0.086	0.418	0.418	0.370	0.661	0.661
0% Safflower herbage		494.46	560.36	371.18	2.69	90.70	8.12	0.81
5% Safflower herbage		457.47	512.99	425.25	2.35	29.89	8.41	0.85
10% Safflower herbage		462.41	517.88	422.91	2.36	32.15	8.44	0.86
20% Safflower herbage		465.46	529.51	408.81	2.45	47.59	8.29	0.83
	SEM	5.58	12.65	7.59	0.04	8.47	0.04	0.01
p value	L	0.056	0.483	0.026	0.016	0.020	0.057	0.057
	Q	0.041	0.301	0.006	0.003	0.005	0.004	0.004
0% Safflower straw		494.46	560.36	371.18	2.69	90.70	8.12	0.81
5% Safflower straw		504.25	542.39	387.66	2.58	74.12	8.44	0.86
10% Safflower straw		480.15	521.02	425.54	2.35	30.65	8.68	0.90
20% Safflower straw		478.33	510.60	448.50	2.23	6.38	8.96	0.94
	SEM	3.90	9.56	9.25	0.05	10.19	0.09	0.01
p value	L	0.014	0.065	<0.001	<0.001	<0.001	<0.001	<0.001
	Q	0.296	0.838	0.242	0.821	0.189	0.731	0.731

GY_{24}: gas yield is total gas volume (mL) produced for g T-DMd at 24 h; MCP: microbial crude protein is produced at 24 h (mg/g DM); ME: metabolic energy as MJ/kg DM; PF_{24}: partial factor is ratio T-DMd to GP24 at 24 h; SFCA: molarities of short chain fatty acid in fermentation fluid at 24 h; T-DMd: *in vitro* true-dry matter disappearance (mg) for g DM at 24 h (mg/g DM); T-OMd: *in vitro* true-organic matter disappearance; SEM: standard error of means; L: linear; Q: quadratic

contents of the safflower grain and safflower straw in the present study were similar to the findings of previous researchers (Asgharzadeh et al., 2013; Sahebi et al., 2011; Stanford et al., 2001). In another study, although aNDFom and ADFom values of safflower herbage grown in arid climatic conditions (in Jordan), were found to be similar to the results of the present study, CP (13.4%), ash (10.8%) values of it were high than those of the present study (Landau et al., 2004). In addition, the calculated ME value of safflower herbage was parallel to that of Asgharzadeh et al. (2013).

Generally, the CP content of safflower herbage, harvested at the pre-flowering stage in the current study, was similar to some meadow-pasture grass (*Dactylis Glomerata*, *Lolium multiflorum* at the end of vegetative). The safflower straw contains the lowest plant cell wall substances than those (70-78% aNDFom and 50-55% ADFom) of wheat straw and similar CP and EE to wheat straw (NRC, 1989).

The differences among the findings of the present study and previous studies can be attributed to variables in the safflower species used and the soil and climatic conditions grown. When evaluated in terms of nutrient content, it can be seen that safflower herbage and safflower straw have potential as alternative forages sources. In addition, the safflower grain may be a good source energy and moderate protein source due to its EE and CP content. The effect of the safflower grain, straw and herbage on the digestibility must be determined.

The *in vitro* fermentation values in horse TMR

In the present study, *in vitro* methane volume produced by aspiration grain, herbage and straw at different levels in horse TMR was 0.22-0.42 mL/g DM at 24 hours of *in vitro* incubation. The *in vitro* methane production of horse TMR was diverse in range from 0.43 to 0.59 mL/g DM by Kara and Baytok (2017). As it is understood from these values, methane is not produced (Ellis et al., 2007) in the digestive tract of horses as much as in ruminants, and it is observed that the contribution of horses to global warming is not as high as ruminants.

The 5% safflower seeds in horse TMR did not affect the *in vitro* total gas production, ME, SCFA, T-DMd and T-OMd during the 48-hour of *in vitro* incubation period. This demonstrates that up to 5% crushed safflower grain can be used in the horse TMR without affecting digestion parameters. However, the use of 10% and 20% crushed safflower grain in horse TMR cannot be recommended due to adverse effects on the *in vitro* total gas production. Neg-

Table 7. Effect of safflower in ruminant TMR on *in vitro* cumulative gas production and methane production

			***In vitro* cumulative gas production (mL/0.2 g DM)**						
Supplementation to TMR		**Methane**	**3 h**	**6 h**	**12 h**	**24 h**	**48 h**	**72 h**	**96 h**
0% Safflower grain		9.35	16.45	32.73	47.39	59.72	70.99	72.41	73.83
5% Safflower grain		9.48	12.09	25.44	43.59	57.83	69.94	71.72	72.43
10% Safflower grain		9.10	12.03	25.31	40.90	53.65	63.21	65.68	67.46
20% Safflower grain		8.29	10.54	23.72	38.47	50.77	61.31	63.41	64.83
	SEM	0.51	2.32	3.65	3.64	3.71	4.51	4.60	4.75
p value	L	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.006
	Q	0.004	<0.001	<0.001	0.400	0.306	0.610	0.613	0.756
0% Safflower herbage		9.35	16.45	32.73	47.39	59.72	70.99	72.41	73.83
5% Safflower herbage		9.83	15.32	30.11	46.87	60.06	71.82	72.89	73.61
10% Safflower herbage		9.30	15.08	28.92	45.39	58.01	69.58	71.32	72.39
20% Safflower herbage		9.30	15.83	33.24	49.41	61.39	72.64	74.74	75.79
	SEM	0.14	0.22	0.55	0.54	0.56	0.71	0.72	0.89
p value	L	0.634	0.252	0.819	0.213	0.528	0.694	0.429	0.603
	Q	0.454	0.390	0.201	0.017	0.167	0.476	0.344	0.373
0% Safflower straw		9.35	16.45	32.73	47.39	59.72	70.99	72.41	73.83
5% Safflower straw		9.00	17.30	31.96	46.44	57.74	69.74	71.15	72.21
10% Safflower straw		9.14	16.85	31.77	47.57	61.26	72.50	73.55	73.91
20% Safflower straw		9.71	16.47	32.06	47.83	60.80	71.67	73.07	73.78
	SEM	0.15	0.15	0.24	0.60	0.62	0.54	0.57	0.73
p value	L	0.391	0.774	0.377	0.699	0.208	0.343	0.430	0.840
	Q	0.163	0.060	0.338	0.668	0.511	0.851	0.752	0.663

L: linear; Q: quadratic; Methane: *In vitro* methane production as ml/g DM at 24; SEM: standard error of means

ative effects of high safflower grain (10% and 20%) on digestion parameters (*in vitro* total gas, ME, SCFA, T-DMd and T-OMd) can be attributed to the husk content and high ADL levels (Blümmel and Orskov, 1993; Getachew et al., 2008; Menke and Steinbgass, 1988). Previous studies have also shown that *in vitro* digestion parameters are negatively correlated with the structural carbohydrate content of the plant (Kara et al., 2016; Kara, 2016).

The *in vitro* digestion parameters (*in vitro* total gas, T-DMd, T-OMd, $GY_{24,}$ ME, SCFA and ruminal pH) show that the safflower herbage can be used up to 20% in instead of some forage (meadow hay and wheat straw) and concentrate (barley and vegetable oil) feeds of DM in horse TMR. Similarly, *in vitro* digestion parameter applies to safflower straw and up to 20% can be used as forage in DM in horse TMR. These effects of safflower herbage and straw could be caused by the lower levels of aNDFom, ADF and ADL that can be included in grass herbage or hay (forages) which is harvested after the seed-binding stage (Kara, 2016; NRC, 1989 and 2001). These results indicate that the herbage and straw of safflower without thorns are the preferred forage for horse TMR.

The *in vitro* fermentation values in beef cattle TMR

Research on the *in vitro* digestion of safflower plant in ruminants is still very limited. In the present study, *the in vitro* cumulative gas production of safflower in ruminants was lower than those of safflower herbage and straw, which is compatible with the findings of Sahebi et al (2011). The present study indicated that the addition of 5% safflower grain to beef TMR does not have a negative effect on the *in vitro* gas production, ME, NE_{L}, SCFA, *in vitro* T-DMd, T-OMd, GY_{24} and PF_{24} values and the safflower grain at this level can be used in ruminant TMR suggesting that studies on safflower grain should be carried out. However, the use of safflower grain at 10% and 20% levels in ruminant TMR does not affect the *in vitro* T-DMd, T-OMd, GY_{24} and PF_{24} values, despite the linear reduction of *in vitro* gas production, ME, NE_{L} and SCFA values. These fermentation results will not reveal a problem on ruminal digestion of beef TMR.

The *in vitro* methane (mL/0.2 g DM) produced by unit DM of beef TMR decreased linearly with increasing rates of safflower grain in beef TMR and is an expected result due to the reduce *in vitro* gas production (Ellis et al., 2007; Kara et al., 2015).

The use of up to 20% safflower herbage in beef TMR did not adversely affect *in vitro* cumulative total gas and methane production levels and *in vitro* T-DMd, T-OMd, GY_{24}, PF_{24}, ME,

Table 8. Effect of safflower in ruminant TMR on *in vitro* fermentation parameters

		T-DMd	T-OMd	GY_{24}	PF_{24}	ME	NEL	SCFA
0% Safflower grain		516.78	549.27	588.06	1.73	11.19	6.95	1.32
5% Safflower grain		654.20	745.24	446.17	2.26	10.94	6.73	1.27
10% Safflower grain		468.84	550.85	572.49	1.74	10.37	6.25	1.18
20% Safflower grain		526.68	577.44	488.84	2.07	9.98	5.92	1.12
	SEM	27.11	28.81	23.62	0.08	0.14	0.12	0.02
p value	L	0.422	0.508	0.328	0.428	<0.001	<0.001	<0.001
	Q	0.362	0.044	0.451	0.459	0.306	<0.001	0.306
0% Safflower herbage		516.78	549.27	588.06	1.73	11.19	6.95	1.32
5% Safflower herbage		572.18	569.79	554.71	1.92	11.24	6.99	1.32
10% Safflower herbage		613.95	685.37	496.27	2.11	10.96	6.75	1.28
20% Safflower herbage		651.90	653.31	477.55	2.11	11.42	7.14	1.35
	SEM	34.55	36.58	33.91	0.11	0.07	0.06	0.01
p value	L	0.207	0.243	0.267	0.261	0.530	0.530	0.530
	Q	0.908	0.738	0.923	0.715	0.168	0.168	0.168
0% Safflower straw		516.78	549.27	588.06	1.73	11.19	6.95	1.32
5% Safflower straw		562.59	518.35	517.00	1.94	10.92	6.72	1.28
10% Safflower straw		561.83	596.87	546.13	1.83	11.40	7.13	1.35
20% Safflower straw		590.31	633.22	530.08	1.95	11.34	7.07	1.35
	SEM	21.21	31.64	21.72	0.07	0.08	0.07	0.01
p value	L	0.318	0.305	0.513	0.445	0.209	0.209	0.209
	Q	0.856	0.631	0.577	0.760	0.512	0.512	0.512

GY_{24}: gas yield is total gas volume (mL) produced for g T-DMd at 24 h; MCP: microbial crude protein is produced at 24 h (mg/g DM); ME: metabolic energy as MJ/kg DM; NEL: net energy lactation as MJ/kg DM; SFCA: molarities of short chain fatty acid in fermentation fluid at 24 h; T-DMd: *in vitro* true-dry matter disappearance (mg) for g DM at 24 h (mg/g DM); T-OMd: *in vitro* true-organic matter disappearance; SEM: standard error of means; L: linear; Q: quadratic

NE_L and SCFA values. This result shows that it could be advisable to use safflower herbage advisable instead of corn silage and wheat straw in the beef cattle TMR. The safflower herbage can be characterized as quality forage due to the values of CP, fibre and ME.

The 5%, 10% and 20% of safflower straw in beef cattle did not change the *in vitro* cumulative total gas and methane production and *in vitro* T-DMd, T-OMd, GY_{24}, PF_{24}, ME, NE_L and SCFA values, indicating it could be preferred instead of wheat straw. However, the safflower plant used in the study was the Dinçer type and did not have thorns. This plant may not cause adverse effects in the *in vivo* feeding experiments in ruminants. It may be advisable to use thorny forms of the plant in goats.

As a result;

- Before flowering, the safflower herbage has ME, CP, aNDFom, ADFom and ADL values, which may contain a moderate/good quality forage,
- Although safflower straw has the equivalent CP content to reference wheat straw, the values of aNDFom, ADFom and ADL are lower than those of wheat straw,
- The husked safflower grain has a high content oil, moderate CP content and high fibre contents,
- The use of safflower grain may be recommended up to 5% in horse TMR and up to 20% in ruminant TMR,
- Up to 20% safflower herbage can be used in high quality forage in horse and beef TMR,
- Moreover, it may be argued that further investigation into the *in vivo* digestibility of these feed sources and the effects on performance and product quality need to be investigated.

Ethics Committee Approval: Ethics committee approval was received for this study from the Local Ethics Committee for Animal Experiments of Erciyes University (ERU-HADYEK), Kayseri-Turkey (Date: January 14, 2015; Decision number: 15/10).

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The Effects of Drinking Water Supplemented with Essential Oils on Performance, Egg Quality and Egg Yolk Fatty Acid Composition in Laying Hens

Özlem KARADAĞOĞLU[1], Bülent ÖZSOY[2], Mükremin ÖLMEZ[3], Özlem DURNA AYDIN[3], Tarkan ŞAHIN[3]

[1]Department of Agricultural and Animal Production, Kars Vocational School, Kafkas University, Kars, 36100, Turkey

[2]Department of Animal Nutrition and Nutritional Disease, Faculty of Veterinary, Mustafa Kemal University, Hatay, 31000, Turkey

[3]Department of Animal Nutrition and Nutritional Disease, Faculty of Veterinary, Kafkas University, Kars, 36100, Turkey

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ORCID IDs of the authors: Ö.K. 0000-0002-5917-9565; B.Ö. 0000-0003-0045-3790; M.Ö. 0000-0002-5003-3383; Ö.D.A. 0000-0003-4532-6795; T.Ş. 0000-0003-0155-2707

Abstract

This study was performed to examine the effect of different concentrations of an essential oil mixture (EOM) added to drinking water on growth performance, internal and external egg quality, and egg yolk fatty acid composition in laying hens. A total of 240 Brown Nick laying hens aged 20 weeks were used in this study. These hens were divided into one control and three treatment groups whose drinking water was supplemented with 0, 0.1, 0.2, and 0.3 ml/L of Oregofarm EOM. Oregofarm is a commercially available product containing peppermint, oregano, and anise oil. Each treatment group was further categorized into 10 subgroups, each consisting of 6 laying hens. The hens were fed basal rations for 16 weeks. For data collection and analysis, a 16-week period was divided into three time periods of 5 weeks. The weights of 25- to 30- and 35-week eggs were recorded weekly. In the groups whose drinking water was supplemented with the EOM, there was a significant improvement in egg production (EP) ($p<0.001$), weekly egg weights during the entire study period ($p<0.001$), and feed conversion ratio ($p<0.05$). Furthermore, egg shell thickness and yolk height were significantly increased ($p<0.05$) at week 35. The Haugh unit was also significantly improved ($p<0.05$) at weeks 25 and 30. However, feed intake and body weight were not affected by the treatment ($p>0.05$). The levels of saturated fatty acids were decreased, whereas the levels of polyunsaturated fatty acids increased with EOM supplementation, although the differences were not statistically significant ($p>0.05$). Therefore, based on the beneficial effects of supplementing drinking water with different concentrations of EOM on egg weight, egg production, feed conversion ratio, and egg yolk fatty acid compositions, it is suggested that EOM can be safely added at concentrations of up to 0.3 ml/L (i.e., the levels used in this study) to drinking water for laying hens.

Keywords: Egg quality, essential oils, laying hens, performance, yolk fatty acids

Introduction

Due to cross and multiple resistance issues, many antibiotic growth promoters in poultry nutrition are restricted by the EU. Consequently, researchers have been exploring the use of phytogenic feed additives (PFA) such as plants, plant extracts, essential oils (EOs) and individual or combined active EOs ingredients (Bozkurt et al., 2014). Essential oils are a mixture of fragile and volatile compounds - often referred to as aromatic - by plant origin and plant rooting. Hence, the chemical composition and concentration of active plant compounds vary greatly dependent on their source (Hippenstiel et al., 2011). Mint (*Mentha piperita*) is a member of the Labiatae family and is probably of East Asian origin. Such herbal medicines are widely used and are believed to be particularly useful in combating the immune system and secondary infections (Akbari et al., 2015). As an aromatic plant growing mostly in the Mediterranean region, Oregano (*Origanum vulgare L.*) EO can be used as a phytogenic additive. The medicinal plant has been used as an anion for digestive, antiparasitic, antibacterial and antifungal stimulant effects (Ertaş et al., 2005).

Address for Correspondence: Özlem KARADAĞOĞLU • **E-mail:** drozlemkaya@hotmail.com

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In some studies, the successful use of EOs as alternative growth promoters has already been proven and has started to play an important role in poultry nutrition. Phytogenic additives found in a wide variety of plants, spices and derivatives have a beneficial effect on the quality of animal products and animal health and are safe for use in the food industry (Ertaş et al., 2005). It has been reported that thyme EOs have various biological activities *in vitro* and *in vivo*, together with antimicrobial, antioxidant and antifungal effects (Espina et al., 2015; Mooyottu et al., 2014). Essential oils using herbs and spices might increase the acceptability of feed because of their flavorful characteristics and therefore, could advance feed intake by being added to poultry diets (Williams and Losa, 2001). Similarly, some studies showed positive effects on performance traits such as egg production rate, egg weight and egg mass output (Aydın et al., 2008; Bozkurt et al., 2012b), however some experiments showed that varying levels of dietary EOM did not significantly affect the performance, damaged eggs and eggshell weight (Olgun and Yıldız, 2014; Özek et al., 2011). There are limited studies reporting the effects of herbs and EOs on egg quality parameters of laying hens with contradictory results (Bölükbaşı et al., 2008 and 2010).

Due to increased dietary fat and increased coronary heart disease, egg yolk fatty acid composition is very important for consumers (Simopoulos and Salem, 1992). Methods of feeding animals can change the fatty acid composition of the eggs (Yi et al., 2014). Essential oils or mixtures added to diets have a positive effect on lipid metabolism (Acamovic and Brooker, 2005). Contrary to this statement, Ding et al. (2017) reported that there was no significant effect on the fatty acid composition of egg yolk in the experimental groups where Enviva EO additions were made and not made. Up to now, although the effects on the performance parameters of essential oil addition to poultry diets have been studied, there have very few reports on the effects of essential oils on egg yolk fatty acid composition. The study was designed to define the effects of essential oil mixture (EOM) (peppermint, oregano, and anise EOs) added to drinking water on growth performance, egg quality and egg yolk fatty acid compositions in laying hens.

Materials and Methods

Animals and experimental design

This study has been reviewed and approved by an ethical or advisory board of Animal Ethics Committee at Kafkas University (KAU-HADYEK/2016-032) (Kars, Turkey). A total of 240 *Brown-Nick* laying hens aged 20 weeks were used in the study. Drinking water was supplemented with EOs (Oregofarm EO, Farmavet International, Feed and Water Additives Specialist, Manisa, Turkey) at concentrations of 0.1, 0.2, and 0.3 mL/L respectively and were provided for 16 weeks (20 to 35 weeks). The EOM components are presented in Table 1. In the experiment, 4 groups of chickens were divided into 1 control group and 3 experimental groups. Ten sub-groups were created for each trial group and each sub group contained 6 laying chickens. In the study, the hens were fed with laying hen rations containing 16% HP and 2750 kcal / kg ME. The basal ration was prepared using maize and soybean meal. The ingredient and nutrient levels of the basal diet as presented in Table 2 met the NRC recommendations (1994).

The rations were applied to all groups as isocaloric and isonitrogenic mixes. Hens were sheltered in a hen house where light-

Table 1. The active componenets of the essential oil mixture

	Components (mg/kg)
Thymol (thyme oil)	2000
B-phellanderene (thymeoil)	1300
Limonene (thyme oil+pepermint oil)	3525
B-pinene (thyme oil+pepermit oil)	1977
Linalool (oreganum oil)	3645
Carcacrol (oreganum oil)	8910
Anethole (anise oil)	10712
Menthole (pepermint oil)	6375

Table 2. Composition and nutrient level of basal diet

Ingredient composition	**Basal Diet, g/kg^{-1}**
Corn	620
Soybean meal	205
Wheat bran	61.8
Oil	10
Limestone	85
Dicalcium phosphate	12.9
Salt	2.5
Vitamin mineral premix[a]	2.5
DL-Methionine	0.3
Calculated nutrient levels	
Metabolisable energy[b] (kcal kg^{-1})	2751
Crude protein	160
Dry matter	904
Ether extract	29.3
Crude ash	125
Crude fibre	28.4
Calcium	35.07
Total phosphorus	5.91
Available phosphorus	6.7
Starch	422.7
Sugar	33.4

[a]Supplied per kilogram of diet: 15372000 IU vitamin A, 6.28 mg vitamin E,0.64 mg vitamin K3, 37.36 mg Mn, 89 mg Fe, 25 mg Zn, 8.76 mg Cu, 0.03 mg Co, 0.05 mg MgI, 0.91 mg Se, 2400.000 IU D_3

[b]Metabolisable energy content of diets was estimated according to the equation of Carpenter and Clegg.

ing, temperature and ventilation were controlled. The temperature was maintained at approximately 22-24°C. The house had controlled lighting (16L: 8D). The experiment was completed in 16 weeks in the layer chicken unit of the Research and Application Farm at Kafkas University. All hens were supplied with diet and water *ad-libitum*. 15 liter water tanks were prepared for each trial group and connected via nipples. The daily water consumption of the animals was estimated. Fresh drinking water and EOM were added to the water tanks daily. The animals were constantly monitored and kept well hydrated throughout the experiment. The nutrient composition of the basal diet was determined according to the AOAC (1995).

Performance and egg quality parameters

In order to determine the changes in body weight, the body weights of the chickens were recorded at the beginning and end of the study. Egg production and weight and FI were registered weekly during the experiment. Egg production and FCR were calculated for each period. The magnitude of production was adjusted according to mortality rates, which were recorded daily. To determine egg quality parameters, in every 5 week period, 20 eggs were randomly selected per treatment (2 eggs per replicate). These were then evaluated in terms of eggshell weight, albumen and yolk weight, yolk height, eggshell thickness and HU. The eggs were weighed with a special instrument (TP-2000A-0.01 g, İstanbul, Turkey). The eggs were broken on a glass table. The eggshell thickness was measured at three different sections (upper-middle and ends) using a micrometer (Standardgage-200mm-8IN, Asia). The height of the yolk was measured with a tripod micrometer (Mitutoya-20mm, Kawasaki, Japan). Albumen quality was measured in terms of the HU calculated from the weight of the egg and the height of the albumen. While calculating HU values for each egg, the following formula was used:

$$HU= 100\log (H-1.7W^{0.37}+7.6)$$

(H is the observed height of the albumen (mm) and W is the weight of the egg (g)).

Fatty acid profile of egg yolk

During the final week of fatty acids analysis, the oil extraction was carried out on 10 egg samples from each group, according to the method laid out by the AOAC (1995), and then processed with methyl esters with Boron Trifluoride. The fatty acid methyl esters were condensed under nitrogen gas and then analyzed in GC-MS (HP 6890/5972). An Agilent HP88 100X250 micro-Mx250 mm column was used in the analysis. The initial temperature of the column was 120°C, while the final temperature was 230°C. The injector and detector temperature was set at 250°C. The injection speed was set at 50:1 and helium was used as a carrier gas.

Statistical analysis

The statistical analysis and the significance of the mean values between the groups were determined by the analysis of variance. The multiple range test was used to determine differences between the trial groups. The statistical analyses were performed with Statistical Package for the Social Sciences 16.0 (SPSS Inc.; Chicago, IL, USA) (Dawson and Trap, 2001).

Results and Discussion

Laying hen performance and egg production

Initial and final BW, FI, FCR and egg production data for the layer hens fed drinking water containing EOM is presented in Table 3. Performance analysis showed that there were no effects on body weight and feed intake in the experimental group. During the experimental period, egg production and FCR improved significantly ($p<0.05$) with the supplementation of 0.2 and 0.3 mL/L EOM. Studies reported beneficial effects on laying performance with EOM supplementation in layer's diet (Bozkurt et al., 2012b; Özek et al., 2011). Radwan et al. (2008) reported that FI and BW were not significantly affected by dietary herbs. No differences in FI were reported in hens when the EO was supplemented to basal diets (Bozkurt et al., 2012a and 2012c; Çabuk et al., 2014). In contrast, Abdo et al. (2010) found that adding green tea EO in the ratio significantly reduced feed intake. Similarly, Bölükbaşı et al. (2008) found that EO supplementation in layer diets lowered FI and FCR was improved when the birds were fed EOM in their diet. Certain feeding experiments with layer hens didn't find any differences in FCR when the oregano EO was added to diets (Florou-Paneri et al., 2005). Similarly, Bozkurt et al. (2012a; 2012c) revealed that dietary inclusion of EO did not improve FCR in white layer hens. However, some beneficial effects on FCR using EO of inclusion level 24 mg/kg feed have been reported for the brown layer strain under hot environmental conditions (Çabuk et al., 2006). Some studies confirmed the positive effects of herbs and their respective EO on the BW of hens. Çabuk et al. (2006) reported that the BW of layer hens fed diets of an EO mixture increased compared to that of the control group over a 20-week period. Similarly, in another study, significant improvements were determined in the BW of hens when they were fed different levels of garlic (Khan et al., 2007). However, no effect was reported on BWG in response to dietary supplementation with tea leaves or green tea EO (Abdo et al., 2010), or dietary oregano EO (Florou-Paneri et al., 2005). Bozkurt et al. (2009) observed that adding an EO mixture to diet had no effect on egg production and weight. Similarly, Bölükbaşı et al. (2010) observed no effect on egg production but there was an increase in egg weight when diets were supplemented with thyme, sage or rosemary over a period of 12 weeks. However, Xianjiing He et al. (2017) found that added oregano EOs in layer diets significantly influenced egg production and average egg weight.

Egg quality parameters

The effects of Oregofarm EO on egg weight and internal and external egg quality are listed in Table 4 and 5. Supplementation of Oregofarm EO significantly increased the egg weight ($p<0.05$) weekly. In all periods, the egg weight was effected significantly

Table 3. The effects of adding EOM on the performance (mean±standard error) to layer hens' drinking water

	Essential oil mixed supplementation (ml/L)				
	0	**0.1**	**0.2**	**0.3**	**p**
Initial body weight, g	1734±17.63	1734±14.44	1771±16.55	1782±15.1	0.62
Final body weight, g	1736±62.91	1661±22.52	1742±21.99	1771±20.44	0.14
Feed intake, g day^{-1} per bird	102.24±1.52	99.94±1.26	103.83±1.61	104.13±1.59	0.191
Egg production, %	90.88±0.44^{b}	89.74±0.48^{b}	92.41±0.41^{a}	92.90±0.40^{a}	<0.001
Feed conversion ratio, kg feed per kg egg	2.01±0.04^{a}	1.97±0.03^{ab}	1.91±0.03^{b}	1.90±0.02^{b}	0.038

$^{a, b}$indicated the difference within a row was significant (p<0.05)

Table 4. The effects on the weekly egg weights of adding EOMs to layer hens' drinking water (g)

	Essential oil mixed supplementation (mL/L)				
Weeks	**0**	**0.1**	**0.2**	**0.3**	**p**
1-wk	55.00±0.59 (n:55)	56.1±0.55 (n:53)	56.36±0.64 (n:58)	56.39±0.71 (n:59)	0.36
2-wk	55.66±0.55^{b} (n:56)	55.81±0.45^{b} (n:56)	57.48±0.55^{a} (n:52)	57.81±0.63^{a} (n:55)	0.007
3-wk	56.8±0.64 (n:52)	57.86±0.49 (n:54)	58.47±0.64 (n:57)	58.38±0.46 (n:55)	0.15
4-wk	56.7±.05 (n:59)	57.07±0.45 (n:57)	58.12±0.57 (n:55)	58.25±0.59 (n:57)	0.103
5-wk	57.16±0.59^{b} (n:58)	56.89±0.43^{b} (n:56)	58.98±0.60^{a} (n:59)	58.40±0.52^{ab} (n:57)	0.019
6-wk	57.17±0.52^{b} (n:58)	57.51±0.50^{b} (n:59)	58.17±0.61^{ab} (n:57)	59.18±0.52^{a} (n:57)	0.047
7-wk	56.90±0.59^{c} (n:48)	57.56±0.47^{bc} (n:52)	59.62±0.65^{a} (n:51)	58.98±0.52^{ab} (n:54)	0.002
8-wk	56.68±0.66^{b} (n:55)	56.93±0.56^{b} (n:58)	59.60±0.66^{a} (n:58)	59.33±0.58^{a} (n:56)	0.001
9-wk	56.38±0.59^{b} (n:48)	56.35±0.57^{b} (n:49)	57.65±0.58^{ab} (n:50)	58.53±0.58^{a} (n:53)	0.019
10-wk	57.44±0.70^{b} (n:51)	56.81±0.64^{ab} (n:46)	58.74±0.57^{a} (n:55)	59.13±0.61^{a} (n:55)	0.037
11-wk	57.50±0.52^{b} (n:47)	58.92±0.61^{ab} (n:49)	59.77±0.63^{a} (n:51)	60.30±0.54^{a} (n:57)	0.005
12-wk	57.75±0.67^{c} (n:44)	58.68±0.57^{bc} (n:51)	60.98±0.64^{a} (n:53)	60.39±0.59^{ab} (n:53)	0.001
13-wk	57.61±0.72^{b} (n:47)	57.06±0.57^{b} (n:47)	60.25±0.52^{a} (n:55)	60.18±0.54^{a} (n:56)	0.000
14-wk	57.87±0.73^{b} (n:57)	58.77±0.69^{ab} (n:50)	60.42±0.59^{a} (n:55)	59.80±0.50^{a} (n:56)	0.024
15-wk	58.24±0.75^{b} (n:51)	57.58±0.54^{b} (n:54)	59.27±0.57^{ab} (n:56)	60.57±0.60^{a} (n:56)	0.003
16-wk	57.55±0.69^{b} (n:54)	57.81±0.63^{b} (n:55)	60.37±0.69^{a} (n:56)	60.27±0.59^{a} (n:56)	0.001
All Periods	56.67±0.16^{b} (n:840)	57.34±0.14^{b} (n:846)	59.17±0.16^{a} (n:878)	59.11±0.15^{a} (n:892)	<0.001

$^{a, b}$indicated the difference within a row was significant (p<0.05)

by essential oil mixes (p<0.05). Bozkurt et al. (2012b) declared that EOM added to layer hen diet significantly enhanced egg production rate and egg weight. Other studies showed no significant change in egg production and egg weight when hens were fed a diet supplemented with EOM (Bozkurt et al., 2012b; Florou-Paneri et al., 2005; Özek et al., 2011). Similarly, Bozkurt et al. (2009) discovered that the addition of EOM at 24 to 48 mg/kg to diet had no effect on the production and weight of eggs for broiler breeders in chickens between the ages of 26 and 46 weeks. This variability in results may be related to the dose of EOs, different application methods, components of EOs or their utilization in different types of poultry.

Eggshell weight increased at week 25 week (p<0.05). At the 30th and 35th weeks of the trial, eggshell weight gradually increased with EOM supplementation, but the difference was not significant (p>0.05). Supplements in drinking water had no effect on albumen and yolk weight (p>0.05) in early period hens at 25 weeks. Eggshell thickness was significantly improved at all periods measured (p<0.05). Adding EOM (0.1, 0.2 or 0.3 mL/L) to drinking water influenced HU at the age of 25 and 30 weeks (p<0.05) The highest HU was obtained in the eggs of layer hens fed drinking water containing 0.3 mL/L EOM. In some studies, it has been observed that the use of different forms of EOs or EOM leads to significant improvements in egg shell weight (Bozkurt et al., 2012b and 2012c). In contrast, Akbari et al. (2015) reported that feeding peppermint and thyme EO had no effect on shell weight in layer hens. In our study, the supplementation of EO significantly increased the eggshell thickness in all weeks (p<0.05). Sim-

Table 5. The effects on the internal and external egg quality (mean± standard error)[1] of adding EOMs to layer hens' drinking water

	Essential oil mixed supplementation (mL/L)				
Item	**0**	**0.1**	**0.2**	**0.3**	**p**
Unit of egg weight					
25 wk	57.18±1.17^{b}	58.13±0.91^{ab}	58.32±0.99^{ab}	60.32±0.83^{a}	0.15
30 wk	57.46±0.74	57.59±0.68	57.85±0.75	58.08±1.03	0.94
35 wk	59.66±0.95	59.39±0.90	61.64±0.65	61.02±0.79	0.10
Eggshell weight (g)					
25 wk	5.95±0.12^{b}	5.97±0.12^{b}	6.03±0.096^{b}	6.37±0.13^{a}	0.04
30 wk	6.03±0.12	6.04±0.12	6.35±0.09	6.21±0.11	0.12
35 wk	5.89±0.09	6.09±0.08	6.10±0.12	5.95±012	0.36
Albumen weight (g)					
25 wk	37.09±0.97	38.48±0.79	37.97±0.69	39.49±0.62	0.19
30 wk	37.91±0.77	36.89±0.84	36.08±0.84	36.14±0.97	0.82
35 wk	38.68±0.93^{a}	38.43±0.89^{a}	40.36±0.78^{a}	40.36±0.75^{a}	0.05
Yolk weight (g)					
25 wk	14.14±0.29	13.68±0.17	14.31±0.31	14.46±0.26	0.19
30 wk	14.48±0.27^{b}	14.66±0.25^{b}	15.42±0.22^{a}	15.72±0.30^{a}	0.003
35 wk	15.09±0.22^{a}	14.86±0.23^{b}	15.18±0.19^{a}	14.71±0.19^{b}	0.05
Yolk height (mm)					
25 wk	28.94±0.70^{a}	28.96±1.16^{a}	26.12±0.35^{b}	26.20±0.41^{b}	0.004
30 wk	26.96±0.27	26.65±0.26	27.08±0.24	26.74±0.29	0.65
35 wk	27.87±0.33^{b}	29.03±0.27^{a}	29.11±0.28^{a}	28.57±0.25^{ab}	0.01
Eggshell thickness (mm)					
25 wk	0.32±0.04^{a}	0.28±0.05^{b}	0.29±0.04^{b}	0.31±0.05^{a}	0.035
30 wk	0.33±0.05^{a}	0.33±0.04^{a}	0.31±0.05^{b}	0.32±0.05^{ab}	0.001
35 wk	0.31±0.03^{b}	0.33±0.03^{a}	0.33±0.08^{a}	0.34±0.06^{a}	0.008
Haugh unit					
25 wk	78.35±1.22^{ab}	75.99±0.89^{b}	78.72±1.04^{ab}	80.95±1.25^{a}	0.024
30 wk	79.26±0.28^{b}	79.15±0.37^{b}	79.86±0.31^{ab}	80.62±0.52^{a}	0.03
35 wk	79.69±0.33	80.31±0.32	80.51±0.58	80.80±0.64	0.43

$^{a, b}$indicated the difference within a row was significant ($p<0.05$)

[1]Means of 20 eggs per treatment

ilarly, Bozkurt et al. (2012b) reported that supplementation of EOM increased egg shell weight, egg shell thickness and shell breaking strength.

Adding oregano EO (50 or 100 mg kg^{-1}) to the diet had no effect on HU at the age of 32 weeks (Florou-Paneri et al., 2005). Similarly, Çabuk et al. (2006) observed that adding EOMs or antibiotics to the diet did not significantly affect the egg quality characteristics HU, shell weight or yolk weight in laying quails. Ding et al. (2017) reported that Enviva EO supplementation in layer hens diet had no significant effect on albumen height and HU. However, Xianjing et al. (2017) observed that the use of oregano EO supplementation had no effect on eggshell ratio but found that it did significantly affect yolk ratio, egg shape index and HU.

Fatty acid compositions

The mean value of the yolk fatty acids percentage in different treatment groups is shown in Table 6. When compared to the control diet, the proportion of DHA (C22:6 n-3) in the egg yolk was significantly decreased in the treatment groups ($p<0.05$). Palmitoleik acid (16:1) increased in drinking water containing EOM when compared to the control group ($p<0.05$). There was no significant effect on total saturated fatty acids and omega 3 and 6 fatty acid in the experimental groups. To our knowledge, there have been few reports about the effects of EO on the fatty acid composition of egg yolk. In the present study, there were significant differences in the treatment groups in α linolenic acid, linolenic acid, palmitoleic acid, arachidonic acid, lignoceric acid and DHA ($p<0.05$). However, Ding et al. (2017) found that EO in

Table 6. The effects on egg yolk fatty acid composition (mean± standard error) of adding EOMs to layer hens' drinking water

	Essential oil mixed supplementation (mL/L)				
Fatty acid (%)	**0**	**0.1**	**0.2**	**0.3**	**p**
C14:0	0.28±0.016^{b}	0.29±0.015^{ab}	0.34±0.015^{a}	0.34±0.024^{a}	0.022
C14:1	0.005±0.003^{b}	0.008±0.004^{b}	0.016±0.005^{b}	0.033±0.009^{a}	0.004
C15:0	0.009±0.005^{b}	0.020±0.006^{b}	0.02±0.006^{b}	0.036±0.002^{a}	0.009
C16:0	24.40±0.20^{b}	23.52±0.42^{b}	24.72±0.21^{a}	24.90±0.34^{a}	0.014
C16:1	2.67±0.09^{c}	2.86±0.16^{b}	3.23±0.14^{ab}	3.48±0.21^{a}	0.033
C17:0	0.21±0.011^{b}	0.21±0.013^{b}	0.21±0.012^{b}	0.28±0.019^{a}	0.005
C17:1	0.21±0.05	0.18±0.03	0.29±0.04	0.28±0.053	0.272
C18:0	9.57±0.20^{a}	9.42±0.35^{a}	8.50±0.38^{b}	7.82±0.30^{b}	0.001
C18:1	41.15±0.71	41.35±0.49	41.32±0.38	41.16±0.70	0.991
C18:2 n6	16.96±0.78	17.64±0.41	17.42±0.44	17.93±0.62	0.696
C18:3 n6	0.50±0.035	0.49±0.052	0.48±0.030	0.48±0.031	0.973
C18:3 n3	0.74±0.058^{b}	0.94±0.059^{a}	0.90±0.041^{a}	0.96±0.044^{a}	0.021
C20:0	0.17±0.029	0.16±0.043	0.13±0.025	0.10 ± 0.017	0.393
C20:1	0.25±0.029	0.30±0.034	0.24±0.018	0.27±0.017	0.420
C20:2 n6	0.22±0.029	0.26±0.040	0.18±0.022	0.19±0.015	0.209
C20:3 n3	0.20±0.033	0.18±0.029	0.19±0.018	0.17±0.018	0.869
C20:4 n6	1.68±0.058^{a}	1.45±0.11^{ab}	1.23±0.14^{bc}	1.07±0.13^{c}	0.004
C22:6 n3	0.61±0.03^{a}	0.51±0.05^{ab}	0.44±0.06^{b}	0.38±0.05^{b}	0.014
C24:0	0.16±0.018^{ab}	0.21±0.03^{a}	0.11±0.014^{b}	0.12±0.014^{b}	0.015
∑SFA	34.81±0.33	33.84±0.32	34.06±0.43	33.60±0.51	0.187
∑MUFA	44.27±0.75	44.69±0.48	45.10±0.46	45.22±0.78	0.710
∑PUFA	20.91±0.86	21.47±0.51	20.84±0.38	21.18±0.73	0.897
∑UFA	65.19±0.33	66.16±0.32	65.94±0.43	66.40±0.51	0.187
∑PUFA/∑SFA	0.60±0.028	0.64±0.018	0.61±0.016	0.63±0.026	0.695
∑UFA/∑SFA	1.88±0.028	1.96±0.027	1.98±0.048	1.94±0.018	0.198
n6	19.37±0.81	19.84±0.46	19.31±0.36	19.68±0.67	0.911
n3	1.54±0.062	1.63±0.083	1.53±0.049	1.50±0.088	0.608
n6/n3	12.60±0.34	12.36±0.49	12.72±0.48	13.29±0.51	0.549
NUTRVALUE	2.08±0.036^{b}	2.17±0.055^{a}	2.02±0.028^{b}	1.97±0.043^{b}	0.014
ATHERNGINX	0.17±0.003^{a}	0.16±0.005^{a}	0.15±0.006^{ab}	0.14±0.006^{b}	0.008
THRINDEX	0.60±0.008	0.58±0.007	0.58±0.010	0.57±0.012	0.191

n=10
^{a,b} indicated the difference within a row was significant ($p<0.05$)
∑SFA= total saturated fatty acids; ∑MUFA= total monounsaturated fatty acids; ∑PUFA= total polyunsaturated fatty acids; n-6: omega-6 fatty acids; n-3: omega-3 fatty acids

layer hen diet had no effect on the fatty acid composition of egg yolk of layer hens. The addition of EOM to the drinking water of layer hens had no effect on the n-6/n-3 ratio in the egg ($p>0.05$). Bölükbaşı et al. (2010) reported that bergamot oil supplementation in layer diets significantly increased the proportion of DHA and n-3 in the egg yolk. While the addition of essential oil to drinking water reduced the SFA concentration, it increased the MUFA concentration, numerically. In agreement with the present study, Ding et al. (2017) reported that SFA concentrations were decreased but PUFA and MUFA concentrations were increased with dietary EO. Bölükbaşı et al. (2008) showed that SFA and PUFA concentrations in leg and breast tissues in broilers were decreased, whereas, MUFA concentrations were increased. To our knowledge, the studies on the addition of essential oils to drinking water are limited. Therefore, further work is needed in order to give more detailed information on this topic in layer hens.

Conclusion

As a result of supplementing peppermint, oregano, and anise essential oils in the amounts of 0.2 mL/L and 0.3 mL/L to drink-

ing water, there were beneficial effects on performance and egg quality without adverse effects on other parameters.

Ethics Committee Approval: All experimental protocols adhered to and approved by the guidelines of Animal Ethics Committee of Kafkas University (KAU-HADYEK/2016-032) (Kars, Turkey).

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Assessment of Temperature and Microbiological Quality of Fresh Sardine, Bouge, Saury and Mackerel Marketed in Tripoli City, Libya

Tawfik Mehdi HASSAN, Amal Abubaker ALHALLUG, Nuri Sahli MADI

Department of Food Science and Technology, University of Tripoli, Faculty of Agriculture, Libya

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Abstract

The aim of this study was to assess the temperature, total aerobic plate count (TAPC), and histamine-producing bacterial count (HPBC) of four types of fish, viz., sardines *(Sardinella aurita)*, bouge *(Boops boops)*, saury *(Trachurus mediterraneus)*, and mackerel *(Scomber scombrus)*, that are sold in three major fish markets in Tripoli's city center. A total of 113 samples of these fish types were collected, both in the morning and in the evening, from July to December in the fishing season. Results showed that the temperature of the collected fish samples ranged from <5°C to 22°C. Of the total 113 fish samples, 5.0%, 52.0%, and 43.0% had temperatures of <5°C, 5°C–14°C, and 15°C–22°C, respectively. The TAPC of all the fish samples ranged from 3.0×10^3 to 3.5×10^7 colony-forming unit/g(cfu/g) of meat (with skin), with a mean of 1.1×10^6 cfu/g. The HPBC ranged from an estimated 5.0×10^2 to 2.7×10^6 cfu/g, with a mean of 1.8×10^5 cfu/g. Statistical analysis of the data showed a weak correlation (r = 0.05) between TAPC and HPBC of all the fish samples collected from the three major markets. The TAPC results revealed that 50%, 46%, 38%, and 17% of the saury, bouge, mackerel, and sardine fish samples, respectively, did not comply with the standard specification limit (10^6 cfu/g) prescribed by the Libyan authorities. A total of 26 isolates of histamine-producing bacteria were identified in this study. The majority of them belonged to the *Enterobacteriaceae family* and were not indigenous to the marine environment. There was a variation in the distribution of these bacterial isolates among all the fish samples during the course of the study. However, *Vibrio fluvialis, Erwinia* spp., and *Klebsiella planticola* were detected in all the fish samples throughout the study period. The high TAPC and HPBC recorded in this study could be attributed to cross-contamination due to the poor quality of the surrounding environment and the poor hygienic practices. Therefore, there is an urgent need for proper control of product handling conditions in the fish markets monitored in this study.

Keywords: Bouge, HPBC, mackerel, sardine, saury, TAPC, Tripoli city

Introduction

Sardine *(Sardinella aurita)*, Bouge (*Boops boops*), Saury (*Trachurus mediterrangus*), and Mackerel (*Scomber scombrus*) are among the most popular fish choices for the Libyan consumer because of their reasonable prices and availability during the fishing season. They represent 50% of the total annual fish catch, which amounts to around 38000 tons (Anonymous, 2014). These fish species have a high nutritive value and they are a source of good quality protein and polyunsaturated fatty acids – in particular, omega 3 and 6 fatty acids. Hassan et al. (2006, 2011), reported that the total lipids of Libyan sardine, bouge and mackerel contain ample quantities of omega 3 fatty acids - 33.11, 28.03 and 20.03% of total fatty acids respectively. In comparison with red meat, fish meat is highly perishable mainly because of its high non-protein nitrogen compound

Address for Correspondence: Tawfik M. HASSAN • **E-mail:** t.hassan@uot.edu.ly

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content, which represents ideal nutrients for spoilage bacteria. In addition, these fish species are particularly sensitive to histamine formation from histamine producing bacteria if they become exposed to poor temperature control when handled, stored or displayed.

Histamine poisoning (also known as scombroid poisoning) occurs following consumption of certain fish species which contain histamine. Symptoms of histamine poisoning appear within anything from a few minutes to 3 hours after consuming these fish whether they are fresh, frozen, canned or dried. Studies and statistical surveys on scombroid poisoning showed that most of the histamine poisoning cases resulted from the consumption of fish belonging to the *scombridae* family - which includes mackerel, *clubeidae* such as sardines (Kim et al., 2004; Moreno et al., 2001) and carangidae -which includes Saury (Brillants et al., 2001; Lokuruka et al., 2004). This is because their muscles are rich in free histidine, the precursor to histamine formation by histamine producing bacteria (Kim et al., 2004; Moreno et al., 2001; Rawles et al., 1996).

Niven et al. (1981) reported that Most of the histamine-producing bacteria isolated from fish muscles belong to mesophilic enteric bacteria, which are not the natural flora of fresh fish. Other studies found the histamine decarboxylase enzyme in some species of bacteria: *Vibrio harveyii, Vibrio alginolyticus, Photobacterium phosphoreum* and *leiognathi spp* which are all indigenous to the marine environment. While they are not potent histamine producers, they were responsible for most of the documented histamine poisoning cases reported (Kanki et al., 2004; Ramesh et al., 1989; Takahashi et al., 2003; Yoshinagu and Frank, 1982).

Due to the lack of local studies on the effect of display conditions in Tripoli fish markets on fish sensitive to histamine production, this study was carried out to determine temperature, total aerobic plate count and histamine producing bacterial count in samples of sardines, saury, bouge and mackerel displayed for sale in three fish markets in Tripoli city during the period July – December of the fishing season.

Materials and Methods

Sample collection
One hundred thirteen samples from fresh sardines, Bouge, Saury and mackerel were collected directly from fish on sale in three main fish markets - A, B and C in Tripoli City, Libya. The samples were collected between 7:00 and 8:00 A.M. and between 12.00 and 13.00 P.M., during the period from July to December of the fishing season. The samples were kept in sterile polyethylene bags and transferred in an icebox, within 15 minutes, to the Microbiology and fish disease laboratory at the marine research center in Tajoura, Libya. The temperature of the displayed fish was taken when the samples were collected.

Sample preparation for bacteriological analysis
From each sample, 5-6 pieces were randomly collected. Meat muscles were cut from the back and sides of each fish body using a sterile knife and then homogenized in a sterile blender. Twenty five grams of the homogenate was then used for bacteriological analysis.

Determination of Total Aerobic Plate Count (TAPC) and Histamine Producing Bacterial Count (HPBC)
Twenty-five grams of minced homogenized fish meat was mixed with 225 mL of 0.1% sterile peptone water in a sterile electric blender for 1 minute. Then, serial dilutions of 10^{-2}, 10^{-4} and 10^{-5} were prepared from the homogenate for TAPC on plate count agar (Oxide Ltd.,Hamsphire, UK), while dilutions of 10^{-4} and 10^{-5} were used for HPBC determination on Niven's medium, according to Swanson et al. (2001). The Niven's medium was prepared according to the procedure of Niven et al. (1981). All plates were incubated inverted at 25°C for 48 $\pm$ 2 hours. Plates were incubated at 25°C, as recommended by Nickelson et al. (2001) for the routine assessment of quality of fresh and frozen seafood products.

Colonies with a purple halo grown on Niven agar were counted, aseptically isolated and then purified with the streaking technique on trypticase soy agar plates (Oxide Ltd. Hamsphire, UK). The plates were incubated at 25°C for 24 hours to obtain isolates. Theses isolates were then restreaked on Niven agar medium plates to confirm that they produced purple halo colonies. Pure isolates were gram stained, and microscopically examined under oil immersion, before identification using analytical profile index 20 E (API 20 E kits) for identification and differentiation of member of the family *Enterobacteriaceae* (Biomerieux Inc Boston MA USA) in accordance with Korashy et al. (2005).

Statistical analysis
The results of the TAPC and HPBC were analyzed with the statistical package Minitab 16 (Minitab Inc. State college Pa USA) using descriptive statistics such as minimum, maximum and mean value. The Correlation coefficient test (r) was performed between the TAPC and HPBC data. Significance was considered where $p<0.05$.

Results and Discussion

Samples temperature
The temperature of fish samples ranged from <5 to 22^{0}C. The percentage of samples that had temperatures <5, 5-14, and 15-22^{0}C were 5.0, 52.0 and 43.0 % respectively out of the total 113 samples (Table 1). It is clear from the results that 95% of the samples collected had a temperature between 5 and 22 ^{0}C. This temperature range is suitable for growth of HPB (Economou et al., 2007; Kim et al., 2009). Therefore, the presence of such bacteria might place the fish samples at risk of histamine formation when displayed for sale at temperatures higher than 5°C.

When the fish samples were classified according to the markets included in this study, the results showed that samples taken from fish market B were the best in terms of temperature. Sev-

enty-two percent (72%) of these samples had temperatures between 5 and 14°C, while the rest of the samples collected from the other two markets had temperatures between 15 and 22°C (Table 2).

The results shown in Table 3 reflect the poor refrigeration conditions of the samples, since the percentage of samples that had a temperature of <5°C did not exceed 5% in the morning and 8% in the afternoon at fish market B. Meanwhile, the highest percentage (60%) of samples that had temperatures between 15 to 22°C was recorded in samples collected at noon. This is probably due to poor refrigeration methods applied in these markets, especially for those who depend solely on ice as it melts by the end of the day. These conditions make fish samples more susceptible to histamine formation.

Total aerobic plate count (TAPC) and Histamine producing bacterial count (HPBC)

The TAPC for samples of Sardines, Bouge, Saury and Mackerel ranged from 5 x 10^3 to 8.9 x 10^6, 1.5 x 10^4 to 5.3 x 10^6, 3.2 x 10^4 to 3.5 x 10^7 and 5 x 10^2 to 3 x 10^6 colony forming unit/gram (cfu/g) meat (with skin) respectively as shown in Table 4. Meanwhile, the range of HPBC for the same samples were from 5 x 10^2 to 5 x 10^6, 6 x 10^2 to 2.6 x 10^6, 3 x 10^3 to 2.6 x 10^6 and 5 x 10^2 to 2.7 x 10^6 cfu/g meat (with skin) respectively as shown in Table 5. It is noted from the results in Table 4 and 5 that the highest average TAPC and HPBC were recorded in the saury fish samples, followed by bouge and the lowest counts were recorded in samples of mackerel.

When comparing the results for TAPC obtained in this study with the standard specification limit - 10^6 cfu/g fish (GSO 1016: 2015) as prescribed by the Libyan authorities, it was found that 50, 46, 38 and 17% of the Saury, Bouge, Mackerel and sardine fish samples did not comply with this limit as shown in Table 6. Remarkably, the total percentage of samples collected from market A that did not comply with this standard did not exceed 11%, whereas the figures for market B and market C were, 52% and 41%, respectively (although market B was equipped with new facilities and utensils). Moreover, the average TAPC in fish market A for all fish types was lower than that recorded in market B and market C.

WHO (2007) indicated that the TAPC rarely reflects the overall quality of fish, but it gives an indication of the risk of spoilage induced since each of these organisms had different ways of affecting health conditions of the consumer of such contaminated fish. The results of TAPC and HPBC obtained from the present study reflect the variations in handling conditions that these fish were exposed to from the time of harvesting until delivered to the fish markets.

The range of averages for TAPC of sardine samples found in this study (from 2.8 X 10^5 to 6.4 x 10^5 cfu/g fish meat) was higher than that reported in fresh Libyan sardines *(Sardinella aurita*) in previous studies carried out by Abuzghia (1990) where the TAPCs were between 7.9 x 10^3 and 8.2 x 10^3 and by Hassan et al. (2008) – between 1.0 x 10^2 and 1.0 x 10^3cfu/g fish meat. The variations in TAPC reported in these studies could be attributed to the variations in handling conditions these fish were exposed to from the time of fishing until they reached the laboratories.

The range of TAPC recorded in this study for sardine and mackerel samples was from 5.0 x 10^3 to 8.9 x 10^5 and 9.0 x 10^3 to 1.0 x 10^6 cfu/g respectively, which is lower than that reported by Korashy et al. (2005) in samples of sardines (*Sardinella gibbosa*), European sardines *(Sardinella pilchards*) and Atlantic mackerel *(Trachurustrachurus)*, where the average counts were, 8.6 x 10^7, 6.5 x 10^6 and 7.0 x 10^7 cfu/g respectively. Furthermore, the results for the same samples showed that HPBCs (2.5 x 10^3, 2.1 x 10^3 and 2.2 x 10^3 cfu/g, respectively) were lower than the results obtained in this study for sardine and mackerel. The range of HPBCs recorded in this study for sardine and mackerel samples was between 5.0 x 10^2 (estimated) and 1.4 x 10^5 and between 5.0 x 10^2 (estimated) and 6.2 x 10^5cfu/ g fish, respectively.

The results from this study were also higher than the results reported by Okuzumi et al. (1982), where the range of TAPCs for fresh sardine (*Sardinella melanostic*) , saury (*Coloabissaira*) and

Table 1. The temperature ranges of fish samples collected from three fish markets in Tripoli, Libya, and the percentage of each range

Temperature range (°C)	Numbers of samples	The percentage of each range
‹ 5	6	5.0%
5 – 14	59	52.0%
15 – 22	48	43.0%

Table 2. Classification of fish samples according to their temperatures and the markets included in the study

Fish samples	Fish market (A)		Fish market (B)		Fish market (C)	
temperature (°C)	Number of samples	% samples	Number of samples	% samples	Number of samples	% samples
‹ 5	-	-	6	12.0	-	-
5 – 14	6	23.0	36	72.0	18	49.0
15 – 22	20	77.0	8	16.0	19	51.0

(-) No samples recorded temperatures within this range.

mackerel (*Scomber japonicas*) in Japan were between 1.1 x 10^4 and 3.0 x 10^4, 1.0 x 10^2 (estimated) and between 4.9 x 10^4 and 2.9 x 105 cfu/g respectively, while the HPBC ranges were, 1.0 to 1.0 x 10^4, 5.7 x 10^3 to 2.1 x 10^5 and 1.0 to 1.0 x 10^2 (estimated) cfu/g. Additionally, Lopez – Sabater et al. (1996) found that the HPBC in mackerel was 3.1 x 10^2 cfu/g fish in Spain, which is lower than the counts recorded in this study.

Statistical analysis of the results showed weak correlation (r=0.05) between TAPC and HPBC in all samples collected from the three markets. This might be related to the randomness of collected samples and the unknown variations in handling conditions that these fish species were exposed to from harvest to delivery to the fish markets.

Identification of HPB isolated from fish samples

The results revealed that twenty-six (26) bacterial types were isolated from the fish samples and identified as HPB. Most of these isolates belong to the family *Enterobacteriacea*e, which are not indigenous to the marine environment, and some belong to *Vibrionaceae* (Table 7). According to these results, the prevalence percentages of *V. fluvialis, Erwinia spp, S. putrefaciens* and *K. planticola* were 18.3, 12.2, 11.9, and 10.0% respectively, while the prevalence percentages of *M. morganii, P. aeruginosa* and *A. baumaii* were almost equal - 6.40, 5.90, and 5.50 respectively. The prevalence percentages of other isolates were lower and ranged between 0.45 and 3.20%.

The prevalence percentages of *S. putrefaciens* and *P. fluorescens* in the sardine samples were 11.9 and 3.2% out of the total isolates, respectively. These percentages are close to those reported by Ababouch et al. (1991) in sardines (*Sardinella pilchardus*) caught off the Atlantic coast (10 and 20%).

The results of this study were compared with a study (Economou et al., 2007) that isolated 77 types of HPB, which accounted for 53% of the total number of bacteria in 30 samples of fresh and frozen albacore tuna (*Thunnus alalonga*) collected from Brazil, Srilanka, The Maldives, Indonesia and Yemen. There was a similarity in types of bacteria isolated from the samples, among which were *P.fluoroscens, P. aeruginosa, E. coli,* and *B. capacia.* However, the differences were in the percent prevalence where their proportion in tuna samples was higher than in the fish samples of this study.

Variations were observed in the prevalence percentages of most types of HPB isolates during the period of the study and even in the same type of bacteria, since the prevalence percentages of *V. fluvialis* during the months of July, August, September, October, November and December were 21.0, 39.0, 30.0, 42.0, 30.0 and 25.0% respectively, While those of *Erwinia spp* were 29.0, 8.0, 21.0, and 9.0 respectively. However, the occurrence of *S. putrefaciens* and *P. aeruginosa* was only recorded

Table 3. Classification of fish samples according to their temperatures and the time of sampling

Sampling time	Morning		Noon	
Temperature Range (°C)	Number of samples	% of samples	Number of samples	% of samples
‹ 5	4	5.0	2	8.0
5 – 14	52	59.0	8	32.0
15 – 22	32	36.0	15	60.0
Total	88	78.0	25	22.0

Table 4. Total aerobic plate counts (cfu/g) for fish samples collected from three fish markets located within Tripoli city center, Libya

	Fish market (A)		Fish market (B)		Fish market (C)	
Fish market	**Range**	**Average**	**Range**	**Average**	**Range**	**Average**
Fish type						
Sardine	3.2 x 10^4 - 9.7 x 10^5	2.8 x 10^5	1.1 x 10^4 - 8.9 x 10^6	8.7 x 10^5	5.0 x 10^3 - 2.8 x 10^6	6.4 x 10^5
Bouge	5.3 x 10^4 - 1.8 x 10^6	4.3 x 10^5	1.5 x 10^4 - 5.3 x 10^6	1.1 x 10^6	5.8 x 10^4 - 2.7 x 10^6	1.1 x 10^6
Saury	1.2 x 10^5 - 2.9 x 10^5	2.1 x 10^5	3.2 x 10^4 - 3.5 x 10^7	4.0 x 10^6	6.4 x 10^4 - 4.6 x 10^6	1.6 x 10^6

Table 5. Histamine producing bacteria counts (cfu/g) for fish samples collected from three fish markets located within Tripoli city, Libya

	Fish market (A)		Fish market (B)		Fish market (C)	
Fish market	**Range**	**Average**	**Range**	**Average**	**Range**	**Average**
Fish type						
Sardine	3.0 x 10^3 - 5.0 x10^6	9.8 x 10^4	5.0 x 10^2 – 8.2 x 10^4	1.9 x 10^4	10 x 10^2 – 2.1 x 10^5	5.1 x 10^4
Bouge	6.0 x10^2 - 1.1 x 10^6	2.0 x 10^5	2.0 x 10^3 – 2.8 x 10^5	7.2 x 10^4	8.0 x 10^3 – 2.6 x 10^6	5.7 x 10^5
Saury	10 x 10^3 – 1.5 x 10^4	3.9 x 10^4	3.0 x 10^3 – 1.3 x 10^6	1.7 x 10^5	1.1 x 10^4 - 2.6 x 10^6	6.0 x 10^6
Mackerel	5.0 x 10^2 - 5.7x 10^4	1.6 x 10^4	5.0 x 10^2 – 2.6 x 10^5	4.2 x 10^4	5.0 x 10^2 – 2.7 x 10^6	3.8 x 10^5

in samples collected during November and December with the percentages being 12.0 and 13.0%, respectively (Figure 1).

Table 6. Numbers and percent of samples (%) having total aerobic plate counts higher than the maximum limit (10^6 cfu/g fish meat) referred to by the standard specification adopted by the Libyan authority

Fish type	Fish market (A)	Fish market (B)	Fish market (C)	Total number of samples (%) > 10^6 cfu /g
Sardine	2 (7)*	1 (12)	2 (10)	5 (17%)
Bouge	1 (6)	9 (14)	3 (8)	13 (46%)
Saury	0 (6)	8 (13)	7 (11)	15 (50%)
Mackerel	0 (9)	7 (9)	3 (8)	10 (38%)

* Numbers between brackets indicate total number of sample examined.

It is clear from the results that the period of fishing has an important effect on the type of bacteria found in fish samples, as confirmed by Yoshinaga et al. (1982) and Kim et al. (2009). The results from this study are also in agreement with the results of the study conducted by Yagoub (2009) in Khartoum, Sudan on fresh fish, which showed that 53.3 of isolated bacteria belong to *Enterobacteriaceae* and the incidence percentages of species belonging to this family during Summer, Autumn and Winter were 60, 33 and 20%, respectively.

Correlation between temperature of fish samples and prevalence percentages (percentage) of HPB isolates

The results illustrated in Figure 2 indicate the incidence of *V. fluvialis, Erwinia spp, S. putrefaciens, P. aeruginosa, P. fluorscens and A. Baumanii* in all samples irrespective of their temperature. The prevalence percentages of these species were 23.0, 26.0, 12.0, 8.0, 4.0, and 8.0% of total isolates for fish samples recorded at

Table 7. The prevalence percentages (%) of histamine producing bacteria (HPB) in fish samples collected from three fish markets located within Tripoli city, Libya

Type of bacteria	Prevalence percentages (%) of HPB in the fish samples according to the markets included in the study			% from total isolates
	Fish market (A)	Fish market (B)	Fish market (c)	
V. fluvialis	23.70	17.70	24.30	18.30
Erwinia spp	13.40	9.70	17.40	13.20
S. putrefaciens	-	14.20	14.50	11.90
K. planticola	9.80	7.08	17.40	10.00
M. morganii	4.90	5.30	8.70	6.40
P. aeruginosa	-	7.96	5.79	5.90
A. baumanii	9.80	4.40	5.80	5.50
P. flouorscens	-	5.31	1.45	3.20
Pantoea spp	-	6.20	-	3.20
K. pneumonia	6.60	0.88	2.90	2.87
E. cloacae	-	1.80	5.80	2.58
A. hydrophila	3.30	3.50	-	2.18
P. mirabilis	-	2.70	1.50	1.78
O. anthropi	-	2.65	1.45	1.71
K. oxytoca	-	2.65	-	1.75
B. cepacia	-	2.65	-	1.30
S. plymuthica	8.10	-	-	0.90
Brucella spp	-	0.90	1.50	0.86
P. vulgaris	-	1.77	-	0.86
S. liquefaciens	4.30	-	-	0.86
S. maltophilia	-	0.90	1.45	0.86
R. aquatilis	-	1.88	-	0.86
P. alcalifacients	-	-	5.41	0.86
P. rettgeri	-	-	3.31	0.45
E. coli	-	-	3.30	0.45
C. freundii	1.45	-	-	0.45

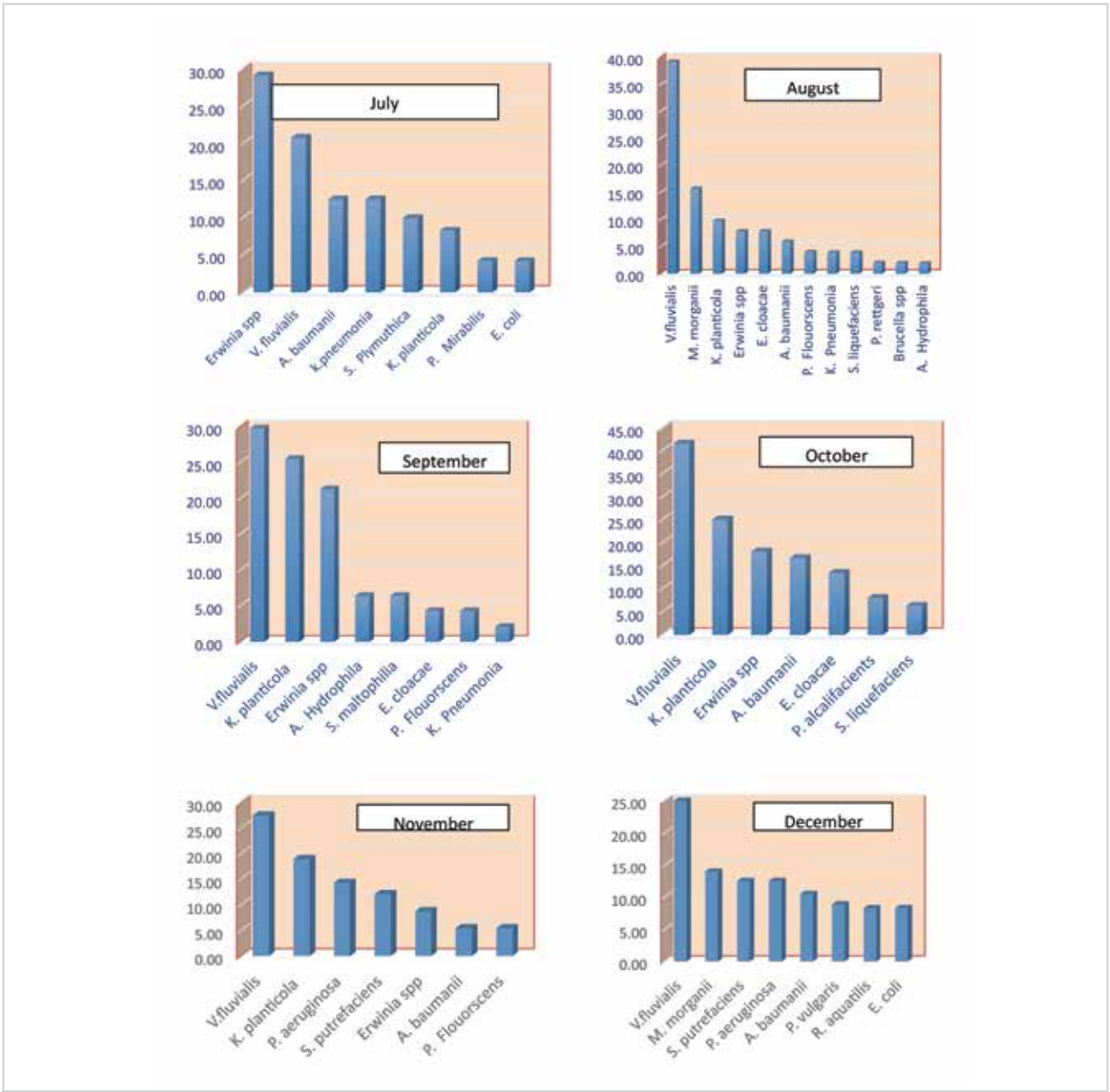

Figure 1. Prevalence percentages (%) of HPB in fish samples collected during the period from July to December

<5°C; 21.0, 8.0, 18.0, 9.0, 2.0, and 5.0% for fish samples recorded between 5 and 14°C; and 30.0, 15.0, 3.0, 2.0, 3.0, and 6.0% for fish samples which had a temperature range between 15 and 22°C, respectively. The Incidence of *M. morganii* was recorded only in fish samples recorded at 5-15 and 15-22°C with prevalence percentages of 8.0 and 11% respectively. The results also showed that *Erwinia spp* made up the highest percentage of isolates from fish samples that had a temperature of <5°C. *V. fluvialis* represented the highest percentage of isolates from fish samples that had a temperature range between 5 and 14^{0}C and 15 and 22°C with prevalence percentages of 20.7 and 30.11% respectively. Furthermore, the prevalence percentages of *Erwinia spp* were 8.49 and 15.12% for fish samples recorded at 5-14 and 15-22°C, respectively.

Conclusion

The results from this study showed that the samples of fish collected from fish markets in Tripoli city were displayed in poor refrigeration conditions. The higher percentage of samples not complying with the standard specification limit prescribed by

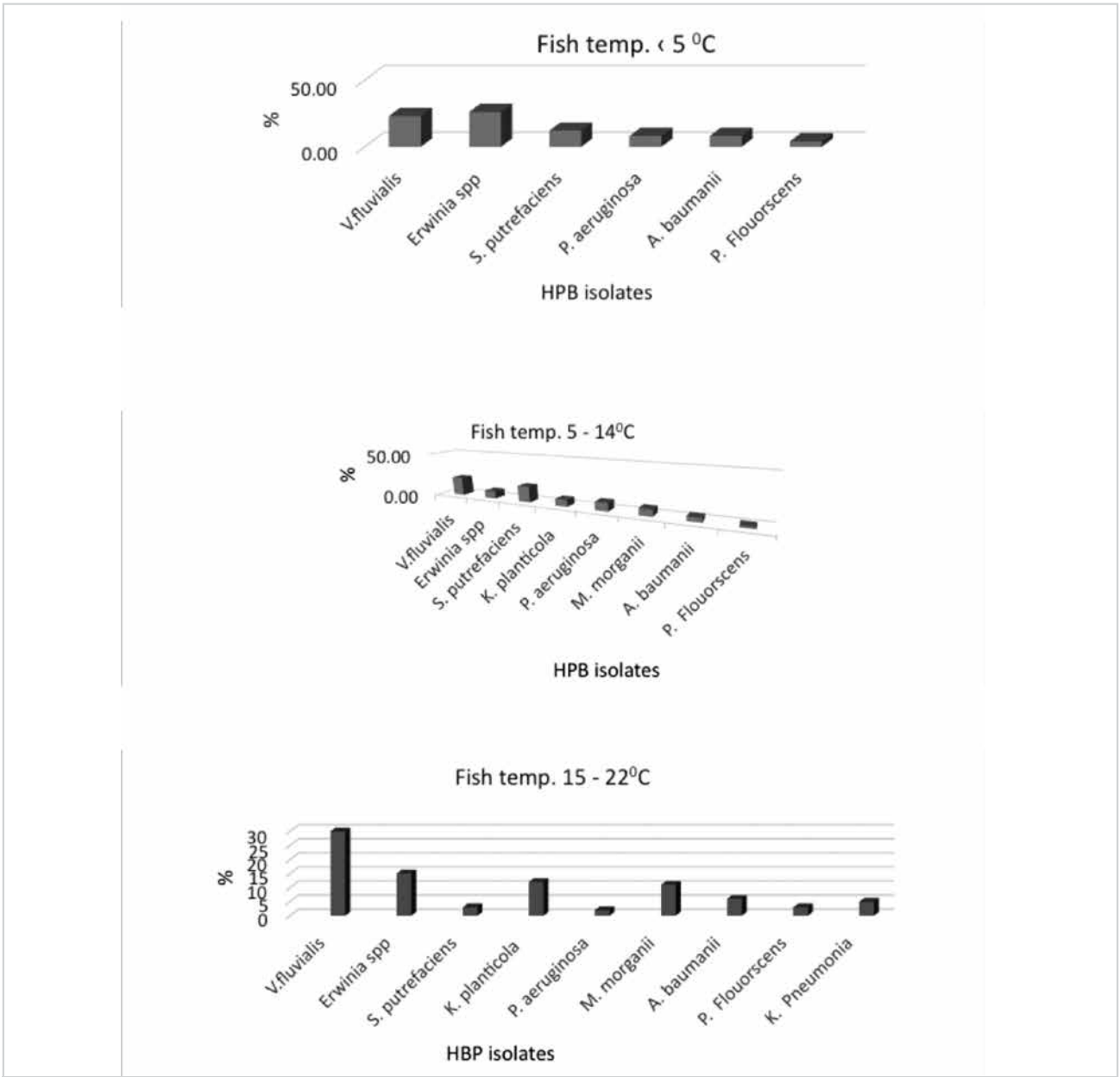

Figure 2. Prevalence percentages (%) of HPB isolates in fish samples according to fish Temperature

the Libyan authorities, and the higher HPBC recorded in this study could be attributed to cross-contamination from the surrounding environment and poor hygiene during handling. Most of the histamine-producing bacteria isolated belong to the family *Enterobacteriaceae* and some belong to *Vibrionaceae*.

These findings represent additional evidence to encourage proper control of handling conditions in those fish markets considered in this study. Since histamine cannot be destroyed by cooking, drying, smoking or freezing, good hygienic practices are the proper way to prevent histamine producing bacteria from growing in fish.

Ethics Committee Approval: This research has been planned and implemented taking into account the contents of the ethics of scientific research document that was issued by University of Tripoli under international number 9799959531551 and it was deposited at The national book house in Libya under a legal number 2017/155.

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A Case of Sebaceous Carcinoma Detected on the Eyelid of a Horse

Aydın GÜREL[1], Damla HAKTANIR[1], İbrahim KURBAN[2], Sevde İSLAMOĞLU[3], Serhat ÖZSOY[4]

[1]Department of Pathology, İstanbul University, Faculty of Veterinary Medicine, İstanbul, Turkey
[2]Equin and Training Programme, Vocational School of Veterinary Faculty, İstanbul University, İstanbul, Turkey
[3]Veterinary Faculty of İstanbul University, 3rd Year Student, İstanbul, Turkey
[4]Department of Surgery, İstanbul University, Faculty of Veterinary Medicine, İstanbul, Turkey

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ORCID IDs of the authors:ORCID IDs of the authors: A.G. 0000-0002-0268-7716; D.H. 0000-0001-5171-1927; İ.K. 0000-0002-8391-905X; S.Ö. 0000-0003-0988-7691

Abstract

This article describes a case of sebaceous carcinoma located on the right lower eyelid of an 11-year-old female Haflinger horse and its surgical, clinical, and histopathological aspects. The Haflinger horse was referred to the clinics of the Department of Surgery with a complaint of a swelling on the right lower eyelid, which had been present for 1 year and began to grow during the past few months. A clinical inspection revealed a soft, multinodular tumoral mass, with the dimensions of 4×5 cm, located in the inferior region of the right lower eyelid and protruding outward. The surgical removal of the mass was decided after the clinical inspection. The excised tumoral mass was submitted to the department of pathology for histopathological evaluation, which revealed well-circumscribed multilobular structures comprising foci of round-to-ovoid and polygonal pleomorphic neoplastic epithelial cells with prominent nuclei and eosinophilic cytoplasm separated by bands of the fibrous tissue of varying thickness. There was prominent cellular pleomorphism; some cells contained cytoplasmic vacuoles of various sizes, whereas some exhibited sebaceous differentiation. Based on these histopathological findings, sebaceous carcinoma of the sebocyte type was diagnosed.

Keywords: Equine, eyelid, histopathology, sebaceous carcinoma

Introduction

The most commonly encountered neoplastic lesions in horses are located in the skin, eye and genital system (Baptiste and Grahan, 2000; Knottenbelt, 2011; Montgomery, 2014). Ocular and periocular tumors of the horses are categorized according to their anatomical locations which are 1) Orbit, 2) Eyelid, adnexa/palpebral conjunctiva, membrana nictitans, 3) Cornea/sclera/bulbar conjunctiva and 4) Intraorbital regions (Lavach and Severin, 1977; Montgomery, 2014).

Tumors that are originated from the eyelid and conjunctiva are the most frequent ocular neoplasms in horses and most common ones are squamous cell carcinoma and sarcoids (Arıkan et al., 1997; Knottenbelt, 2011; Rooney and Robertson, 1996). Papilloma, melanoma and lymphoma though not as frequent as the above mentioned tumors also occur in these regions. Adenoma, adenocarcinoma, Meibomian epithelioma, angioma, basal cell carcinoma, fibroma, fibrosarcoma and mast cell tumors were also reported despite their rarity (Baker and Leyland, 1975; Baptiste and Grahan, 2000; Brooks, 1999; Choi et al., 2013; Cotchin, 1977; Giulino, 2011; Knottenbelt, 2011). While the majority of these tumors are primary tumors, secondary tumors such as lymphomas may be encountered in the eyelid or the orbita by metastases (Knottenbelt, 2011; Lavach and Severin, 1977; Montgomery, 2014; Rooney and Robertson, 1996). Ocular neoplasms may be grossly confused with infectious or non-infectious lesions such as foreign body reactions, conjunctival infections, trauma, and cystic glandular changes of the eyelid. Determina-

Address for Correspondence: Aydın GÜREL • **E-mail:** Agurel@istanbul.edu.tr
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tion of the prognosis and accurate and rapid treatment of these lesions depends on the definitive histopathological diagnosis (Knottenbelt, 2011; Montgomery, 2014).

A case of sebaceous carcinoma located on the right lower eyelid of a Haflinger horse was described with its surgical, clinical and histopathological aspects.

Case Report

An 11-year-old female Haflinger horse was referred to the clinics of the Department of Surgery with the complaint of a swelling on the right lower eyelid (Figure 1a). The swelling occurred for a year and started to grow during the last few months. Clinical inspection revealed a soft, multinodular tumoral mass with the dimensions of 4x5 cm, located in the inferior region of the right lower eyelid protruding outwards. The mass was restricted to the conjunctiva and the lesioned area was covered with a thick purulent exudate (Figure 1b). Surgical removal of the mass was decided after the clinical inspection. A wide excision was done to remove the total mass under local anesthesia (Figure 1c) Postoperatively, the conjunctiva and the affected area were treated topically with sterile serum physiologic solution and antibiotic pomade for 7 days. The patient was discharged on the 10th postoperative day (Figure 1d). The owner reported that there was no recurrence approximately 1 year after the treatment but the horse was tested positive for glanders and euthanized.

The excised tumoral mass was submitted to the Department of Pathology for histopathological evaluation. The specimen was fixed in 10% buffered formalin, routinely processed, embedded in paraffin and cut at about 4-5 µm thickness and then stained with Hematoxylin and eosin (HE) to be evaluated by light microscopy.

Histopathology revealed well circumscribed multilobular structures composed of foci of round to ovoid and polygonal pleomorphic neoplastic epithelial cells with prominent nuclei and eosinophilic cytoplasm. The tumoral mass was separated into lobular structures by bands of fibrous tissue of varying thickness. There was prominent cellular pleomorphism and some cells contained cytoplasmic vacuoles of various sizes and some showed sebaceous differentiation (Figure 2a-d). Some lobules showed wide central caseification necrosis (Figure 2b) and some contained inflammatory cell infiltrations including neutrophil leukocytes and mononuclear cells extending from the center to the interlobular areas (Figure 2a). These neoplastic epithelial cells were larger than basaloid reserve cells and euchromatic (Figure 2c). Moreover, foci of neoplastic cells showed squamous differentiation and metaplasia in some areas with the formation of keratin pearls. There was an average of 2-4 mitotic figures in the different foci of atypical

Figure 1. a-d. (a) Swelling on the right lower eyelid. (b) Prominent accumulation of purulent exudate in the lesioned area. (c) Multinodular structure of the surgically removed mass. (d) Postoperative appearance of the operation site

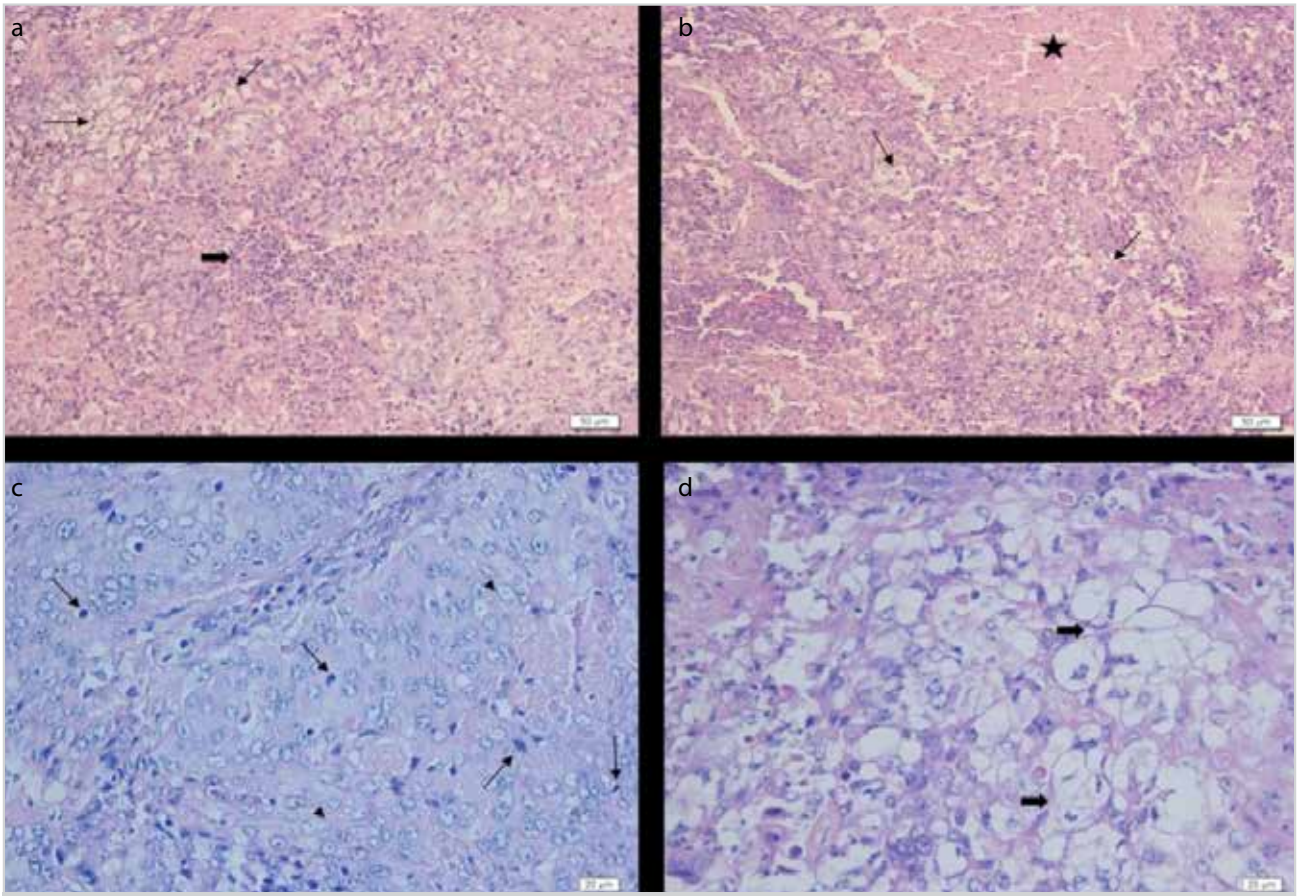

Figure 2. a-d. (a) Epithelial cells showing sebaceous differentiation (thin arrows), inflammatory reaction -lymphocyte and neutrophil leukocyte infiltration (thick arrow)-within the tumoral mass. H.E. Bar=50µm. (b) Epithelial cells showing sebaceous differentiation (thin arrows), tumor necrosis (star). H.E. Bar=50µm. (c) Basaloid cells with euchromatic nuclei of varying sizes (arrow heads), numerous mitotic figures (thin arrows). H.E. Bar=20 µm. (d) Different atypical cells showing sebaceous differentiation (thick arrows). H.E. Bar=20 µm

epithelial cells per high power field (40x) (Figure 2c). On the basis of histopathological features a diagnosis of sebaceous carcinoma of sebocytic type was rendered.

Discussion

Neoplastic lesions constitute 1-3% of all surgical cases in horses. (Arıkan et al., 1997; Cotchin, 1977). Ocular and periocular tumors account for approximately 10% of all equine tumors and around 80% show characteristics of malignancy (Baker and Leyland, 1975; Lavach and Severin, 1977; Montgomery, 2014). The most frequently seen tumors in this region are squamous cell carcinoma and sarcoids and although other types of tumors were occasionally reported (Baker and Leyland, 1975; Choi et al., 2013; Montgomery, 2014; Rachel et al., 2011; Rooney and Robertson, 1996), there is no data available with respect to the occurrence of sebaceous carcinoma originating from the conjunctiva of the horses. Therefore, this report was designed to be a contribution to the literature and described with its clinical and morphological features. To our knowledge this case was the first reported case of conjunctival sebaceous carcinoma in a horse in Turkey and even in Europe.

Sebaceous carcinoma which has been reported in cats and dogs and rarely encountered in other species is a malignant tumor originating from the sebaceous cells (namely meibomian cells) in the eyelid and is also referred to as meibomian carcinoma (Goldschmidt and Goldschmidt, 2017; Gross et al., 2015). Only one case of meibomian epithelioma has been previously reported in the eyelid of a horse (Choi et al., 2013). Since there is no report available regarding equine sebaceous carcinoma, the relevant case was comparatively evaluated based on the features of equine meibomian epithelioma and canine and feline sebaceous carcinomas of the eyelid.

Sebaceous carcinomas in dogs and cats are known to be originating from the sebaceous cells in the eyelid. In our case, we consider that epithelial cells of the tarsal gland in the eyelid (Meibomian gland) were the cellular origin of sebaceous carcinoma (Banks, 1986). The incidence of sebaceous carcinoma

among feline and canine skin tumors was reported to be approximately 0.7%. These tumors are usually solitary ulcerated nodular lesions that can reach up to 7-8 cm in diameter (Goldschmidt and Goldschmidt, 2017; Gross et al., 2015). Our case was a diffusely ulcerated multinodular mass with the dimensions of 4x6. While canine and feline sebaceous carcinomas were reported to have developed usually on the head and the eyelids (Goldschmidt and Goldschmidt, 2017; Gross et al., 2015), the most frequently affected site was reported to be the eyelids in their human counterparts (Gross et al., 2015). This type of tumors was reported to be locally invasive and aggressive entities with rare incidences of distant metastasis in cats and dogs. Local metastasis and lymph node involvement were also noted (Goldschmidt and Goldschmidt, 2017; Gross et al., 2015). On the contrary, sebaceous carcinomas usually recur in humans (Gross et al., 2015). Our case was a locally invasive tumor but there was no evidence of recurrence after one year follow-up. However the horse was euthanized due to glanders. The microscopic findings detected in our case were consistent with those of canine sebaceous carcinomas and partly included some of the histomorphological features of meibomian epithelioma reported in a horse (Choi et al., 2013; Goldschmidt and Goldschmidt, 2017; Gross et al., 2015). Sebaceous epithelioma, which is another type of tumor arising from sebaceous glands, may be confused with sebaceous carcinomas due to their mild malignant changes. Therefore, an accurate histopathological evaluation is of great importance ((Choi et al., 2013; Goldschmidt and Goldschmidt, 2017; Gross et al., 2015). In our case, severe cellular pleomorphic changes such as prominent anisocytosis and anisonucleosis in numerous basal cells, sebocytic differentiations in fewer cells and presence of mitotic figures led us to the diagnosis of sebaceous carcinoma. Sebaceous carcinomas are divided into two groups as sebocytic sebaceous carcinomas and epitheliomatous sebaceous carcinomas according to their histomorphological features. (Gross et al., 2015). Our case was a sebocytic type sebaceous carcinoma. The distinct histomorphological features of the tumor and its consistency with canine and feline sebaceous carcinomas (Goldschmidt and Goldschmidt, 2017; Gross et al., 2015) excluded the necessity of immunohistochemistry. While primary neoplasms on the eyelid are usually unilateral, secondary neoplasms show bilateral involvement (Konnetbelt, 2011). We consider that our case was a primary sebaceous carcinoma originating from the tarsal gland (sebaceous gland) of the eyelid (Banks, 1986).

Surgical excision, laser therapy, radiotherapy, cryotherapy and chemotherapy are the most commonly preferred and most efficient therapeutic approaches of the tumors of the eyelid and periocular region in horses (Arıkan et al., 1997; Brooks, 1999). In this case, our treatment of choice was the total surgical removal of the mass due to the well circumscribed nature of the tumor within the conjunctiva, the owner's refusal of chemotherapy, inconvenience of cryotherapy and the lack of possibility of laser therapy and radiotherapy.

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