ISSN 1300 - 6045
e-ISSN 1309 - 2251

# KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Journal of the Faculty of Veterinary Medicine, Kafkas University

Published Bi-monthly

http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Volume: 24 | Issue: 5 SEPTEMBER - OCTOBER | Year: 2018

ISSN: 1300-6045
e-ISSN: 1309-2251

# KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

## JOURNAL OF THE FACULTY OF VETERINARY MEDICINE, KAFKAS UNIVERSITY

(SEPTEMBER - OCTOBER)

Volume: 24 Issue: 5 Year: 2018

**This journal is indexed and abstracted by Thomson Reuters Services beginning with Volume 13 (1) 2007 in the followings:**

- **Science Citation Index Expanded (also known as SciSearch®)**
- **Journal Citation Reports/Science Edition**

**This journal is also indexed and abstracted in:**

- **Academic Search Premier**
- **CAB Abstracts**
- **DOAJ**
- **EBSCO**
- **Elsevier - Scopus**
- **EMBASE**
- **Thomson Reuters - Zoological Record**
- **TÜBİTAK - ULAKBİM Yaşam Bilimleri Veri Tabanı**
- **Türkiye Atıf Dizini**
- **Veterinary Science Database**

**YAZIŞMA ADRESİ (ADDRESS FOR CORRESPONDENCE)**

Kafkas Üniversitesi Veteriner Fakültesi Dergisi Editörlüğü
36040 - Kars / TÜRKİYE
Phone: +90 474 2426807-2426836/5228
Fax: +90 474 2426853
E-mail: vetdergi@kafkas.edu.tr

**ELEKTRONİK BASKI (ELECTRONIC EDITION)**
http://vetdergikafkas.org

**ONLINE MAKALE GÖNDERME (ONLINE SUBMISSION)**
http://submit.vetdergikafkas.org

Bu dergi Kafkas Üniversitesi Veteriner Fakültesi tarafından iki ayda bir yayımlanır
This journal is published bi-monthly, by the Faculty of Veterinary Medicine, University of Kafkas



**BASKI (Print)**
ESER OFSET MATBAACILIK
BOSNAHERSEK CAD. ALTUNALEM YAPI KOOP. ZEMİN KAT - ERZURUM
Tel: +90 442 2334667 E-mail: eserofset25@hotmail.com

## Bu Sayının Hakem Listesi *(alfabetik sıra)*
## The Referees List of This Issue *(in alphabetical order)*

| | |
|---|---|
| AKBAŞ Aykut Asım | Mehmet Akif Ersoy Üniversitesi, Veteriner Fakültesi |
| AKSOY Aksem | Kafkas Üniversitesi, Mühendislik Mimarlık Fakültesi |
| AKYOL İsmail | Kahramanmaraş Sütçü İmam Üniversitesi, Ziraat Fakültesi |
| ALBAYRAK Harun | Ondokuz Mayıs Üniversitesi, Veteriner Fakültesi |
| ALTUNBAŞ Korhan | Afyon Kocatepe Üniversitesi, Veteriner Fakültesi |
| AYAŞAN Tugay | Doğu Akdeniz Tarımsal Araştırma Enstitüsü |
| AYDIN Erol | Kafkas Üniversitesi, Veteriner Fakültesi |
| BARAN Vedat | Kafkas Üniversitesi, Veteriner Fakültesi |
| BEYTUT Enver | Kafkas Üniversitesi, Veteriner Fakültesi |
| BİLGİLİ Hasan | Ankara Üniversitesi, Veteriner Fakültesi |
| COŞKUN Alparslan | Cumhuriyet Üniversitesi, Veteriner Fakültesi |
| ÇALIK Ali | Ankara Üniversitesi, Veteriner Fakültesi |
| ÇARLI Tayfun | Uludağ Üniversitesi, Veteriner Fakültesi |
| ÇELEBİ Özgür | Kafkas Üniversitesi, Veteriner Fakültesi |
| DALĞIN Duygu | Ondokuz Mayıs Üniversitesi, Veteriner Fakültesi |
| DEPREM Turgay | Kafkas Üniversitesi, Veteriner Fakültesi |
| DURMUŞ Ali Said | Fırat Üniversitesi, Veteriner Fakültesi |
| ERAT Serkan | Kırıkkale Üniversitesi Veteriner Fakültesi |
| ERKAN Mehmet Emin | Dicle Üniversitesi, Veteriner Fakültesi |
| ERTÜRK Mehmet Mustafa | Akdeniz Üniversitesi, Ziraat Fakültesi |
| ESİN Evren | Çukurova Üniversitesi, Ceyhan Veteriner Fakültesi |
| EVRIGH Nemat Hedayet | University of Mohaghegh Ardabili, Iran |
| GÖKÇE Ramazan | Pamukkale Üniversitesi, Mühendislik Fakğltes |
| GÜL SANCAK İrem | Ankara Üniversitesi, Veteriner Fakültesi |
| GÜMÜŞOVA Semra | Ondokuz Mayıs Üniversitesi, Veteriner Fakültesi |
| GÜRLER Hande | Ondokuz Mayıs Üniversitesi, Veteriner Fakültesi |
| GÜVENÇ Dilek | Ondokuz Mayıs Üniversitesi, Veteriner Fakültesi |
| HALICI Zekai | Atatürk Üniversitesi, Tıp Fakültesi |
| İNAL Şeref | Selçuk Üniversitesi Veteriner Fakültesi |
| KALAYCIOĞLU Atilla Taner | Karadeniz Teknik Üniversitesi, Eczacılık Fakültesi |
| KAMALAK Adem | Kahramanmaraş Sütçü İmam Üniversitesi, Ziraat Fakültesi |
| KARAYİĞİT M. Önder | Cumhuriyet Üniversitesi, Veteriner Fakültesi |
| KENAR Beytullah | Afyon Kocatepe Üniversitesi, Veteriner Fakültesi |
| KILIÇ Servet | Namık Kemal Üniversitesi, Veteriner Fakültesi |
| KURAR Ercan | Selçuk Üniversitesi, Veteriner Fakültesi |
| KUTSAL Osman | Ankara Üniversitesi, Veteriner Fakültesi |
| LAÇİN Ekrem | Atatürk Üniversitesi, Veteriner Fakültesi |
| MİDİLLİ Mustafa | Bolu Abant İzzet Baysal Üniversitesi, Tarım Bilimleri Fakültesi |
| MURATOĞLU Karlo | İstanbul Üniversitesi - Cerrahpaşa, Veteriner Fakültesi |
| OK Mahmut | Selçuk Üniversitesi, Veteriner Fakültesi |
| OTLU Salih | Kafkas Üniversitesi, Veteriner Fakültesi |
| ÖZAK Ahmet | Ondokuz Mayıs Üniversitesi, Veteriner Fakültesi |
| ÖZEN Hasan | Balıkesir Üniversitesi, Veteriner Fakültesi |
| PALABIYIK Sezin | Atatürk Üniversitesi, Eczacılık Fakültesi |
| POLAT Bülent | Atatürk Üniversitesi, Veteriner Fakültesi |
| SABUNCUOĞLU ÇOBAN Nilüfer | Atatürk Üniversitesi, Veteriner Fakültesi |
| SEVİMLİ Alper | Afyon Kocatepe Üniversitesi, Veteriner Fakültesi |
| ŞEKER İbrahim | Fırat Üniversitesi, Veteriner Fakültesi |
| ŞENTÜRK Berrin | Ondokuz Mayıs Üniversitesi, Veteriner Fakültesi |

## Bu Sayının Hakem Listesi *(alfabetik sıra)*
## The Referees List of This Issue *(in alphabetical order)*

| | |
|---|---|
| TEKİN Mehmet Emin | Selçuk Üniversitesi, Veteriner Fakültesi |
| UÇAN Uçkun Sait | Selçuk Üniversitesi, Veteriner Fakültesi |
| UYARLAR Cangir | Afyon Kocatepe Üniversitesi, Veteriner Fakültesi |
| ÜNSALDI Emine | Fırat Üniversitesi, Veteriner Fakültesi |
| YAĞCI Artay | İstanbul Üniversitesi - Cerrahpaşa, Veteriner Fakültesi |
| YALÇIN Sakine | Ankara Üniversitesi, Veteriner Fakültesi |
| YAŞAR Aşkın | Selçuk Üniversitesi, Veteriner Fakültesi |
| YAVUZ Handan Hilal | Ondokuz Mayıs Üniversitesi, Veteriner Fakültesi |
| YAYLA Sadık | Kafkas Üniversitesi, Veteriner Fakültesi |
| YILDIRIM Yakup | Mehmet Akif Ersoy Üniversitesi, Veteriner Fakültesi |
| YILDIZ Ahmet | Atatürk Üniversitesi, Veteriner Fakültesi |
| YİĞİT Ali | Kafkas Üniversitesi, Veteriner Fakültesi |
| ZIK Berrin | Uludağ Üniversitesi, Veteriner Fakültesi |

# İÇİNDEKİLER (Contents)

Kafkas Univ Vet Fak Derg
24 (5): 633-638, 2018
DOI: 10.9775/kvfd.2018.19415

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# The Impact of Periodontal Disease on the Heart and Kidneys in Dogs

Izabela POLKOWSKA [1] Aleksandra SOBCZYŃSKA-RAK [1] Tomasz SZPONDER [1]
Beata ŻYLIŃSKA [1] Urszula ORZĘDAŁA-KOSZEL [2] Igor CAPIK [3] Łukasz MATUSZEWSKI [4]

[1] University of Life Sciences in Lublin, Department and Clinic of Animal Surgery, Lublin, POLAND
[2] Medical University of Lublin, Chair and Departament of Oral Surgery, Lublin, POLAND
[3] University of Veterinary Medicine and Pharmacy in Kosice , Small Animals Clinic, Kosice, SLOVAKIA
[4] Medical University of Lublin, Children's Orthopaedic Clinic and Rehabilitation Department, Lublin, POLAND

**Article Code: KVFD-2018-19415 Received: 30.01.2018 Accepted: 25.07.2018 Published Online: 27.07.2018**

**How to Cite This Article**

**Polkowska I, Sobczyńska-Rak A, Szponder T, Żylińska B, Orzędała-Koszel U, Capik I, Matuszewski Ł:** The impact of periodontal disease on the heart and kidneys in dogs. *Kafkas Univ Vet Fak Derg,* 24 (5): 633-638, 2018. DOI: 10.9775/kvfd.2018.19415

## Abstract

Periodontal disease is one of the most common canine diseases that can have serious systemic consequences. The aim of the study was the identification of the bacterial microflora causing periodontitis as well as its influence on the pathological changes in heart and kidneys. The study was performed on the group of 19 dogs (10 males and 9 females, aged between 6-15 years) sectioned at the Pathological Anatomy Department in Lublin, Poland. In all dogs the periodontal disease (third stage in 5 dogs and the fourth stage in 14 dogs) was diagnosed. A culture of aerobic bacteria from clinical material (swab of gingival, heart and kidneys) and histopathological examination on heart and kidney were performed. In the material the presence of bacteries such as: *Escherichia coli, Streptococcus* spp., *Streptococcus pyogenes, Streptococcus equi, Staphylococcus epidermidis, Staphylococcus* spp. and *Corynebacterium* spp. The results of this study showed the advanced pathological changes in kidney glomeruli and the anterior wall of the left heart in all animals, especially in the dogs with the fourth stage of periodontitis. In histopathological examination of preparations from the anterior wall of the left heart ventricle the intense interstitial cardiac oedema and interstitial fibrosis was diagnosed. Additionally, subepicardially between the muscle fibres and around the vessels single adipocytes were found. The study reveals the important correlations between chronic inflammatory lesions within periodontal tissues and heart and kidneys.

***Keywords:*** *Periodontal disease, Dog, Bacteriological culture, Heart, Histopathological examination, Kidney*

# Köpeklerde Kalp ve Böbrekler Üzerine Periodontal Hastalığın Etkisi

## Öz

Periodontal hastalık sistemik sonuçları olabilen en yaygın köpek hastalıklarından biridir. Bu çalışmanın amacı periodontitise neden olan bakteriyel mikrofloranın tespiti ve aynı zamanda kalp ve böbreklerdeki patolojik değişiklikler üzerine etkilerini araştırmaktır. Çalışma Lublin, Polanya'daki Patolojik Anatomi Departmanında disekte edilen 19 köpek (10 erkek ve 9 dişi, 6-15 yaşlı) üzerinde gerçekleştirildi. Tüm köpeklerde (5 köpekte 3. seviyede ve 14 köpekte 4. seviyede) periodontal hastalık tespit edildi. Klinik materyalden (diş eti, kalp ve böbreklerden svab) aerobik bakteri kültürü yapıldı ve kalp ile böbreklerde histopatolojik muayene gerçekleştirildi. Materyallerde tespit edilen bakteriler: *Escherichia coli, Streptococcus* spp., *Streptococcus pyogenes, Streptococcus equi, Staphylococcus epidermidis, Staphylococcus* spp. ve *Corynebacterium* spp. Çalışmanın sonuçları tüm köpeklerin, özellikle dördüncü seviye periodontitisli olan, böbrek glomeruluslarında ve sol kalbin anterior duvarında şiddetli patolojik değişiklikler bulunduğunu gösterdi. Kalbin sol ventrikülünden hazırlanan preparatların histopatolojik muayenesinde şiddetli interstisyel kardiyak ödem ve interstisyel fibrozis tespit edildi. Kas fibrilleri arasında ve damar çevresinde tek adipositler gözlemlendi. Bu çalışma periodontal dokulardaki kronik yangısal lezyonlar ile kalp ve böbreklerdeki lezyonlar arasında önemli bağlantı olduğunu göstermektedir.

***Anahtar sözcükler:*** *Periodontal hastalık, Köpek, Bakteriyolojik kültür, Kalp, Histopatolojik muayene, Böbrek*

## INTRODUCTION

The oral cavity in human and animal is a reservoir of pathogenic bacteria that cause not only local infections, but also systemic diseases [1]. A number of human and canine disease affecting organs located outside the oral cavity may be initiated by inflammatory mediators, toxaemia or recurring bacteraemia caused by chronic periodontal diseases-PD [2]. Chronic periodontal disease is one of the most dangerous chronic infection in dogs. It has been observed that 85% of dogs over three years of age suffer from periodontopathic lesions of varying

**İletişim (Correspondence)**
+48 81 4456193
olsob2@gmail.com

intensity [3-5]. Peridonthopathy or chronic periodontal disease is described as an inflammatory condition that affects tissues surrounding and supporting the teeth. The underlying cause of this disease is bacterial infection.

It is generally accepted that the mouth of a dog, with its steady temperature, humidity and recesses that cannot be self-cleaned, offers good conditions for the proliferation of microorganisms. The inflammatory process results in damage to the gingival attachment and the penetration of bacteria and their toxins into the circulatory system. In many dogs, periodontal disease remains untreated for many years, and the resulting chronic inflammation may be conductive to permanent bacteraemia [6]. The inflammatory process taking place in the mouth, and especially in the periodontium, is the same as in other body parts and causes an acute or chronic inflammatory response [7].

Research conducted in the field of dentistry yields substantial information about the cause and effect relationship between periodontopathies and the risk factors for cardiovascular diseases, renal diseases and diabetes [8,9]. Until the early 20th century, focal diseases of dental origin were not a particular point of interest to dentists and general practitioners. It was only after the death of President Theodore Roosevelt in 1919 due to odontogenic sepsis that researchers became interested in the topic [10]. Currently it is generally accepted that the mechanism of inflammatory-allergic reactions in the endocardium, kidneys, skin and joints involves tissue sensitization by bacterial antigens from inflammatory foci in the mouth [11].

Microbiological examinations allow us to determine the microbiological profile of odontogenic infections, which are usually of a multibacterial character, with mixed aerobic-anaerobic flora. The pathogens which usually initiate the infectious process are aerobic bacteria, mainly streptococci, which penetrate into the deeper layers of the tissues and initiate the inflammatory process [12].

The immune system of the animal can prevent these bacterial infections thanks to local barriers as well as humoral and cellular immune responses. Undamaged layers of the skin and mucosa prevent the penetration of bacteria deeper into the tissues. In the mouth, the protective barrier is broken more often than in other places of the body due to periodontal disease, tooth extraction, periapical abscess or mechanical injuries in this area [13]. It has been shown that pathogenic periodontal bacteria may penetrate to the periodontal tissues and then to the blood stream, through which they may reach distant organs [14].

The aim of the study was the identification of the bacterial microflora causing periodontitis as well as its influence on the pathological changes in heart and kidneys

## MATERIAL and METHODS

Among 210 dogs treated dentally for periodontal disease between 2006 and 2010, at the Department and Clinic of Animal Surgery of the Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Poland, fifty-six dogs were diagnosed with stage 3 or 4 periodontal disease. The age of these 56 dogs ranged between 6 and 15 years (37 male, 19 female).

All study protocols were approved by the Local Ethics Committee of the University of Life Sciences in Lublin (No. 15/2009, 10 II 2009). Dogs qualified for the study were selected relative to systemic diseases based on medical history and clinical examination. Patients suffering from diseases of the endocrine system, cancer or generalized infectious diseases were excluded from the study.

The periodontal health status of each dog was determined following the Wiggs & Lobprise scoring system [15]. The diagnosis of the stage of the disease was made on the basis of clinical and radiological examinations. The following parameters were assessed: connective tissue attachment (the distance from the cemento-enamel-junction to the bottom of the pocket) and periodontal pocket depth. The examination of these indicators was performed with the use of a calibrated periodontal probe.

All the patients were provided with periodontal treatment appropriate for their respective stage of periodontal disease. During the 4-year observation and ongoing treatment period, a study group *(post mortem* group) was selected, comprising 19 dogs (10 males and 9 females) which underwent euthanasia due to poor general condition. The group consisted of 5 dogs in the third stage and 14 dogs in the fourth stage of the disease. From the gingival margin of each dog (targeting the teeth believed to be most often affected by PD: upper 103,104, 108 at right side and lower 404, 408 and 409 at right side) swabs were taken to perform microbiological examinations. In order to specify what kind of bacterial flora is responsible for periodontitis in the selected group of 56 dogs material was collected from the gingival margin by sterile swab friction and scraping with a sterile curette.

The pathomorphological and bacteriological examination was performed on the dogs condemned to euthanasia for medical reasons.

During the necropsy, swab material was collected once more from the gingival margin additionally from heart and kidney of the dogs. The samples were inoculated on solid media (Columbia Agar with Sheepblood), McConkey Agar, Enterococci Bile Azide Agar, Sabouraund Glucose Selective Agar, Mannitol Salt Agar (Chapman). The plates were incubated at 35-36ºC for 24-48 h. Bacteria were at first identified by colony morphology, haemolytic pattern and Gram staining (Color Gram 2 kit, bioMérieux, F-69280

Marcy-l'Etoile, France). For Gram-positive cocci, a catalase test was carried out to differentiate catalase-negative streptococci from catalase-positive staphylococci. A CAMP (Christie, Atkins, Munch-Petersen) test were used to differentiate *Str. agalactiae* from other streptococci. Enterococci were initially identified with the use of bile esculin agar (Biocorp, Parc technologique Lavaur la Béchade 63500 Issoire, France). Gram-negative bacilli were identified based on the cultural and morphological characteristics, growth on MacConkey agar (Oxoid, Wade Road Basingstoke Hampshire RG24 8PW United Kingdom), indole production and oxidase test (bioMérieux, Marcy-l'Etoile, France) [16]. Coryneform bacteria were initially identified based on colony morphology and microscopic view. When pure cultures were isolated, the further identification was performed using the diagnostic tests GP and AST -P644, and in the case of *Streptococcus pyogenes/equi* GP and AST- ST03 with the use of bioMerieux Poland sets (VITEK® MS microbial identification system) [17,18].

During the post mortem examination, samples were also taken from the anterior wall of the left ventricle (a section through the whole thickness of the ventricle) and kidney. Collected tissues were fixed in 10% buffered formalin (pH 7.2) for 24 (48 h) and embedded in paraffin blocks which were used to obtain 4 µm thick samples. Standard hematoxylin and eosin (HE) stain was used, as well as a specific staining: periodic acid-Schiff reaction (PAS) and Van Gieson stain, Masson's Trichrome staining, silver staining of reticular fibres [19] and the Nielsen-Selye staining method were used to evaluate recent cardiomyocyte necrosis [20]. The histopathological examination was performed at the Department of Clinical Pathomorphology of the Medical University of Lublin.

### *Statistical Analysis*

The $\chi^2$ test was used to determine whether the aerobic bacteria counts in the heart, kidneys and gingiva were the same or different in patients in the third and fourth stages of periodontal disease.

With a statistical significance of $P<0.05$, the differences between the studied groups were significant. The authors used the STATISTICA 8.0 software for calculations.

## RESULTS

### *Culture*

Bacteria most commonly isolated from the gingival margin in dogs from study group dignosed with third stage of PD included *Streptococcus* spp., *Escherichia coli,* and *Staphylococcus epidermidis*. Bacteria isolated from kidney tissue most commonly included: *Streptococcus* spp., and *Staphylococcus* spp; and from the anterior wall of the left heart ventricle: *Staphylococcus epidermidis*, *Streptococcus spp.*, and *Streptococcus pyogenes* (Table 1).

**Table 1.** *Results of the bacteriological examination of the gingival margin, kidney and heart of patients in the third stage of periodontal disease in the study group (post mortem)*

| Isolated Microorganisms (CFU/mL) | Gingival Margin | Kidney | Heart |
|---|---|---|---|
| *Escherichia coli* | 14 | 1 | 0 |
| *Streptococcus* spp. | 16 | 3 | 2 |
| *Streptococcus pyogenes* | 1 | 1 | 2 |
| *Streptococcus equi* | 0 | 1 | 1 |
| *Staphylococcus epidermidis* | 3 | 1 | 3 |
| *Staphylococcus* spp. | 2 | 2 | 0 |
| *Corynebacterium* spp. | 1 | 1 | 0 |
| Total | 37 | 10 | 8 |

**Table 2.** *Results of the bacteriological examination of material from the gingival margin, kidney and heart in patients in the fourth stage of periodontal disease in the study group (post mortem)*

| Isolated Microorganisms (CFU/mL) | Gingival Margin | Kidney | Heart |
|---|---|---|---|
| *Escherichia coli* | 5 | 2 | 1 |
| *Streptococcus* spp. | 17 | 4 | 6 |
| *Streptococcus pyogenes* | 7 | 3 | 5 |
| *Streptococcus equi* | 2 | 3 | 4 |
| *Staphylococcus epidermidis* | 5 | 3 | 7 |
| *Staphylococcus* spp. | 5 | 6 | 0 |
| Total | 41 | 21 | 23 |

**Table 3.** *Number of microorganisms (CFU/mL) isolated from the gingival margin, kidney and heart in the study group (post mortem)*

| Sample | Third Stage of Periodontal Disease | Fourth Stage of Periodontal Disease |
|---|---|---|
| Gingival margin | 37 | 41 |
| $\chi^2=0.21$ (-) | P>0.05 | |
| Kidney | 10 | 21 |
| $\chi^2=3.84$ (*) | P<0.05 | |
| Heart | 8 | 23 |
| $\chi^2=7.06$ (**) | P<0.01 | |

Bacteria most commonly isolated from the gingival margin in dogs from study group diagnosed with fourth stage of PD included: *Streptococcus* spp., *Streptococcus pyogenes, Staphylococcus epidermidis*, and *Staphylococcus* spp. Bacteria isolated from the kidneys were *Staphylococcus* spp., *Streptococcus* spp., *Streptococcus pyogenes, Streptococcus equi,* and *Staphylococcus epidermidis;* while bacteria from the anterior wall of the heart ventricle were- *Staphylococcus epidermidis*, *Streptococcus* spp., *Streptococcus pyogenes,* and *Streptococcus equi* (Table 2).

No significant difference was observed in terms of the number of bacteria isolated from the periodontal pockets in the third and fourth stages of periodontal disease. The

percentages of bacteria in both groups were similar, approx. 43.4% in the third stage and 56.6% in the fourth stage [$\chi^2$=2.76 ; P>0.05].

The number of bacteria isolated from the kidneys was significantly higher in patients in the fourth stage of periodontal disease-65.91%, compared to 34.09 % in the group of third stage animals [$\chi$2=4.45; P<0.05].

The $\chi^2$ test revealed that microorganisms isolated from the dogs' cardiac tissues occurred much more frequently in the group in the fourth stage of periodontal disease-70.59%, compared to 29.41% in patients in the third stage of periodontal disease [$\chi^2$=5.76; P<0.01].

The statistical analysis of bacterial counts in the periodontal pockets in the third and fourth stages of periodontal disease has been shown in *Table 3*. Together with results of number of bacteria in heart and kidneys.

### *Histopathological Examination on Heart and Kidney Samples Obtained from Dogs in The Study Group*

In the standard hematoxylin and eosin (HE) stain of the material collected from the anterior wall of the left ventricle, single cardiomyocytes with a more homogeneous eosinophilic character were observed focally, with the disappearance of transverse striation and hyperchromatic nuclei (third and fourth stage). In fourth stage between the muscular fibres and around vessels mononuclear cells -limfocytes, either single or in small groups, were visualised *(Fig. 1)*. However, the Nielsen-Selye staining method revealed segmental necrosis of single cardiomyocytes in several samples *(Fig. 2)*. The Van Gieson's and Masson's Trichrome stains revealed fragmentation of the cardiac fibres and subepicardial parenchymal fibrosis *(Fig. 3)*. Subepicardially, between the muscle fibres and around the vessels, single adipocytes were found. The lesions were characteristic of endocarditis.

In the material collected from the kidneys, focal infiltrations of mononuclear cells (located near renal tubules and glomeruli) were observed *(Fig. 4)*. Foci of intensified interstitial hyalinization, dilated tubules with homogeneous fluid, tubule atrophy (the so-called "thyroid-like area"), intense infiltrations of mononuclear cells (usually in clusters), numerous solidifying and hyalinising glomeruli with clearly marked periglomerular fibrosis were also observed *(Fig. 5)*. The identified pathomorphological lesions were characteristic of pyelonephritis.

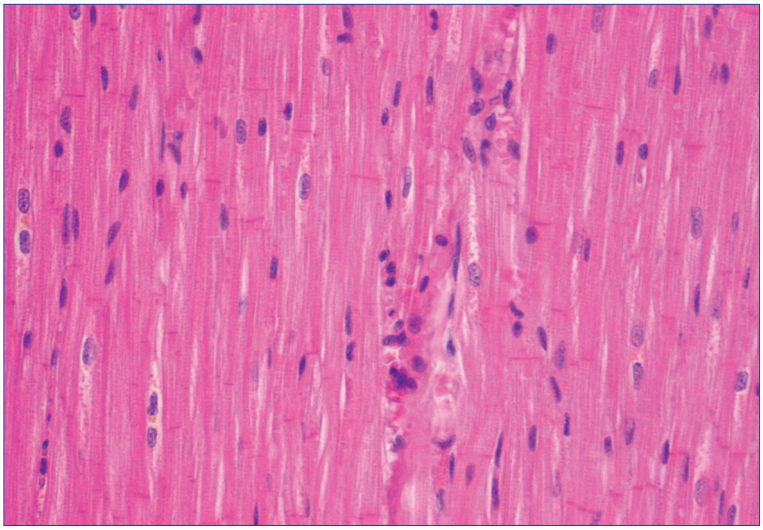


**Fig 1.** Mononuclear cells between cardiomyocytes. HE staining (20X)

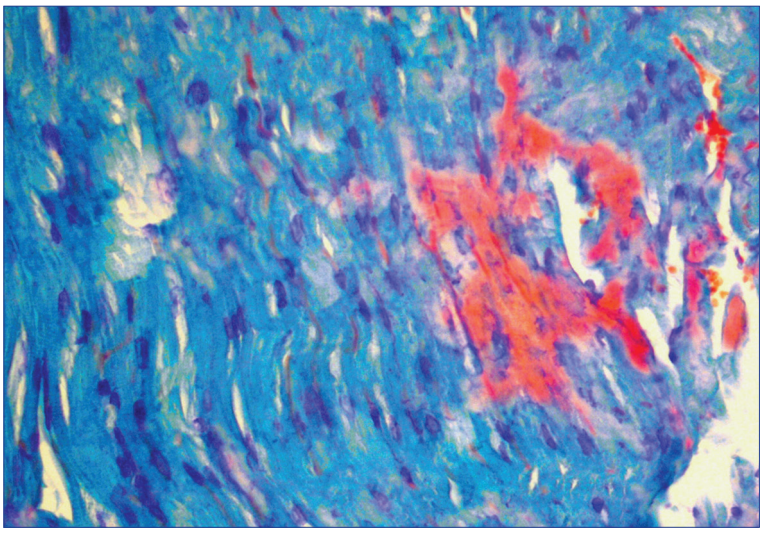


**Fig 2.** Cardiomyocyte necrosis. Nielsen- Selye's staining (20X)

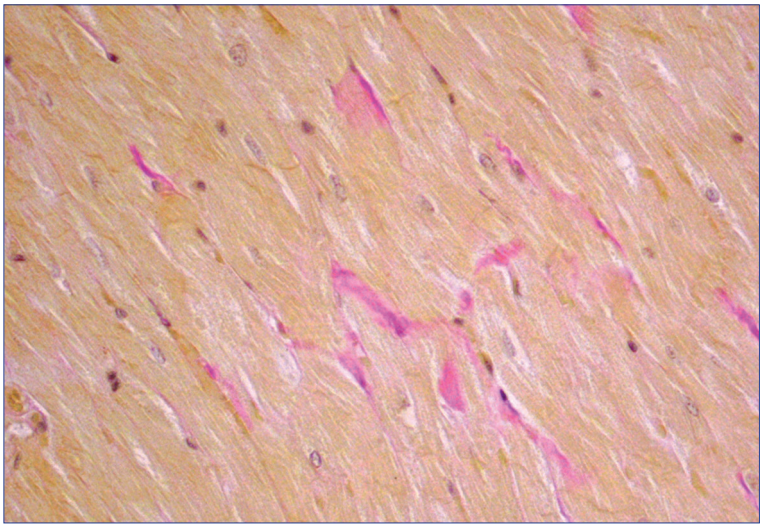


**Fig 3.** Connective tissue - red-stained between cardiomyocytes. Interstitial fibrosis. Periodic acid-Schiff reaction (PAS) and Van Gieson stain (20X)

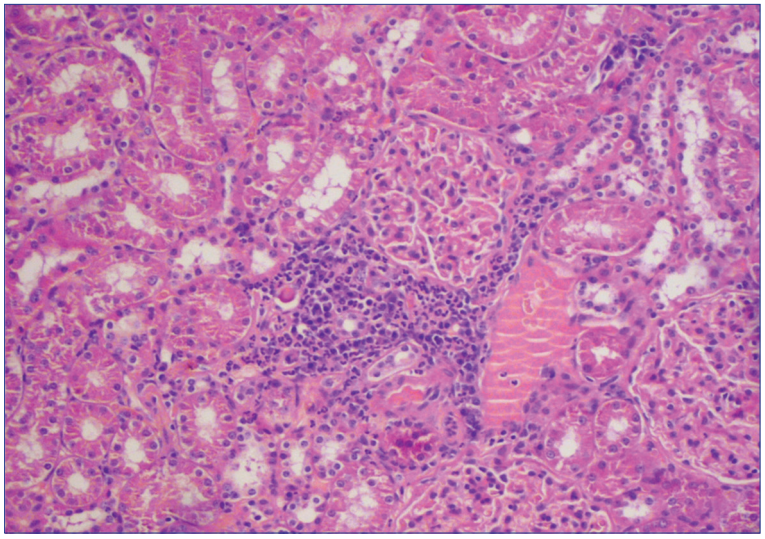


**Fig 4.** Focal infiltrations of mononuclear cells. HE staining (20X)

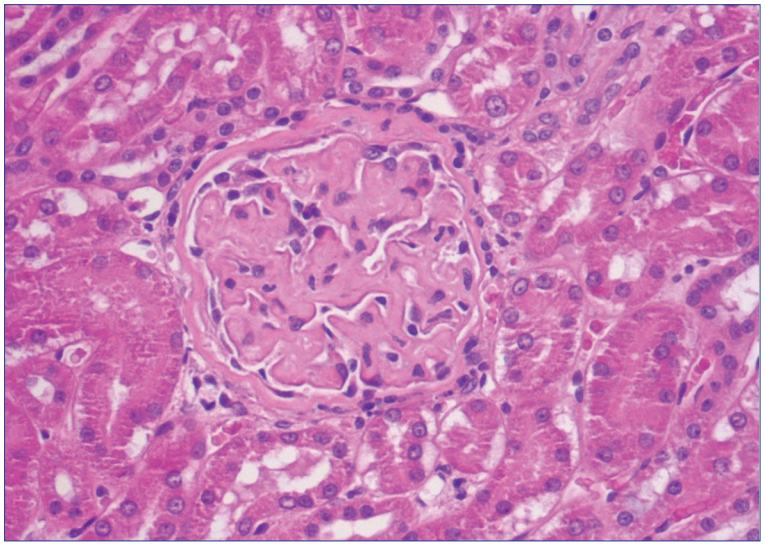


**Fig 5.** Glomerular hyalinization and periglomerular fibrosis. HE staining (20X)

## DISCUSSION

The most common chronic bacterial infection, both in humans and dogs is periodontal disease. *Gingivitis* is an initial and reversible form of periodontopathy which, when untreated, leads to *periodontitis* and, in consequence, to the destruction of the alveolar bone. Identification of bacteria associated with periodontopathy and seeking evidence for this association, as well as with generic diseases, is a subject of both dental and general medical research. There are some premises allowing the possibility to associate periodontitis with the pathophysiology of chronic systemic inflammatory diseases. Ongoing research indicates that long-term untreated periodontopathies negatively affect animal health [6,21].

Nowadays, periodontopathies are not considered a problem related solely to the oral cavity. Recent research results indicate that there is an association between periodontitis and systemic diseases, such as cardiovascular diseases, kidney diseases, atherosclerosis, and respiratory system diseases in dogs [22,23]. Immunological kidney diseases, especially glomerulonephritis, are considered a potential result of chronic bacteraemia and toxaemia associated with periodontal disease in humans and dogs [22-24]. Pavlica, in his study on 44 poodles, identified a correlation between periodontal disease and renal impairment, indicating the impact of periodontopathies on occurring lesions, probably caused by repeated or chronic infections [6]. Other researchers also confirmed these results in their studies in canines [25,26]. The 4-year results of the authors' own studies performed on a group of dogs with periodontal disease confirm Pavlica's observations stating that glomerulonephritis was associated with a sustained inflammatory factor in the oral cavity. It could be concluded that, in the studied dogs, frequent bacteremia occurred due to repeated minor injuries in the area of the affected periodontium. In our own research the lesions identified by anatomopathological examination prove the development of a chronic glomerulonephritis associated with an oral cavity infection. The studies show important correlations between chronic inflammatory lesions in periodontium and increases in the number of microorganisms in the kidney and heart tissues. These results may be a confirmation that periodontopathies affect not only the teeth but also can have a negative influence on the whole organism, leading to systemic diseases.

It was indicated that in risk group humans, repeated bacteraemia in the oral cavity may constitute an important causal factor of infective endocarditis or pyelonephritis [27-29]. Increasingly often, periodontopathies are regarded as an independent risk factor for ischemic heart disease [30]. Studies have showed that in a group of people with chronic periodontitis, the risk of developing ischemic heart disease is twice as high as in healthy people [31].

In dogs statistical analysis of our results revealed that the microorganism counts in the periodontal pockets in the third and fourth stages of periodontal disease did not differ significantly. However, a significant increase in the number of microorganisms was noted with exacerbation of inflammatory processes in the material collected from the hearts and kidneys. The results show that the development and advancement of periodontal disease significantly increases the number of bacteria in distant organs, such as the heart and kidneys.

In conclusion, the results gained from the above study confirm the association between periodontopathies and the pathology of organs; however, other factors should also be taken into account, such as: age of the animal, nutritional problems and compromised immune system, which may at the same time affect both the development of periodontopathy and the pathology of remote organs. Despite credible traces, irrefutable evidence for the direct impact of peridontopathologies on the development of diseases of remote organs is still being sought.

## REFERENCES

**1. Lommer MJ:** Oral inflammation in small animals. *Vet Clin North Am Small Anim Pract,* 43 (3): 555-571, 2013. DOI: 10.1016/j.cvsm.2013.02.004

**2. Rawlinson JE, Goldstein RE, Reiter AM, Attwater DZ, Harvey CE:** Association of periodontal disease with systemic health indices in dogs and the systemic response to treatment of periodontal disease. *J Am Vet Med Assoc,* 238 (5): 601-609, 2011. DOI: 10.2460/javma.238.5.601

**3. Harvey CE, Shofer FS and Laster L:** Association of age and body weight with periodontal disease in North American dogs. *J Vet Dent*, 11 (3): 94-105, 1994.

**4. Hoffmann TH, Gaengler P:** Clinical and pathomorphological investigation of spontaneously ocuring periodontal disease in dogs. *J Small Anim Pract,* 37, 471-479, 1996. DOI: 10.1111/j.1748-5827.1996.tb01743.x

**5. Kouki M, Papadimitriou SA, Kazasko GM, Savas I, Bitchava D:** Periodontal disease as a potential factor for systemic inflammatory response in the dog. *J Vet Dent,* 30, 26-29, 2013. DOI: 10.1177/089875641303000103

**6. Pavlica Z, Petelin M, Juntes P, Erzen D, Crossley DA, Skaleric U:** Periodontal disease burden and pathological changes in organs of dogs. *J Vet Dent,* 25 (2): 97-105, 2008. DOI: 10.1177/089875640802500210

**7. Linden GJ, Lyons A, Scannapieco FA:** Periodontal systemic associations: Review of the evidence. *J Clin Periodontol*, 40 (14 Suppl): S8-S19, 2013. DOI: 10.1111/jcpe.12064

**8. Scannapieco FA:** Systemic effects of periodontal disease. *Dent Clin North Am,* 49 (3): 533-550, 2005. DOI: 10.1016/j.cden.2005.03.002

**9. Ricardo AC, Athavale A, Chen J, Hampole H, Garside D, Marucha P, Lash JP:** Periodontal disease, chronic kidney disease and mortality: Results from the third national health and nutrition examination survey. *BMC Nephrol,* 16:97, 2016. DOI: 10.1186/s12882-015-0101-x

**10. Niedzielska I, Wziątek-Kuczmik D:** The effects of dentogenic infection foci on internal organ disease-literature review. *Chir Pol,* 9, 92-96, 2007.

**11. Peddle GD, Sleeper M:** Canine bacterial endocarditis: A review. *J Am Anim Hosp Assoc,* 43 (5): 258-63, 2007. DOI: 10.5326/0430258

**12. Daly CG, Mitchell DH, Highfield JE, Grossberg DE, Stewart D:** Bacteremia due to periodontal probing: A clinical and microbiological investigation. *J Periodontol,* 72 (2): 210-14, 2001. DOI: 10.1902/jop.2001.72.2.210

**13. Schuster GS:** Oral flora and pathogenic organisms. *Infect Dis Clin North Am,* 13 (4): 757-774, 1999. DOI: 10.1016/S0891-5520(05)70107-0

**14. Kalicka A, Walasek L:** The role of inflammation and infection in the pathogenesis of atherosclerosis. *Post Med Klin Wojsk,* 10 (2): 31-35, 2005.

**15. Wiggs RB, Lobprise HB:** Periodontology. **In,** Wiggs RB, Lobprise HB (Eds): Veterinary Dentistry, Principles and Practice.186-231, Philadelphia: Lippincot-Raven, USA, 1997.

**16. Murray PR, Rosenthal KS, Pfaller MA:** Microbiology. Elsevier Urban&Partner, Wrocław, Poland, 2011.

**17. Luo Y, Siu GKH, Yeung ASF, Chen JHK, Ho PL, Leung KW, Tsang JLY, Cheng VCC, Guo L, Yang J, Ye L, Yam WC:** Performance of the VITEK MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for rapid bacterial identification in two diagnostic centres in China. *J Med Microbiol,* 64 (1): 18-24, 2015. DOI: **1**0.1099/jmm.0.080317-0

**18. Dubois D, Grare M, Prere MF, Segonds C, Marty N, Oswald E:** Performances of the Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for rapid identification of bacteria in routine clinical microbiology. *J Clin Microbiol,* 50 (8): 2568-2576, 2012. DOI: 10.1128/JCM.00343-12

**19. Schwint OA, Labraga M, Cervino CO, Haffar M, Sequeiros PH, Marcos HJA:** Modification of the staining technique of reticular fibres for image analysis of the cardiac collagen network. *Cardiovasc Pathol*, 13 (4): 213-220, 2004. DOI: 10.1016/S1054-8807(03)00153-4

**20. Rzepecka-Woźniak E, Konieczna M, Bolechała F:** Myocardial ischemia of the driver as a cause of a traffic road accident. Immuno-histochemical C9 staining method in diagnostics of early myocardial infarction. *Arch Med Sadowej Kryminol*, 56 (2): 110-114, 2006.

**21. Barbudo-Selmi GR, Carvalho MB, Selmi AL, Martins SEC:** Periodontal disease characterization in dogs with normal renal function or chronic renal failure. *Cienc Rural,* 34 (1): 113-118, 2004. DOI: 10.1590/S0103-84782004000100017

**22. Debowes LJ, Mosier D, Logan E, Harvey CE, Lowry S, Richardson DC:** Association of periodontal disease and histologic lesions in multiple organs from 45 dogs. *J Vet Dent,* 13 (2): 57-60, 1996.

**23. Glickman LT, Glickman NW, Moore GE, Lund EM, Lantz GC, Pressler BM:** Association between chronic azotemic kidney disease and the severity of periodontal disease in dogs. *Prev Vet Med,* 99 (2-4): 193-200, 2011. DOI: 10.1016/j.prevetmed.2011.01.011

**24. Grubbs V, Vittinghoff E, Beck JD, Kshirsagar AV, Wang W, Griswold ME, Powe NR, Correa A, Young B:** The association between periodontal disease and kidney function decline in African Americans: The Jackson heart study. *J Periodontol,* 86 (10): 1126-1132, 2015. DOI: 10.1902/jop.2015.150195

**25. Nemec A, Verstraete FJM, Jerin A, Šentjurc M, Kass PH, Petelin M, Pavlica Z:** Periodontal disease, periodontal treatment and systemic oxide in dogs. *Res Vet Sci,* 94 (3): 542-544, 2013. DOI: 10.1016/j.rvsc.2012.10.017

**26. O'Neill DG, Elliott J, Church DB, McGreevy PD, Thomson PC Brodbelt DC:** Chronic kidney disease in dogs in UK veterinary practices: Prevalence, risk factors, and survival. *J Vet Intern Med,* 27 (4): 814-821, 2013. DOI: 10.1111/jvim.12090

**27. Hartzell JD, Torres D, Kim P, Wortmann G:** Incidence of bacteremia after routine tooth brushing. *Am J Med Sci,* 329 (4): 178-180, 2005.DOI: 10.1097/00000441-200504000-00003

**28. Han SS, Shin N, Lee SM, Lee H, Kim DK, Kim YS:** Correlation between periodontitis and chronic kidney disease in Korean adults. *Kidney Res Clin Pract,* 32 (4): 164-170, 2013. DOI: 10.1016/j.krcp.2013.09.001

**29. Salimi S, Ng N, Seliger SL, Parsa A:** Periodontal disease, renal dysfunction and heightened leukocytosis. *Nephron Clin Pract,* 128 (1-2): 107-114, 2014. DOI: 10.1159/000366445

**30. Latronico M, Segantini A, Cavallini F, Mascolo A, Garbarino F, Bondanza S, Debbia EA, Blasi G:** Periodontal disease and coronary heart disease: An epidemiological and microbiological study. *New Microbiol,* 30 (3): 221-228, 2007.

**31. Mattila KJ, Pussinen PJ, Paju S:** Dental infections and cardiovascular diseases: A review. *J Periodontol,* 76 (11S): 2085-2088, 2005. DOI**:** 10.1902/jop.2005.76.11-S.2085

Kafkas Univ Vet Fak Derg
24 (5): 639-645, 2018
DOI: 10.9775/kvfd.2018.19523

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Effect of Different Dietary Lysine Regimens on Meat Quality Attributes in Varieties of Indigenous Aseel Chicken

Tahira BATOOL [1] Salman FAROOQ [1] Nabila ROOHI [1] Athar MAHMUD [2]
Muhammad USMAN [2] Abdul GHAYAS [2] Sohail AHMAD [2]

[1] Department of Zoology, University of the Punjab, Lahore, 54000, PAKISTAN
[2] Department of Poultry Production, Faculty of Animal Production and Technology, University of Veterinary and Animal Sciences, Lahore, 54000, PAKISTAN

**Article Code: KVFD-2018-19523 Received: 10.02.2018 Accepted: 01.06.2018 Published Online: 03.06.2018**

**How to Cite This Article**

**Batool T, Farooq S, Roohi N, Mahmud A, Usman M, Ghayas A, Ahmad S:** Effect of different dietary lysine regimens on meat quality attributes in varieties of indigenous Aseel chicken. *Kafkas Univ Vet Fak Derg,* 24 (5): 639-645, 2018. DOI: 10.9775/kvfd.2018.19523

## Abstract

This study was planned to evaluate the effect of different dietary lysine regimens on sensory attributes and meat quality in Lakha, Mianwali, Mushki and Peshawari varieties of indigenous Aseel. A total of 240 day-old chicks, 60 per each variety were casually selected and sub-divided equally into A, B and C groups. Three lysine regimens namely L1, L2 and L3 were offered to these birds, L1 comprising of 1.3% lysine was served from 0-6$^{th}$ week to birds of group A, and L2 constituting1.4 and 1.2% lysine, where 1.4% lysine from 0-3$^{rd}$ week and 1.2% lysine from 4-6$^{th}$ week was offered to group B. L3 having 1.5, 1.3 and 1.1% lysine was offered as 1.5 from 0-2$^{nd}$, 1.3 from 3-4$^{th}$ and 1.1% lysine from 5-6$^{th}$ week, respectively to group C. For evaluation of organoleptic and meat quality traits, 72 birds counting 18 per each variety were randomly selected and slaughtered. The breast and thigh meat pieces were separated and their pH was determined at various intervals. The cooked meat color, taste, flavor, tenderness and juiciness were checked to calculate overall acceptability of panelists on nine hedonic scale points between extreme liking and disliking. The findings of this study revealed L3 lysine regimen to be the best for nourishing the birds having good quality meat with respect to color, taste, flavor tenderness, juiciness and overall acceptability towards its consumers.

***Keywords:*** *Aseel varieties, Lysine regimens, Meat quality, Meat pH, Sensory attributes*

# Yerel Aseel Tavuklarında Farklı Miktarlarda Diyetsel Lizin Uygulamalarının Et Kalite Özelliklerine Etkisi

## Öz

Bu çalışma Lakha, Mianwali, Mushki ve Peshawari yerel Aseel tavuklarında farklı miktarlarda diyetsel lizin uygulamalarının duyusal özellikler ve et kalitesi üzerine etkisini araştırmak amacıyla gerçekleştirildi. Toplam 240 adet bir günlük civciv her deneysel uygulamada 60 adet olacak şekilde seçildi ve A, B ve C olmak üzere gruplara ayrıldı. Üç farlı lizin rejimi oluşturuldu; L1: Grup A'daki civcivlere 0-6. haftalar arasında %1.3 lizin verildi, L2: Grup B'deki civcivlere 0-3. haftalar arasında %1.4 ve 4-6. haftalar arasında %1.2 lizin verildi, L3: Grup C'deki civcivlere 0-2. haftalar arasında %1.5, 3-4. haftalar arasında %1.3 ve 5-6. haftalar arasında %1.1 lizin verildi. Organoleptik ve et kalite özelliklerini değerlendirmek amacıyla her bir varyantta 18 adet olacak şekilde 72 civciv rastgele seçildi ve kesime sevk edildi. Göğüs ve but etlerinin pH değerleri farklı aralıklarla ölçüldü. Pişmiş et rengi, tadı, lezzeti, pişkinliği ve suyu 9 hedonik skala puanlaması kullanılarak panelistlerin sevme ve sevmemeleri değerlendirildi. Çalışma sonuçları L3 lizin uygulamasının et rengi, tadı, lezzeti, pişkinliği, suyu ve genel olarak tüketicilerin memnuniyeti bakımından en iyi uygulama olduğunu gösterdi.

***Anahtar sözcükler:*** *Aseel varyantları, Lizin rejimi, Et kalitesi, Et pH'sı, Duyusal nitelikler*

## INTRODUCTION

The poultry meat is valued for its high quality protein source, less fat content, high digestibility and superior organoleptic traits. These nutritional characteristics makes the poultry meat, especially of the chicken more appreciable by consumers [1]. At market level, the breast part of chicken is considered to be the best among the whole carcass due to its muscle fiber conformation and development and even a mild change in its yield could have a substantial

**İletişim (Correspondence)**
+92 313 4288381
sohail.ahmad@uvas.edu.pk

economic impact [2]. That's why it is very essential to keep the chicken industry up to the running culinary standards by maintenance and improvement in the chemical composition and technical properties of carcass especially the breast muscle through performance evaluation [3]. As chicken carcass or meat quality of pectoral muscles is highly dependent on its biophysical, biochemical and histological properties which intern are influenced by age, sex, inheritance, nutrition and environmental factors [2]. In some studies, a positive correlation among biochemical, histological characteristics and meat quality of breast muscles has been found [4], while a negative impact of bird's age on meat quality has been revealed, especially when breast muscle fiber increases in size with the increase in age [5,6]. However, higher pH values with dark colored meat of pectoral muscle fibers along with increased in size and diameter among fast-growing chicken have also been reported [7]. The fast-growing commercial chicken meat have larger muscle fiber diameter which reduces its tenderness but the slow growing native breeds of chicken have shorter muscle fiber diameter and hence is comparatively more tender and soft [8]. Lot of variations have been found in meat quality of slow and fast-growing chicken with respect to breed and breeding system [9]. A slow-growing chicken (indigenous breeds) being reared under free range in natural environment and slaughtered at mature age might provide a meat with higher quality traits up to the consumer's taste and sensory quality expectations as compared to fast-growing (like broilers, leghorns as well as products of their crosses) chicken [10]. There is an emerging trend for the conservation and development of native chicken breeds as they are being important with respect to historical, traditional as well as socio-economical perspective [11]. The free-ranged and outdoor organic chicken models have significantly more sensory scores of panelists for juiciness of their breast meat as well as overall acceptability. Moreover, their breast and thigh meat have higher percentages of cooking and shear loss values with low fat content [12]. Another study has also revealed that free-ranged chicken had significantly higher breast meat percentages, CP (crude protein) content, values of shear force, chewiness with significantly less fat part, hardiness and factorability as compared to commercial fenced chicken [13,14]. Indigenous Aseel with better growth, carcass, blood biochemical profile, immunity, egg production and persistency have been found when lysine was supplemented as per growth requirements of birds [15]. With the hope that improved growth and carcass of Aseel will ultimately led to better quality, the current experiment was designed to evaluate the comparative sensory traits and meat quality among varieties of indigenous Aseel chicken been reared under three different dietary lysine regimens.

# MATERIAL and METHODS

### *Place of Experiment and Animal Care*

The present experiment was conducted at Indigenous Chicken Genetic Resource Center (ICGRC), University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan, by keeping in view the standard instructions for the care and welfare of the experimental birds. All the procedures used in this study were in accordance with the guidelines and code of practice of University of Veterinary and Animal Sciences, Lahore, Pakistan. Before conducted this study, ethical approval was granted.

### *Experimental Animals and Design*

This experiment was started by securing 240 day-old Aseel chicks (34±2 g) counting 60 per each of the four varieties including Lakha, Mianwali, Mushki and Peshawari. These randomly selected birds were divided into three sub-groups A, B and C, each with 20 birds of each variety. These bird were positioned in equally spaced blocks following standard conditions under Randomized Complete Block Design (RCBD) with a factorial arrangement of 3 (lysine regimens/treatments) × 4 (varieties) × 20 (replicates) with one bird in each replicate.

### *Experimental Feed and Feeding Program*

Three lysine regimens namely L1, L2 and L3 were offered to these Aseel birds, wherein L1 comprising 1.3% lysine was served from 0-6$^{th}$ week (in one phase) to birds of group A, and L2 constituting 1.4% and 1.2% lysine, where 1.4% lysine from 0-3$^{rd}$ week and 1.2% lysine from 4-6$^{th}$ week (in two phases) was offered to group B. Whereas, L3 having 1.5%, 1.3% and 1.1% lysine was offered as 1.5 from 0-2$^{nd}$, 1.3 from 3-4$^{th}$ and 1.1% lysine from 5-6$^{th}$ week (in three phases), respectively to group C. *Table 1* and *Table 2* represent the ingredients and nutrients composition of experimental feed. After six weeks of rearing under three lysine regimens, all these birds were equally offered the normal broiler grower feed prepared as per standards of National Research Council [16].

### *Meat Quality and Sensory Evaluation Parameters*

After eighteenth week of growth, 72 birds per each variety and 6 birds per treatment group were indiscriminately selected, exposed to fast for 12 h and slaughtered rendering to the Halal Muslim manner. Afterwards, the slaughtered birds were de-feathered and their breasts and thighs pieces were separated from the main body to evaluate the meat quality and organoleptic traits as per method adopted by Adedeji *et al.*[17]. The pH of each breast and thigh sample at various intervals was determined by direct probe and thrusting the digital pH meter into breast and thigh muscle. The meat samples were enfolded in impervious polythene wrappers which could be destroyed by cooking. The breast and thigh meat samples after 20 min of cooking in boiling water bath without using any spices were presented to a panel of ten experts for evaluation of color, taste, flavor, tenderness, juiciness and overall acceptability. The assessment was based on nine-point hedonic scale constituting maximum (9) for extremely like and the minimum (1) for poorest score of dislike.

**Table 1.** *Chemical composition of experimental diets*

| Ingredients | Dietary Lysine Levels (%) | | | | |
|---|---|---|---|---|---|
| | 1.1 | 1.2 | 1.3 | 1.4 | 1.5 |
| Corn | 59.08 | 59.08 | 59.08 | 59.08 | 59.08 |
| Sunflower meal (24%) | 18.90 | 18.90 | 18.90 | 18.90 | 18.90 |
| Soya bean meal (44%) | 7.04 | 7.04 | 7.04 | 7.04 | 7.04 |
| Rapeseed meal (36%) | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Fish meal (52%) | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Poultry by-product meal (50%) | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Molasses | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Limestone | 1.14 | 1.14 | 1.14 | 1.14 | 1.14 |
| Lysine sulphate | 0.75 | 0.80 | 0.90 | 1.00 | 1.10 |
| Mono calcium phosphate | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 |
| Vitamin-mineral premix* | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Sodium chloride | 0.18 | 0.18 | 0.18 | 0.18 | 0.18 |
| Alimet (novus) | 0.17 | 0.17 | 0.17 | 0.17 | 0.17 |
| Betaine HCl | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| Threonine | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 |

** Vit-Min premix supplied per 1 kg of diet: Vit. A 12.000 IU; Vit. $D_3$ 2.200 ICU; Vit. E 10 mg; Vit. $K_3$ 2 mg; Vit. $B_1$ 1 mg; Vit. $B_2$ 4 mg; Vit. $B_6$ 1.5 mg; Vit. $B_{12}$ 10 µg; nicotinic acid 20 mg; folic acid 1 mg; pantothenic acid 10 mg; biotin 50 µg; choline chloride 500 mg; copper 10 iron 30 mg; manganese 55 mg; zinc 50 mg; iodine 1 mg; selenium 0.1 mg*

### Statistical Analysis

Prior to analysis, degree of uniformity and homogeneity of variance was tested and verified for the normality, collected data were analyzed by two-way Analysis of Variance (ANOVA) and General Linear Model of SAS [18] software and the outcomes (results) were indicated as least square means and their standard errors. Duncan's Multiple Range test [19] was used to compare the treatment means and they were considered to be significant at P≤0.05.

## RESULTS

Significant (P≤0.05) variations were found in different organoleptic properties of thigh meat in Aseel birds reared on different lysine regimens and both L3 (1.5-1.3-1.1%) as well as L2 (1.4-1.2%) were found to be the better for nourishing the birds with moderately liked color, taste, juiciness and overall acceptability of panelists towards organoleptic properties of cooked meat. While, flavor and tenderness of meat was better in L3 followed by L2 and L1 lysine regimens. Non-significant variations were found among different varieties of Aseel for organoleptic properties of thigh meat *(Table 3)*. Likewise, significant variations were also shown by rating the breast meat organoleptic properties including very much liking color, taste, flavor and tenderness among lysine regimens, wherein, L3 found to be better followed by L2 and L1 feeding regimens. While juiciness and overall acceptability of breast meat was better in both L3 and L2 (non-significant among themselves) lysine regimens than L1 as was expressed by panelists during sensory evaluation. Non-significant variations were found among different varieties of Aseel for organoleptic properties of

**Table 2.** *Calculated nutritional composition of experimental diets*

| Nutrients (%) | Dietary Lysine Level % | | | | |
|---|---|---|---|---|---|
| | 1.1 | 1.2 | 1.3 | 1.4 | 1.5 |
| Metabolize energy (k calories/kg) | 2746.99 | 2753.69 | 2760.39 | 2767.09 | 2773.79 |
| Dry matter | 87.17 | 87.36 | 87.56 | 87.76 | 87.96 |
| Crude protein | 17.06 | 17.18 | 17.29 | 17.40 | 17.51 |
| Crude fiber | 6.93 | 6.93 | 6.93 | 6.93 | 6.93 |
| Ash | 4.09 | 4.09 | 4.09 | 4.09 | 4.09 |
| Either extract | 3.59 | 3.59 | 3.59 | 3.59 | 3.59 |
| Calcium | 0.84 | 0.84 | 0.84 | 0.84 | 0.84 |
| Chloride | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 |
| Sodium | 0.16 | 0.16 | 0.16 | 0.16 | 0.16 |
| Total phosphorus | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 |
| Potassium | 0.71 | 0.71 | 0.71 | 0.71 | 0.71 |
| Digestible phosphorus | 0.36 | 0.36 | 0.36 | 0.36 | 0.36 |
| Linoleic acid | 1.42 | 1.42 | 1.42 | 1.42 | 1.42 |
| Lysine | 1.1 | 1.2 | 1.3 | 1.4 | 1.5 |
| Methionine | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 |
| Methionine + Cystine | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |
| Digestible arginine | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 |
| Digestible tryptophan | 0.14 | 0.14 | 0.14 | 0.14 | 0.14 |
| Digestible threonine | 0.57 | 0.57 | 0.57 | 0.57 | 0.57 |
| Digestible lysine | 0.99 | 1.09 | 1.20 | 1.31 | 1.41 |
| Digestible methionine | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 |
| Digestible methionine + Cysteine | 0.67 | 0.67 | 0.67 | 0.67 | 0.67 |
| Threonine | 0.67 | 0.67 | 0.67 | 0.67 | 0.67 |
| Tryptophan | 0.19 | 0.19 | 0.19 | 0.19 | 0.19 |
| Arginine | 1.10 | 1.10 | 1.10 | 1.10 | 1.10 |
| Cysteine | 0.32 | 0.32 | 0.32 | 0.32 | 0.32 |
| Digestible cysteine | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 |
| Valine | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 |
| Digestible valine | 0.71 | 0.71 | 0.71 | 0.71 | 0.71 |
| Histidine | 0.43 | 0.43 | 0.43 | 0.43 | 0.43 |
| Digestible histidine | 0.37 | 0.37 | 0.37 | 0.37 | 0.37 |
| Phenylalanine | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |
| Digestible phenylalanine | 0.67 | 0.67 | 0.67 | 0.67 | 0.67 |
| Leucine | 1.44 | 1.44 | 1.44 | 1.44 | 1.44 |
| Digestible leucine | 1.21 | 1.21 | 1.21 | 1.21 | 1.21 |
| Isoleucine | 0.66 | 0.66 | 0.66 | 0.66 | 0.66 |
| Digestible isoleucine | 0.58 | 0.58 | 0.58 | 0.58 | 0.58 |

***Table 3.*** *Various parameters involving sensory evaluation of thigh meat in varieties of indigenous Aseel at 18th week of age*

| Variables | | Color | Taste | Flavor | Tenderness | Juiciness | Overall Acceptability |
|---|---|---|---|---|---|---|---|
| **Lysine levels (%)/Regimens (LR)** | | | | | | | |
| 1.3 (L1) | | 7.21±0.13$^{b}$ | 6.50±0.26$^{b}$ | 5.96±0.18$^{c}$ | 5.79±0.16$^{c}$ | 5.79±0.13$^{b}$ | 6.75±0.12$^{b}$ |
| 1.4-1.2 (L2) | | 7.79±0.13$^{a}$ | 7.25±0.15$^{a}$ | 7.21±0.13$^{b}$ | 7.08±0.15$^{b}$ | 6.83±0.17$^{a}$ | 7.58±0.15$^{a}$ |
| 1.5-1.3-1.1 (L3) | | 8.08±0.18$^{a}$ | 7.63±0.19$^{a}$ | 7.88±0.16$^{a}$ | 7.54±0.13$^{a}$ | 7.17±0.18$^{a}$ | 7.75±0.11$^{a}$ |
| **Aseel varieties (AV)** | | | | | | | |
| Lakha | | 7.83±0.17 | 7.00±0.26 | 7.00±0.29 | 6.72±0.25 | 6.50±0.22 | 7.39±0.18 |
| Mianwali | | 7.61±0.22 | 7.22±0.30 | 6.78±0.24 | 6.89±0.24 | 6.61±0.22 | 7.44±0.18 |
| Mushki | | 7.61±0.23 | 7.22±0.22 | 7.11±0.28 | 6.67±0.24 | 6.61±0.28 | 7.28±0.18 |
| Peshawari | | 7.72±0.16 | 7.06±0.26 | 7.17±0.25 | 6.94±0.25 | 6.67±0.21 | 7.33±0.18 |
| **Lysine levels (%)/Regimens × Aseel varieties (LR × AV)** | | | | | | | |
| 1.3 (L1) | Lakha | 7.50±0.22 | 6.33±0.49 | 5.67±0.33$^{c}$ | 5.67±0.33$^{c}$ | 5.67±0.21$^{d}$ | 6.83±0.17$^{bc}$ |
| | Mianwali | 7.00±0.26 | 6.50±0.76 | 5.83±0.31$^{c}$ | 6.00±0.37$^{bc}$ | 5.83±0.31$^{cd}$ | 6.67±0.21$^{c}$ |
| | Mushki | 7.00±0.37 | 6.50±0.43 | 6.00±0.37$^{c}$ | 5.67±0.33$^{c}$ | 5.67±0.21$^{d}$ | 6.67±0.33$^{c}$ |
| | Peshawari | 7.33±0.21 | 6.67±0.42 | 6.33±0.42$^{bc}$ | 5.83±0.31$^{c}$ | 6.00±0.37$^{bcd}$ | 6.83±0.31$^{bc}$ |
| 1.4-1.2 (L2) | Lakha | 7.83±0.31 | 7.00±0.37 | 7.33±0.21$^{ab}$ | 6.83±0.31$^{ab}$ | 6.83±0.31$^{abc}$ | 7.67±0.42$^{ab}$ |
| | Mianwali | 7.67±0.21 | 7.33±0.33 | 7.00±0.26$^{ab}$ | 7.00±0.37$^{a}$ | 6.83±0.31$^{abc}$ | 7.67±0.21$^{ab}$ |
| | Mushki | 7.83±0.31 | 7.33±0.21 | 7.33±0.33$^{ab}$ | 7.00±0.26$^{a}$ | 6.83±0.48$^{abc}$ | 7.50±0.22$^{abc}$ |
| | Peshawari | 7.83±0.31 | 7.33±0.33 | 7.17±0.31$^{ab}$ | 7.50±0.22$^{a}$ | 6.83±0.31$^{abc}$ | 7.50±0.34$^{abc}$ |
| 1.5-1.3-1.1 (L3) | Lakha | 8.17±0.31 | 7.67±0.33 | 8.00±0.37$^{a}$ | 7.67±0.21$^{a}$ | 7.00±0.37$^{ab}$ | 7.67±0.21$^{ab}$ |
| | Mianwali | 8.17±0.48 | 7.83±0.17 | 7.50±0.34$^{a}$ | 7.67±0.21$^{a}$ | 7.17±0.31$^{a}$ | 8.00±0.26$^{a}$ |
| | Mushki | 8.00±0.45 | 7.83±0.31 | 8.00±0.37$^{a}$ | 7.33±0.33$^{a}$ | 7.33±0.49$^{a}$ | 7.67±0.21$^{ab}$ |
| | Peshawari | 8.00±0.26 | 7.17±0.60 | 8.00±0.26$^{a}$ | 7.50±0.34$^{a}$ | 7.17±0.31$^{a}$ | 7.67±0.21$^{ab}$ |
| **Source of variation** | | ***P*-value** | | | | | |
| LR | | 0.0009 | 0.0016 | <.0001 | <.0001 | <.0001 | <.0001 |
| AV | | 0.7991 | 0.8847 | 0.4827 | 0.6391 | 0.9443 | 0.8865 |
| LR×AV | | 0.9683 | 0.9382 | <.0001 | <.0001 | 0.0013 | 0.0026 |

*Values have been mentioned as Mean ± SE and various superscripted alphabets show significant (P≤0.05) differences among them (order of significance is as: a>b>c…….); Rating scale score points = 9; Dislike (extremely = 1, very much = 2, moderately = 3, slightly = 4); Neither dislike nor like = 5; Like (slightly = 6, moderately = 7, very much = 8, extremely = 9)*

breast meat *(Table 4)*. As far as interactions among lysine regimens and Aseel varieties are concerned, inconsistent results of organoleptic properties of both thigh and breast meat were observed between moderately and very much liked. Non-significant variations were found among pH of both thigh and breast meat at various intervals (0, 20 and 120 min) after slaughtering in lysine regimens, Aseel varieties and their interaction (P>0.05) *(Table 5)*.

## DISCUSSION

As nutrition plays a vital role in muscle growth and meat quality characters of poultry birds, particularly, the muscle development and yield may largely be determined by protein intake which intern affects many molecular pathways with substantial consequences on post mortem metabolism of muscles and eventually the meat quality [20]. Moreover, the meat quality can be modulated and improved more efficiently by strategically supplying the protein and amino acid during early growth [21]. This study is in close lines with our study and its findings, wherein, lysine was supplemented in various dietary regimens and three phase feeding lysine regimen (L3) was found to be very effective with respect to organoleptic and meat quality characteristics. Meat quality is a combination of biochemistry, muscle morphology, muscle fiber configuration [22]. However, genetic factors (sex, breed and strain) may also have great contribution in specific quality traits of meat consistency storing and processing ability [23]. In our findings of organoleptic traits and meat quality characteristics with respect to Aseel varieties, a variable pattern was observed which might also be due to genetic impact. The birds susceptible to fattening possess less sensitivity towards dietary variations than those which are leaner [24]. As far as, the change in post mortem pH of breast and thigh meat is concerned, a large number of factors are involved with respect to birds, feed and feeding processes [25]. Pre-slaughter management has direct relationship with meat

**Table 4.** *Various parameters involving sensory evaluation of breast meat in varieties of indigenous Aseel at 18th week of age*

| Variables | | Color | Taste | Flavor | Tenderness | Juiciness | Overall Acceptability |
|---|---|---|---|---|---|---|---|
| **Lysine levels (%)/Regimens (LR)** | | | | | | | |
| 1.3 (L1) | | 6.75±0.14$^{c}$ | 7.00±0.16$^{c}$ | 6.75±0.12$^{c}$ | 7.13±0.14$^{c}$ | 6.83±0.17$^{b}$ | 6.96±0.18$^{b}$ |
| 1.4-1.2 (L2) | | 7.46±0.10$^{b}$ | 7.67±0.17$^{b}$ | 7.67±0.14$^{b}$ | 7.79±0.13$^{b}$ | 7.88±0.20$^{a}$ | 7.63±0.15$^{a}$ |
| 1.5-1.3-1.1 (L3) | | 7.96±0.14$^{a}$ | 8.17±0.16$^{a}$ | 8.13±0.13$^{a}$ | 8.33±0.12$^{a}$ | 8.25±0.18$^{a}$ | 7.96±0.15$^{a}$ |
| **Aseel varieties (AV)** | | | | | | | |
| Lakha | | 7.44±0.18 | 7.33±0.20 | 7.56±0.20 | 7.50±0.19 | 7.61±0.26 | 7.56±0.18 |
| Mianwali | | 7.44±0.18 | 7.67±0.23 | 7.50±0.23 | 7.72±0.23 | 7.72±0.25 | 7.56±0.20 |
| Mushki | | 7.22±0.19 | 7.83±0.20 | 7.50±0.20 | 7.83±0.17 | 7.78±0.29 | 7.61±0.22 |
| Peshawari | | 7.44±0.20 | 7.61±0.23 | 7.50±0.19 | 7.94±0.17 | 7.50±0.23 | 7.33±0.23 |
| **Lysine levels (%)/Regimens × Aseel varieties (LR × AV)** | | | | | | | |
| 1.3(L1) | Lakha | 6.83±0.31$^{bc}$ | 6.83±0.31$^{c}$ | 7.00±0.26$^{bcde}$ | 7.00±0.37$^{de}$ | 6.67±0.21$^{cd}$ | 7.00±0.26$^{bc}$ |
| | Mianwali | 6.83±0.31$^{bc}$ | 7.00±0.45$^{c}$ | 6.50±0.22$^{e}$ | 6.83±0.31$^{e}$ | 7.17±0.48$^{abcd}$ | 7.17±0.40$^{abc}$ |
| | Mushki | 6.50±0.22$^{c}$ | 7.17±0.31$^{bc}$ | 6.67±0.21$^{de}$ | 7.33±0.21$^{cde}$ | 7.00±0.37$^{abcd}$ | 6.83±0.31$^{c}$ |
| | Peshawari | 6.83±0.31$^{bc}$ | 7.00±0.26$^{c}$ | 6.83±0.31$^{cde}$ | 7.33±0.21$^{cde}$ | 6.50±0.22$^{d}$ | 6.83±0.48$^{c}$ |
| 1.4-1.2 (L2) | Lakha | 7.50±0.22$^{ab}$ | 7.50±0.43$^{abc}$ | 7.50±0.34$^{bcde}$ | 7.50±0.22$^{bcde}$ | 7.83±0.48$^{abc}$ | 7.67±0.21 |
| | Mianwali | 7.50±0.22$^{ab}$ | 7.67±0.21$^{abc}$ | 7.67±0.21$^{abc}$ | 7.83±0.31$^{abcd}$ | 8.00±0.45$^{ab}$ | 7.67±0.33$^{abc}$ |
| | Mushki | 7.33±0.21$^{abc}$ | 7.83±0.31$^{abc}$ | 7.83±0.31$^{ab}$ | 7.83±0.31$^{abcd}$ | 8.00±0.52$^{ab}$ | 7.83±0.31$^{abc}$ |
| | Peshawari | 7.50±0.22$^{ab}$ | 7.67±0.42$^{abc}$ | 7.67±0.33$^{abc}$ | 8.00±0.26$^{abc}$ | 7.67±0.21$^{bcde}$ | 7.33±0.33$^{abc}$ |
| 1.5-1.3-1.1 (L3) | Lakha | 8.00±0.26$^{a}$ | 7.67±0.21$^{abc}$ | 8.17±0.31$^{a}$ | 8.00±0.26$^{abc}$ | 8.33±0.33$^{a}$ | 8.00±0.37$^{ab}$ |
| | Mianwali | 8.00±0.26$^{a}$ | 8.33±0.33$^{a}$ | 8.33±0.33$^{a}$ | 8.50±0.22$^{a}$ | 8.00±0.37$^{ab}$ | 7.83±0.31$^{abc}$ |
| | Mushki | 7.83±0.31$^{a}$ | 8.50±0.22$^{a}$ | 8.00±0.26$^{a}$ | 8.33±0.21$^{ab}$ | 8.33±0.49$^{a}$ | 8.17±0.31$^{a}$ |
| | Peshawari | 8.00±0.37$^{a}$ | 8.17±0.40$^{ab}$ | 8.00±0.21$^{a}$ | 8.50±0.22$^{a}$ | 8.33±0.33$^{a}$ | 7.83±0.31$^{abc}$ |
| **Source of variation** | | ***P*-value** | | | | | |
| LR | | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | 0.0003 |
| AV | | 0.6837 | 0.3263 | 0.9926 | 0.2095 | 0.8221 | 0.7454 |
| LR × AV | | 0.0004 | 0.0073 | <.0001 | <.0001 | 0.0041 | 0.0519 |

*Values have been mentioned as Mean ± SE and various superscripted alphabets show significant (P≤0.05) differences among them (order of significance is as: a>b>c……..); Rating scale score points = 9; Dislike (extremely = 1, very much = 2, moderately = 3, slightly = 4); Neither dislike nor like = 5; Like (slightly = 6, moderately = 7, very much = 8, extremely = 9)*

color and pH, which may directly affect the ability of myoglobin to bind water and meat quality attributes [26]. Contradictory study also reported non-significant variation regarding meat pH among the indigenous genotypes of India [27]. Moreover, other scientists observed that heavy birds did not struggle much and their pH decline was very slow [28]. Studies on indigenous chickens revealed higher pH value in higher weight birds as compared to lower ones [27]. The normal range of pH for broiler chickens were reported 5.80 to 6.29. Higher pH leads to dark, firm and dry (DFD) meat with poor storage quality due to accelerated microbial growth whereas low pH improves the shelf life of meat but pale coloration [28]. Meat pH has great effect on its color and water holding capacity, drip loss and tenderness which intern are dependent upon lysine supply and intake [21]. Breast muscle glycogen storage might also be greatly responsible for meat pH variations. High ultimate pH levels can be observed in breast meat when lysine intake is reduced along with decreased glycogen storage, while low pH when lysine supply is in excess and more energy storage in the form of muscle glycogen [29]. The present overall better acceptability of panelists towards breast meat in terms of color, taste, flavor, tenderness and juiciness as compared to thigh might be due to the presence of more inosine-5-monophosphate (IMP), generally the key nucleotide in muscles which imparts taste and flavor to cooked meat [30] and lysine content of breast (7% of breast meat), as the proper intake of protein in the form of lysine efficiently controls the molecular regulation of breast muscle growth and reinforce the modulation of meat quality characteristics [31] However, opposite to our study, some studies have also indicated that food programs and lysine supplementation had no impact on pork meat quality attributes i.e., pH, drip loss, water holding capacity and shear force [32].

The present study revealed that the change of nutritional strategy does effect the ultimate muscle growth, pH and

***Table 5.*** *pH values of breast and thigh meat at different time intervals (minutes) after slaughtering among four varieties of indigenous Aseel at 18th week of age*

| Variables | | 0 min | | 20 min | | 120 min | |
|---|---|---|---|---|---|---|---|
| | | Breast | Thigh | Breast | Thigh | Breast | Thigh |
| **Lysine Levels (%)/Regimens (LR)** | | | | | | | |
| 1.3 (L1) | | 6.53±0.09 | 6.75±0.07 | 6.16±0.07 | 6.45±0.08 | 5.80±0.06 | 6.09±0.06 |
| 1.4-1.2 (L2) | | 6.59±0.08 | 6.73±0.08 | 6.27±0.07 | 6.29±0.06 | 5.76±0.05 | 6.02±0.05 |
| 1.5-1.3-1.1 (L3) | | 6.49±0.08 | 6.79±0.08 | 6.19±0.06 | 6.43±0.07 | 5.88±0.05 | 6.07±0.06 |
| **Aseel varieties (AV)** | | | | | | | |
| Lakha | | 6.46±0.11 | 6.65±0.08 | 6.14±0.05 | 6.27±0.02 | 5.74±0.07 | 6.06±0.07 |
| Mianwali | | 6.50±0.07 | 6.82±0.09 | 6.28±0.08 | 6.44±0.01 | 5.88±0.04 | 6.01±0.08 |
| Mushki | | 6.70±0.10 | 6.73±0.05 | 6.21±0.01 | 6.49±0.09 | 5.73±0.04 | 6.06±0.07 |
| Peshawari | | 6.50±0.09 | 6.83±0.02 | 6.20±0.07 | 6.37±0.06 | 5.89±0.01 | 6.12±0.09 |
| **Lysine levels (%)/Regimens × Aseel varieties (LR × AV)** | | | | | | | |
| 1.3 (L1) | Lakha | 6.29±0.12 | 6.55±0.13 | 6.11±0.12 | 6.15±0.11 | 5.65±0.11 | 6.03±0.11 |
| | Mianwali | 6.54±0.14 | 6.87±0.13 | 6.22±0.18 | 6.56±0.13 | 5.95±0.12 | 5.99±0.12 |
| | Mushki | 6.70±0.23 | 6.76±0.08 | 6.07±0.19 | 6.61±0.18 | 5.67±0.11 | 6.05±0.12 |
| | Peshawari | 6.62±0.20 | 6.84±0.14 | 6.23±0.11 | 6.49±0.15 | 5.95±0.13 | 6.29±0.12 |
| 1.4-1.2 (L2) | Lakha | 6.48±0.01 | 6.54±0.13 | 6.22±0.23 | 6.26±0.12 | 5.67±0.11 | 6.06±0.11 |
| | Mianwali | 6.60±0.13 | 6.74±0.20 | 6.39±0.14 | 6.19±0.11 | 5.72±0.11 | 6.10±0.11 |
| | Mushki | 6.71±0.18 | 6.71±0.18 | 6.19±0.11 | 6.19±0.11 | 5.74±0.10 | 5.74±0.10 |
| | Peshawari | 6.57±0.12 | 6.91±0.13 | 6.31±0.12 | 6.30±0.12 | 5.91±0.11 | 5.91±0.12 |
| 1.5-1.3-1.1 (L3) | Lakha | 6.62±0.24 | 6.86±0.13 | 6.10±0.12 | 6.41±0.14 | 5.92±0.12 | 6.10±0.12 |
| | Mianwali | 6.35±0.12 | 6.85±0.15 | 6.24±0.12 | 6.57±0.13 | 5.97±0.11 | 5.95±0.12 |
| | Mushki | 6.70±0.14 | 6.69±0.16 | 6.39±0.12 | 6.43±0.15 | 5.79±0.11 | 6.07±0.11 |
| | Peshawari | 6.31± 0.12 | 6.76±0.17 | 6.05±0.11 | 6.31±0.12 | 5.83±0.11 | 6.16±0.11 |
| **Source of variation** | | ***P*-value** | | | | | |
| LR | | 0.7127 | 0.8590 | 0.4895 | 0.2060 | 0.3472 | 0.7079 |
| AV | | 0.2849 | 0.4133 | 0.6744 | 0.2244 | 0.1742 | 0.7167 |
| LR × AV | | 0.5996 | 0.7199 | 0.6517 | 0.4048 | 0.4752 | 0.4388 |

other physico-chemical traits of meat and L3 lysine regimen found to be better for producing the meat with superior sensory attributes. The present findings also disclosed the new ways of research to define the requirement of amino acid (s) and the metabolic reasons involved in breast and thigh muscle pH variations in relation to protein and carbohydrate metabolism among slow growing breeds like Aseel.

## Acknowledgements

Prof. Dr. Muhammad Akram (Late), Ex-Dean, Faculty of Animal Production and Technology, UVAS, Lahore, is greatly acknowledged for his marvelous contribution during the planning of present research project and cooperation extended by UVAS administration to provide the research facilities at Indigenous Chicken Genetic Resource Centre, Department of Poultry Production, Ravi Campus, Pattoki.

## Disclosure Statement

The content matter of this article is based on findings of original research work and there is no any personal conflict and interest on the part of any author.

## REFERENCES

**1. Mueller S, Kreuzer M, Siegrist M, Mannale K, Messikommer RE, Gangnat IDM:** Carcass and meat quality of dual-purpose chickens (Lohmann Dual, Belgian Malines, Schweizerhuhn) in comparison to broiler and layer chicken types. *Poult Sci*, 2018. DOI: 10.3382/ps/pey172

**2. Scheuermann GN, Bilgili SF, Tuzun S, Mulvaney DR:** Comparison of chicken genotypes: Myofibre number in pectoralis muscle and myostatin ontogeny. *Poult Sci*, 83, 1404-1412, 2004. DOI: 10.1093/ps/83.8.1404

**3. Mir NA, Rafiq A, Kumar F, Singh, Shkla V:** Determinants of broiler chicken meat quality and factors affecting them: A review. *J Food Sci Tech.* 54 (10): 2997-3009, 2017. DOI: 10.1007/s13197-017-2789-z

**4. Rehfeldt CH, Fiedler I, Stickland NC:** Number and size of muscle fibres in relation to meat production. **In,** Pas MFW, Everts ME, Haagsman HP (Eds): Muscle Development of Livestock Animals: Physiology, Genetics, and Meat Quality. Cambridge (MA), CABI Publisher, 2004. DOI: 10.1079/9780851998114.0000

**5. Chen XD, Ma QG, Tang MY, Ji C:** Development of breast muscle and meat quality in Arbor Acres broilers, Jingxing 100 crossbred chickens and Beijing fatty chickens. *Meat Sci*, 77 (2): 220-227, 2007. DOI: 10.1016/j.meatsci.2007.03.008

**6. Choi YM, Kim BC:** Muscle fiber characteristics, myofibrillar protein isoforms, and meat quality. *Livest Sci*, 122 (2-3): 105-118, 2009. DOI: 10.1016/j.livsci.2008.08.015

**7. Tumova E, Teimouri A:** Chicken muscle fibres characteristics and meat quality: A review. *Scientia Agric Bohem*, 40 (4): 253-258, 2009.

**8. Guan RF, Lyu F, Chen XQ, Ma JQ, Jiang H, Xiao CG:** Meat quality traits of four Chinese indigenous chicken breeds and one commercial broiler stock. *J Zhejiang Univ Sci B*, 14 (10): 896-902, 2013. DOI: 10.1631/jzus.B1300163

**9. Ayaşan T, Okan F:** Effects of diets containing different levels of threonine and lysine aminoacids on fattening performance of broiler chicks. *Süleyman Demirel Üniv Ziraat Fak Derg*, 5 (1): 36-43, 2010.

**10. Le Bihan-Duval E:** Genetic variability within and between breeds of poultry technological meat quality. *Worlds Poult Sci J*, 60, 331-340, 2004. DOI: 10.1079/WPS200321

**11. De Marchi M, Cassandro M, Lunardi E, Baldan G, Siegel PB:** Carcass characteristics and qualitative meat traits of the Padovana breed of chicken. *Int J Poult Sci*, 4 (4): 233-238, 2005. DOI: 10.3923/ijps.2005.233.238

**12. Castellini C, Mugnai C, Bosco AD:** Effect of organic production system on broiler carcass and meat quality. *Meat Sci,* 60 (3): 219-225, 2002. DOI: 10.1016/S0309-1740(01)00124-3

**13. Cheng FY, Huang CW, Wan TC, Liu YT, Lin LC, Lou Chyr CY:** The effects of free-range farming, compared to a conventional production system, on carcass and meat qualities were studied using black-feathered Taiwan native chickens. *Asian-Australas J Anim Sci,* 21 (8): 1201-1206, 2008. DOI: 10.5713/ajas.2008.80080

**14. Thutwa K, Nsoso SJ, Kgwatalala PM, Moreki JC:** Comparative live weight, growth performance, feed intake, carcass traits and meat quality in two strains of Tswana chickens raised under intensive system in south east district of Botswana. *Int J Appl Poult Res*, 1 (1): 21-26, 2012.

**15. Batool T, Roohi A, Roohi N, Mahmud A:** Impact of different dietary lysine regimens on blood biochemical profile and immune response in indigenous Aseel varieties. *Pak Vet J,* 37 (4): 393-398, 2017.

**16. NRC:** Nutrient Requirements of Poultry. 9th edn., Washington (DC): National Academy Press, 1994.

**17. Adedeji OS, Amao SR, Oguntunde MM, Dada ID:** Evaluation of general performance and carcass qualities of organically raised broiler chickens from day old to 12 weeks of age. *IJAIR*, 2 (4): 466-471, 2014.

**18. SAS Institute Inc:** SAS/STAT User's Guide: Statistics.Version 9.1. SAS Inst. Inc., Cary, NC; 2002-2003.

**19. Duncan DB:** Multiple range and multiple F-tests. *Biometrics*, 11 (1): 1-42, 1955. DOI: 10.2307/3001478

**20. Berri C, Besnard J, Relandeau C:** Increasing dietary lysine increases final pH and decreases drip loss of broiler breast meat. *Poult Sci*, 87 (3): 480-484, 2008. DOI: 10.3382/ps.2007-00226

**21. Lessire M, Primot Y, Corrent E, Fraysse P, Tesseraud S, Berri C:** Lysine supply in finishing broilers: Effect on performances and meat quality. **In,** Oltjen JW, Kebreab E, Lapierre H (Eds): Energy and Protein Metabolism and Nutrition in Sustainable Animal Production. 134, 209-210, Wageningen Academic Publishers, Wageningen 2013.

**22. Saxena VK, Sachdev AK, Gopal R, Pramod AB:** Roles of important candidate genes on broiler meat quality. *Worlds Poult Sci J*, 65 (1): 37-50, 2009. DOI: 10.1017/S0043933909000038

**23. Le Bihan-Duval E, Debut M, Berri C, Sellier N, Sante-Lhoutellier V, Jego Y, Beaumont C:** Chicken meat quality: Genetic variability and relationship with growth and muscle characteristics. *BMC Genet,* 9 (1): 53, 2008. DOI: 10.1186/1471-2156-9-53

**24. Jlali M, Gigaud V, Métayer-Coustard S, Sellier N, Tesseraud S, Le Bihan-Duval E, Berri C:** Modulation of glycogen and breast meat processing ability by nutrition in chickens: Effect of crude protein level in 2 chicken genotypes. *J Anim Sci*, 90 (2): 447-455, 2012. DOI: 10.2527/JAS.2011-4405

**25. Zhao JP, Zhao GP, Jiang RR, Zheng MQ, Chen JL, Liu RR, Wen J:** Effects of diet-induced differences in growth rate on metabolic, histological, and meat-quality properties of 2 muscles in male chickens of 2 distinct broiler breeds. *Poult Sci*, 91 (1): 237-247, 2012. DOI: 10.3382/PS.2011-01667

**26. Dadgar S, Lee ES, Leer TLV, Crowe TG, Classen HL, Shand PJ:** Effect of acute cold exposure, age, sex and lairage on broiler breast meat quality. *Poult Sci*, 90 (2): 444-457, 2011. DOI: 10.3382/ps.2010-00840

**27. Rajkumar U, Muthukumar M, Haunshi S, Niranjan M, Raju MVLN, Rama Rao SV, Chatterjee RN:** Comparative evaluation of carcass traits and meat quality in native Aseel chickens and commercial broilers. *Br Poult Sci*, 57 (3): 339-347, 2016. DOI: 10.1080/00071668.2016.1162282

**28. Sarsenbek A, Wang T, Zhao JK, Jiang W:** Comparison of carcass yields and meat quality between Baicheng-You chickens and Arbor Acres broilers. *Poult Sci,* 92 (10): 2776-2782, 2013. DOI: 10.3382/ps.2012-02841

**29. Zhuang H, Savage EM:** Comparisons of sensory descriptive flavor and texture profiles of cooked broiler breast fillets categorized by raw meat color lightness values. *Poult Sci,* 89 (5): 1049-1055, 2010. DOI: 10.3382/PS.2009-00422

**30. Jayasena DD, Ahn DU, Nam KC, Jo C:** Flavour chemistry of chicken meat: A review. *Asian-Austtralas J Anim Sci,* 26 (5): 732-742, 2013. DOI: 10.5713/ajas.2012.12619

**31. Berri C:** Poultry meat: Expectations for quality that diversify and specific defects to correct. *INRA Prod Anim*, 28 (2): 115-118, 2015.

**32. Apple JK, Maxwell CV, Brown DC, Friesen KG, Musser RE, Johnson ZB, Armstrong TA:** Effects of dietary lysine and energy density on performance and carcass characteristics of finishing pigs fed ractopamine. *J Anim Sci*, 82 (11): 3277-3287, 2004. DOI: 10.2527/2004.82113277x

Kafkas Univ Vet Fak Derg
24 (5): 647-654, 2018
DOI: 10.9775/kvfd.2018.19596

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Some Characteristics of Erzincan Tulum Cheese Produced Using Different Probiotic Cultures and Packaging Material

Oktay TOMAR [1,a] Gökhan AKARCA [2,b] Mehmet BEYKAYA [3] Abdullah ÇAĞLAR [4,c]

[1] Food Engineering Department, Faculty of Engineering, Afyon Kocatepe University, TR-03200 Afyonkarahisar - TURKEY
[2] Food Engineering Department, Faculty of Engineering, Afyon Kocatepe University, TR-03200 Afyonkarahisar - TURKEY
[3] Food Engineering Department, Graduate School of Natural and Applied Science, Afyon Kocatepe University, TR-03200 Afyonkarahisar - TURKEY
[4] Food Engineering Department, Faculty of Engineering, Afyon Kocatepe University, TR-03200 Afyonkarahisar - TURKEY
[a] ORCID: 0000-0001-5761-7157; [b] ORCID: 0000-0002-5055-2722; [c] ORCID: 0000-0002-9716-8795

**Article Code: KVFD-2018-19596 Received: 28.02.2018 Accepted: 12.06.2018 Published Online: 13.06.2018**

**How to Cite This Article**

**Tomar O, Akarca G, Beykaya M, Çağlar A:** Some characteristics of Erzincan tulum cheese produced using different probiotic cultures and packaging material. *Kafkas Univ Vet Fak Derg,* 24 (5): 647-654, 2018. DOI: 10.9775/kvfd.2018.19596

## Abstract

In this study, Erzincan tulum cheeses produced using pasteurized sheep milk and probiotic bacterial cultures were filled in different packaging materials (skin bag, intestine, and appendix) and stored for 90 days. Subsequently, the chemical, microbiological and quality criteria of the samples were examined on the 2nd, 30th, 60th and 90th days of the storage period. Although the acidity values of cheese samples increased during the storage period, the pH and water activity values decreased. Similarly, TAMB, TAPB, yeast/mold, lactic acid bacteria, lipolytic bacteria, proteolytic bacteria, *Lactococcus* spp. and *Pseudomonas* spp. counts increased; however, the total coliform and *Enterobacteriaceae* counts decreased. The probiotic culture counts added in cheese production also decreased during storage. Moreover, no *Escherichia coli, Staphylococcus aureus, Salmonella* spp., *Listeria* spp. and *Brucella* spp. development was determined in the samples.

***Keywords:*** *Erzincan tulum cheese, Probiotic bacteria, Appendix, Small intestine, Quality criteria*

# Farklı Probiyotik Kültür ve Ambalaj Malzemesiyle Üretilen Erzincan Tulum Peynirlerinin Bazı Özelliklerinin İncelenmesi

## Öz

Bu çalışmada, pastörize koyun sütleri kullanılarak ve probiyotik bakteri kültürleri ilave edilerek üretilen Erzincan tulum peynirleri farklı ambalaj materyallerine doldurularak (tulum, ince bağırsak, kör bağırsak, 90 gün süre ile depolanmıştır. Depolamanın 2., 30., 60., ve 90. günlerinde peynirlerin bazı kimyasal ve mikrobiyolojik, kalite kriterleri incelenmiştir. Depolama süresi boyunca peynir örneklerinin % asitlik değerleri artış göstermesine karşın, pH ve aw değerlerinde azalma olduğu belirlenmiştir. Benzer şekilde depolama süresince TAMB, TAPB, maya/küf, laktik asit, lipolitik bakteri, proteolitik bakteri sayıları, *Lactococcus* spp. ve *Pseudomonas* spp. cinsi bakteri sayılarında artış tespit edilmesine karşın, Toplam koliform ve *Enterobacteriaceae* sayılarında ise azalma olduğu belirlenmiştir. Peynir üretiminde ilave edilen probiyotik kültür sayıları da yine depolama süresince azalmıştır. Ayrıca peynirlere yapılan *Escherichia coli, Staphylococcus aureus, Salmonella* spp, *Listeria* Spp. *Brucella* spp. analizleri sonucunda herhangi bir gelişme tespit edilememiştir.

***Anahtar sözcükler:*** *Erzincan tulum peyniri, Probiyotik bakteri, kör bağırsak, İnce bağırsak, Kalite kriterleri*

## INTRODUCTION

Cheese was first produced 8000 years ago, and approximately, 4000 different types of cheese are currently found in the world [1]. Turkey produces approximately 200 types of cheese [2]. The method of cheese production, the mixtures added to the cheese and fermentation process and other variables in this process differentiate the cheese types from each other. Cheese is an extensive microbial ecosystem, and the complex microbiota directly influences the formation of various kinds of cheese [3].

Tulum is the most consumed cheese after the white cheese and kashar cheese types in Turkey. Moreover, tulum cheese

**İletişim (Correspondence)**
+90 272 2281423 Fax: +90 272 2281422
oktomar@aku.edu.tr

has the highest consumption rate among the traditionally produced cheese types [4]. Although tulum cheese is mostly produced in small family businesses in Turkey, in recent years its production has expanded to large factories [2,4].

Tulum cheese is produced in all regions in Turkey except Trakya region and is referred to by different names according to the areas where they are produced [5]. The most common tulum cheese types are Erzincan tulum cheese, Çimi tulum cheese (Antalya), Divle tulum cheese (Karaman), Afyon tulum cheese (Afyon), Kargı tulum cheese (Çankırı, Çorum), Isparta tulum cheese (Isparta), Selçuklu tulum cheese (Konya) and İzmir tulum cheese (ripened in brine). The production and storage conditions of tulum cheese are very different from other tulum cheese types [6].

Among these cheese types, Erzincan tulum cheese is the most produced and consumed tulum cheeses in Turkey and has a significantly higher economic value much higher than most cheese types [7]. So far, the Erzincan tulum cheese has no standard production method and is mostly produced in small dairy plants by traditional methods using fatty sheep milk, sometimes mixed with cow and goat milk [8]. Milk is processed with no fat standardization [9]. The milk used is renneted at its temperature following milking or heated to 30-32°C with no pasteurization application [10] and no starter culture addition [11,12]. Tulum cheese is marketed by traditionally filling in skin bags mostly obtained from the skins of small cattle [13]. However, in recent years, excessive microbial load in such packaging has had a negative impact on the consumer and economical aspects; therefore, various materials have been used in the production of cheeses such as plastic drums, artificial bags and intestines [14-16].

The intestinal systems of humans and warm-blooded animals are complex ecosystems where 400 different microorganism species live together. The microorganisms found in this ecosystem are defined as "natural flora" and are divided into two groups as "beneficial" and "harmful" [17]. The functions of beneficial microorganisms in the intestinal system include assisting digestion of foods, protecting the host from pathogenic microorganisms, and promoting the defense mechanism of the host. In this context, microorganisms that have therapeutic effects on the health of the host by regulating the intestinal flora are described as "probiotics". The most important of these microorganisms include the genera *Lactobacillus, Bifidobacterium*, and *Enterococcus* [18].

A substantial increase in studies on the addition of probiotic microorganisms into various foods has been noted in recent years. The food groups to which the probiotic microorganisms are added the most include milk and dairy products [19]. The proportion of probiotic dairy products in total dairy production worldwide increases every year and is expected to continue increasing in the following years [20].

This study aimed to investigate the physicochemical and microbiological properties of Erzincan tulum cheeses, produced using different probiotic bacteria and packaging materials, during the storage period.

# MATERIAL and METHODS

### *Production of Tulum Cheeses*

In the production of tulum cheese, the production method applied by Dikici [21] has been modified. Sheep milk needed to produce cheese used in the research was obtained from the sheep farmers in the Erzincan provincial highlands and brought to a milk factory also operating in Erzincan province borders under cold chain with the milk tanks. After passing through the separator, the milk was cleaned and heat treated at 75°C for 15 sec. Then, pasteurized milk was coagulated with calf rennet (Mayasan Inc., Istanbul, Turkey) having a coagulation strength of 1:16000 MCU $mL^{-1}$ at 33°C for 90 min.

After the coagulation and the appropriate curding time, the curds were broken to the size of chickpea and filled in bags produced from cheesecloth-with an average weight of 5 kg and placed in a 20-22°C storage. Following the filling process, the cotton bags filled with curd were whirled around at regular intervals (1 h), and the whey was drained more easily and quickly.

After 24 h olmalı, the cotton bags were opened, and the curd was removed carefully without breaking it and was stuffed in tighter and roughly woven cotton cloths for 6-7 days of storage at 20-22°C. At the end of storage period, the curd was crushed to the size of a chickpea by hand and granulated Erzincan rock salt was added at 3.5% and stirred thoroughly until a homogenous form was obtained. Following this procedure activated probiotic bacterial cultures such as *Lactobacillus acidophilus* (Pro Lafti L-10) and *Bifidobacterium animalis* spp. *lactis* (Pro Lafti B-94) were added separately as $10^7$ cfu/mL minimum and filled in special bags that could contain 45-50 kg cheese. The cheeses were then pre-ripened in storage at 22-23°C for three days. Then, the cheese was thoroughly crumbled and filled in small skin bags or appendix or intestines.

The cheese bags were stored for 90 days at -1°C at 75-80% relative humidity. On the $2^{nd}$, $30^{th}$, $60^{th}$, and $90^{th}$ days of ripening, the physicochemical and microbiological analyses were conducted, and the results were evaluated comparatively.

## Methods

### *Physiochemical Analysis*

Titratable acidity was measured according to AOAC [22]. The pH was measured in a homogenate prepared by blending 10 g of a sample with 90 mL of distilled water for 30 s. pH values were obtained with glass electrode attached to a

Hanna pH meter (Model 2215, Hanna Instruments, USA). Water activity of the samples was recorded by Novasina TH-500 $a_w$ Sprint (Novasina, Axair Ltd., Switzerland).

### Microbial Analyses

Samples of 10 g were taken from the tulum cheese aseptically. A sterilized ringer solution at a dilution of 1:9 (w/v) was added, and the samples were homogenized for 3 min in a stomacher Lab-Blender 400 (London, UK). The serial decimal dilutions were sterilized and plated for bacterial counts [23,24].

Total aerobic mesophilic bacteria (TAMB), Total aerobic psychrophilic bacteria (TAPB) yeast/mold, lipolytic bacteria, proteolytic bacteria, Lactic Acid bacteria, *Lactoccocus* spp., *Listeria* spp., *Salmonella* spp., *Pseudomonas* spp., Total coliform, *Enterobacteriaceae*, *Staphylococcus aureus, Escherichia coli, Lactobacillus acidophilus* and *Bifidobacterium animalis* spp. *lactis* were counted using a spread plate technique *(Table 1, Table 2)*.

### Statistical Analysis

The research design was completely randomized having a factorial structure (3 x 3 x 4). The factors were probiotic *(Lactobacillus acidophilus*, *Bifidobacterium animalis* spp. *lactis,* and *Lactobacillus acidophilus* + *Bifidobacterium animalis* spp. *lactis)*, packaging (Skin bag, appendix, and intestine), and storage time (2, 30, 60 and 90 day). Three-way ANOVA was applied to data using procedure of the SPSS statistical package program (SPSS Inc., Chicago, IL) to do this analysis. Lsmeans values were generated and corresponding Duncan multiple comparison test. The treatment structure was completely randomized with 2 replications.

***Table 1.*** *Pre-enrichment broths used in microbiological analysis, incubation conditions and methods used*

| Microorganisms | Broth | Supplement | Incubation Conditions | Method Used |
|---|---|---|---|---|
| *Listeria* spp. | Fraser (Merck 1. 10398) | FLSS (Merck 1.0092)<br>FLAIS (Merck 1.0093) | 30°C - 24 h - aerobic | ISO 11290-1:2017 [25]<br>ISO 11290-2:2017 [26] |
| *Salmonella* spp. | NB (Merck 1.05443)<br>RVS (Merck 1.07700) | -- | 37°C - 24 h - aerobic<br>42°C - 24 h - aerobic | [27]<br>ISO 6579-1:2017 [28] |

***FLSS:*** *Fraser Listeria Selective Supplement;* ***FLAIS:*** *Fraser Listeria Ammonium Iron (III) Supplement;* ***NB:*** *Nutrient Broth;* ***RVS:*** *Rappaport Vassiliadis Salmonella Enrichment Broth*

***Table 2.*** *Analysis of the microorganism groups and reproduction condition*

| Microorganisms | Medium | Incubation Conditions | Method Used |
|---|---|---|---|
| TAMB | Plate Count Agar (Merck 1.05463) | 30°C - 48/72 h - aerobic | ISO 4833-2:2013 [29]<br>ISO 4833-2:2013 [30] |
| TAPB | Plate Count Agar (Merck 1.05463) | 4°C - 5/7 day - aerobic | [24] |
| Yeast/Mold | Potato Dextose Agar (Merck 1.10130) | 22°C - 4/5 day - aerobic | [24] |
| *Salmonella* spp. | Brilliant Green Phenol Red Lactose Sucrose Agar (Merck 1.10747.0500) | 37°C - 24/48 h - aerobic | ISO 6579-1:2017 [27,31] |
| *Pseudomonas* spp. | *Pseudomonas* Selective Agar Base (PSA) (Merck 1.07620) | 37°C - 24/48 h - aerobic | ISO 13720:2010 [32] |
| *Esherichia coli* | Chromocult TBX Agar (Merck 1.16122) | 44°C - 24/48 h - aerobic | ISO 16649-1:2001 [33]<br>ISO 16649-2:2001 [34]<br>ISO 16649-3:2015 [35] |
| *Enterobacteriaceae* | Eosin Methylene-Blue Lactose Sucrose Agar (Merck 1.01347) | 30°C - 24/48 h - aerobic | ISO 21528-2:2004 [36] |
| Total Coliform Group | Violet Red Bile Agar (Merck 1.01406) | 30°C - 24/48 h - aerobic | ISO 4832 [37] |
| *Staphylococcus aureus* | Baird Parker Agar (Merck 1.05406) | 37°C - 24/48 h - aerobic | ISO 6888-1 [38] |
| Lipolytic Bacteria | Tributyrin Agar (Merck 1.01957) | 30°C - 48/72 h - aerobic | [24] |
| Proteolytic Bacteria | Plate Count Skim Milk Agar (Merck 1.15338) | 21°C - 72 h - aerobic | [24] |
| *Lactobacillus acidophilus* | MRS-sorbitol Agar (Merck 1.10660) | 30°C - 24/48 h - anaerobic | [24] |
| *Bifidobacterium animalis spp. lactis* | Propionate agar (Merck 1.00043) | 30°C - 24/48 h - anaerobic | [24] |
| Lactic Acit Bacteria | MRS (Man Rogasa) Agar (Merck 1.10661) | 30°C - 24/48 h - anaerobic | [24] |
| *Lactococcus* spp. | M17 Agar (Merck 1.15108) | 30°C - 24/48 h - aerobic | [24] |
| *Listeria* spp. | Oxford (Merck 1.07004) | 37°C - 24/48 h - aerobic | ISO 11290-1:2017 [25]<br>ISO 11290-2:2017 [26] |
| *Brucella spp.* | Farrell's Agar (Oxoid CM 169) Brucella Sellective Supplement (Oxoid SR 83) | 37°C - 21 day 6% $CO_2$ | [24] |

***TAMB:*** *Total Aerobic Mesophilic Bacteria,* ***TAPB:*** *Total Aerobic Psychrophilic Bacteria*

## RESULTS

Probability values of physiochemical and microbiological analyses (P-values) for all main effects and interactions source of variation are presented in *Table 3* and *Table 4*. Chemical analysis results of Erzincan tulum cheese produced with the addition of probiotic bacteria packed using different packaging materials are shown in *Table 5* while microbiological analysis results are shown in *Table 6* and *Table 7*. During the three-month-storage period, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella spp.*, *Listeria* spp. and *Brucella* spp. were not detected. *Enterobacteriaceae* counts were <2 log cfu/g during the storage period.

***Table 3.*** *Probability values of physiochemical and microbiological analyses (p-values) for all main effects and interactions source of variation*

| Source of Variation | Acidity | pH | $a_w$ | TAMB | TAPB | Mold-Yeast | LAB |
|---|---|---|---|---|---|---|---|
| Probiotic | 0.049 | 0.422 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| Packaging | 0.427 | 0.193 | <.0001 | <.0001 | <.0001 | 0.037 | <.0001 |
| Storage time | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| Probiotic x Packaging | 0.186 | 0.012 | 0.271 | <.0001 | <.0001 | <.0001 | <.0001 |
| Probiotic x Storage Time | 0.031 | 0.047 | <.0001 | 0.057 | 0.199 | <.0001 | 0.075 |
| Packaging x Storage Time | 0.703 | 0.905 | <.0001 | 0.112 | 0.129 | 0.007 | 0.007 |
| Probiotic x Packaging x Storage Time | 0.208 | 0.233 | 0.005 | 0.024 | 0.022 | 0.010 | <.0001 |

***TAMB:*** *Total Aerobic Mesophilic Bacteria,* ***TAPB:*** *Total Aerobic Psychrophilic Bacteria,* ***LAB:*** *lactic Acid Bacteria*

***Table 4.*** *Probability values of microbiological analyses (p-values) for all main effects and interactions source of variation*

| Source of Variation | *Lactococcus* spp. | *Pseudomonas* spp. | Lipolytic Bacteria | Proteolytic Bacteria | Total Coliform | *Bifidobacterium animalis* spp. *lactis* | *Lactobacillus acidophilus* |
|---|---|---|---|---|---|---|---|
| Probiotic | <.0001 | <.0001 | <.0001 | <.0001 | -- | <.0001 | <.0001 |
| Packaging | <.0001 | 0.022 | 0.138 | 0.107 | -- | <.0001 | <.0001 |
| Storage Time | <.0001 | <.0001 | <.0001 | <.0001 | -- | <.0001 | <.0001 |
| Probiotic x Packaging | <.0001 | 0.017 | <.0001 | 0.663 | -- | <.0001 | <.0001 |
| Probiotic x Storage time | 0.070 | <.0001 | <.0001 | 0.001 | -- | 0.006 | 0.005 |
| Packaging x Storage Time | 0.009 | 0.817 | 0.061 | 0.480 | -- | 0.042 | 0.704 |
| Probiotic x Packaging x Storage Time | <.0001 | 0.799 | 0.344 | 0.798 | -- | 0.618 | 0.447 |

***Table 5.*** *Changes in the physicochemical and microbiological analysis results of tulum cheese samples during storage*

| Samples | % Acidity | | | | pH | | | | $a_w$ | | | | TAMB | | | | TAPB | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Storage Time (Day) | | | | Storage Time (Day) | | | | Storage Time (Day) | | | | Storage Time (Day) | | | | Storage Time (Day) | | | |
| | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 |
| TCBS | 0.84$^{d}$ | 0.92$^{c}$ | 0.98$^{b}$ | 1.19$^{a}$ | 5.08$^{a}$ | 4.91$^{b}$ | 4.80$^{c}$ | 4.64$^{d}$ | 0.934$^{a}$ | 0.926$^{b}$ | 0.911$^{c}$ | 0.897$^{d}$ | 5.14$^{d}$ | 5.34$^{c}$ | 5.55$^{b}$ | 5.63$^{a}$ | 3.18$^{d}$ | 3.38$^{c}$ | 3.47$^{b}$ | 3.67$^{a}$ |
| TCBC | 0.85$^{d}$ | 0.90$^{c}$ | 0.98$^{b}$ | 1.33$^{a}$ | 5.06$^{a}$ | 4.92$^{b}$ | 4.80$^{c}$ | 4.54$^{d}$ | 0.929$^{a}$ | 0.910$^{b}$ | 0.906$^{c}$ | 0.898$^{d}$ | 4.12$^{d}$ | 4.70$^{c}$ | 5.03$^{b}$ | 5.32$^{a}$ | 2.16$^{d}$ | 2.74$^{c}$ | 3.07$^{b}$ | 3.36$^{a}$ |
| TCBİ | 0.86$^{d}$ | 0.92$^{c}$ | 0.98$^{b}$ | 1.33$^{a}$ | 5.03$^{a}$ | 4.92$^{b}$ | 4.77$^{c}$ | 4.54$^{d}$ | 0.931$^{a}$ | 0.912$^{b}$ | 0.899$^{c}$ | 0.886$^{c}$ | 5.19$^{d}$ | 5.41$^{c}$ | 5.61$^{b}$ | 5.68$^{a}$ | 3.23$^{d}$ | 3.36$^{c}$ | 3.65$^{b}$ | 3.72$^{a}$ |
| TCLS | 0.85$^{d}$ | 0.91$^{c}$ | 0.98$^{b}$ | 1.21$^{a}$ | 5.07$^{a}$ | 4.89$^{b}$ | 4.78$^{c}$ | 4.63$^{d}$ | 0.933$^{a}$ | 0.914$^{b}$ | 0.899$^{c}$ | 0.883$^{d}$ | 5.25$^{d}$ | 5.43$^{c}$ | 5.58$^{b}$ | 5.69$^{a}$ | 3.22$^{d}$ | 3.41$^{c}$ | 3.58$^{b}$ | 3.75$^{a}$ |
| TCLC | 0.85$^{d}$ | 0.90$^{c}$ | 0.97$^{b}$ | 1.04$^{a}$ | 5.04$^{a}$ | 4.97$^{b}$ | 4.82$^{c}$ | 4.74$^{d}$ | 0.930$^{a}$ | 0.906$^{b}$ | 0.894$^{c}$ | 0.881$^{d}$ | 5.21$^{d}$ | 5.41$^{c}$ | 5.57$^{b}$ | 5.71$^{a}$ | 3.25$^{d}$ | 3.45$^{c}$ | 3.61$^{b}$ | 3.75$^{a}$ |
| TCLİ | 0.86$^{d}$ | 0.91$^{c}$ | 0.99$^{b}$ | 1.17$^{a}$ | 5.00$^{a}$ | 4.85$^{b}$ | 4.76$^{c}$ | 4.65$^{d}$ | 0.930$^{a}$ | 0.904$^{b}$ | 0.887$^{c}$ | 0.849$^{d}$ | 5.21$^{d}$ | 5.40$^{c}$ | 5.56$^{b}$ | 5.66$^{a}$ | 3.25$^{d}$ | 3.45$^{c}$ | 3.60$^{b}$ | 3.69$^{a}$ |
| TCMS | 0.86$^{d}$ | 0.90$^{c}$ | 0.97$^{b}$ | 1.27$^{a}$ | 4.96$^{a}$ | 4.88$^{b}$ | 4.78$^{c}$ | 4.60$^{d}$ | 0.933$^{a}$ | 0.916$^{b}$ | 0.904$^{c}$ | 0.893$^{d}$ | 4.83$^{d}$ | 5.10$^{c}$ | 5.32$^{b}$ | 5.58$^{a}$ | 2.87$^{d}$ | 3.14$^{c}$ | 3.36$^{b}$ | 3.62$^{a}$ |
| TCMC | 0.85$^{d}$ | 0.91$^{c}$ | 0.98$^{b}$ | 1.06$^{a}$ | 5.07$^{a}$ | 4.83$^{b}$ | 4.80$^{c}$ | 4.76$^{d}$ | 0.925$^{a}$ | 0.908$^{b}$ | 0.899$^{c}$ | 0.893$^{d}$ | 5.25$^{d}$ | 5.42$^{c}$ | 5.63$^{b}$ | 5.77$^{a}$ | 3.35$^{d}$ | 3.52$^{c}$ | 3.72$^{b}$ | 3.86$^{a}$ |
| TCMİ | 0.85$^{d}$ | 0.90$^{c}$ | 0.97$^{b}$ | 1.09$^{a}$ | 5.08$^{a}$ | 4.97$^{b}$ | 4.79$^{c}$ | 4.71$^{d}$ | 0.940$^{a}$ | 0.921$^{b}$ | 0.885$^{c}$ | 0.858$^{d}$ | 5.06$^{d}$ | 5.24$^{c}$ | 5.40$^{b}$ | 5.56$^{a}$ | 3.21$^{d}$ | 3.39$^{c}$ | 3.54$^{b}$ | 3.71$^{a}$ |

***TCBS:*** *Tulum Cheese Bifidobacterium animalis spp. lactic Skin Bag,* ***TCBC:*** *Tulum Cheese Bifidobacterium animalis spp. lactic appendix,* ***TCBİ:*** *Tulum Cheese Bifidobacterium animalis spp. lactic intestine,* ***TCLS:*** *Tulum Cheese Lactobacillus acidophilus Skin Bag,* ***TCLC:*** *Tulum Cheese Lactobacillus acidophilus appendix,* ***TCLİ:*** *Tulum Cheese Lactobacillus acidophilus intestine,* ***TCMS:*** *Tulum Cheese Mix Skin Bag,* ***TCMC:*** *Tulum Cheese Mix appendix,* ***TCMİ:*** *Tulum Cheese Mix intestine,* ***a - d (→):*** *Values with the same capital letters in the same rows for each analysis differ significantly (P<0.05)*

**Table 6.** *Changes in the microbiological analysis results of tulum cheese samples during storage*

| Samples | Mold-Yeast | | | | Lactic Acid Bacteria | | | | *Lactococcus* spp. | | | | *Pseudomonas* spp. | | | | Lipolytic Bacteria | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Storage Time (Day) | | | | Storage Time (Day) | | | | Storage Time (Day) | | | | Storage Time (Day) | | | | Storage Time (Day) | | | |
| | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 |
| TCBS | 3.70$^{d}$ | 4.29$^{c}$ | 4.76$^{b}$ | 5.01$^{a}$ | 4.98$^{d}$ | 5.12$^{c}$ | 5.29$^{b}$ | 5.43$^{a}$ | 3.95$^{d}$ | 4.10$^{c}$ | 4.26$^{b}$ | 4.41$^{a}$ | 2.76$^{d}$ | 3.35$^{c}$ | 3.82$^{b}$ | 4.06$^{a}$ | 4.03$^{d}$ | 4.60$^{c}$ | 4.90$^{b}$ | 5.22$^{a}$ |
| TCBC | 3.55$^{d}$ | 4.23$^{c}$ | 4.66$^{b}$ | 4.89$^{a}$ | 3.50$^{d}$ | 4.14$^{c}$ | 4.61$^{b}$ | 4.94$^{a}$ | 2.47$^{d}$ | 3.11$^{c}$ | 3.58$^{b}$ | 3.91$^{a}$ | 2.61$^{d}$ | 3.28$^{c}$ | 3.71$^{b}$ | 3.95$^{a}$ | 4.11$^{d}$ | 4.42$^{c}$ | 4.74$^{b}$ | 5.06$^{a}$ |
| TCBİ | 3.48$^{d}$ | 4.11$^{c}$ | 4.58$^{b}$ | 4.72$^{a}$ | 4.99$^{d}$ | 5.27$^{c}$ | 5.52$^{b}$ | 5.66$^{a}$ | 3.96$^{d}$ | 4.24$^{c}$ | 4.49$^{b}$ | 4.63$^{a}$ | 2.53$^{d}$ | 3.17$^{c}$ | 3.63$^{b}$ | 3.77$^{a}$ | 4.09$^{d}$ | 4.42$^{c}$ | 4.63$^{b}$ | 5.03$^{a}$ |
| TCLS | 2.48$^{d}$ | 3.64$^{c}$ | 4.29$^{b}$ | 4.81$^{a}$ | 4.88$^{d}$ | 5.17$^{c}$ | 5.42$^{b}$ | 5.62$^{a}$ | 3.85$^{d}$ | 4.15$^{c}$ | 4.39$^{b}$ | 4.59$^{a}$ | <2 | 2.70$^{c}$ | 3.34$^{b}$ | 3.86$^{a}$ | 3.86$^{d}$ | 4.52$^{c}$ | 4.90$^{b}$ | 5.11$^{a}$ |
| TCLC | 1.89$^{d}$ | 3.79$^{c}$ | 4.31$^{b}$ | 4.74$^{a}$ | 5.06$^{d}$ | 5.29$^{c}$ | 5.50$^{b}$ | 5.62$^{a}$ | 4.04$^{d}$ | 4.26$^{c}$ | 4.47$^{b}$ | 4.60$^{a}$ | <2 | 2.85$^{c}$ | 3.37$^{b}$ | 3.80$^{a}$ | 3.91$^{d}$ | 4.50$^{c}$ | 4.86$^{b}$ | 5.28$^{a}$ |
| TCLİ | 2.05$^{d}$ | 3.87$^{c}$ | 4.40$^{b}$ | 4.73$^{a}$ | 5.09$^{d}$ | 5.33$^{c}$ | 5.50$^{b}$ | 5.61$^{a}$ | 4.06$^{d}$ | 4.31$^{c}$ | 4.47$^{b}$ | 4.58$^{a}$ | <2 | 2.93$^{c}$ | 3.46$^{b}$ | 3.79$^{a}$ | 4.00$^{d}$ | 4.79$^{c}$ | 5.02$^{b}$ | 5.21$^{a}$ |
| TCMS | 3.41$^{d}$ | 3.85$^{c}$ | 4.54$^{b}$ | 4.88$^{a}$ | 4.52$^{d}$ | 4.77$^{c}$ | 5.05$^{b}$ | 5.41$^{a}$ | 3.49$^{d}$ | 3.74$^{c}$ | 4.02$^{b}$ | 4.38$^{a}$ | 3.77$^{d}$ | 4.22$^{c}$ | 4.24$^{b}$ | 4.64$^{a}$ | 4.14$^{d}$ | 4.95$^{c}$ | 5.12$^{b}$ | 5.27$^{a}$ |
| TCMC | 3.99$^{d}$ | 4.48$^{c}$ | 4.88$^{b}$ | 5.16$^{a}$ | 5.01$^{d}$ | 5.33$^{c}$ | 5.52$^{b}$ | 5.62$^{a}$ | 3.99$^{d}$ | 4.30$^{c}$ | 4.49$^{b}$ | 4.59$^{a}$ | 3.66$^{d}$ | 4.07$^{c}$ | 4.44$^{b}$ | 4.62$^{a}$ | 4.23$^{d}$ | 4.88$^{c}$ | 5.24$^{b}$ | 5.31$^{a}$ |
| TCMİ | 3.97$^{d}$ | 4.61$^{c}$ | 4.83$^{b}$ | 5.07$^{a}$ | 4.98$^{d}$ | 5.16$^{c}$ | 5.31$^{b}$ | 5.40$^{a}$ | 3.95$^{d}$ | 4.13$^{c}$ | 4.28$^{b}$ | 4.37$^{a}$ | 3.59$^{d}$ | 3.95$^{c}$ | 4.22$^{b}$ | 4.54$^{a}$ | 4.38$^{d}$ | 5.02$^{c}$ | 5.19$^{b}$ | 5.32$^{a}$ |

***TCBS:*** *Tulum Cheese Bifidobacterium animalis spp. lactic Skin Bag,* ***TCBC:*** *Tulum Cheese Bifidobacterium animalis spp. lactic appendix,* ***TCBİ:*** *Tulum Cheese Bifidobacterium animalis spp. lactic intestine,* ***TCLS:*** *Tulum Cheese Lactobacillus acidophilus Skin Bag,* ***TCLC:*** *Tulum Cheese Lactobacillus acidophilus appendix,* ***TCLİ:*** *Tulum Cheese Lactobacillus acidophilus intestine,* ***TCMS:*** *Tulum Cheese Mix Skin Bag,* ***TCMC:*** *Tulum Cheese Mix appendix,* ***TCMİ:*** *Tulum Cheese Mix intestine,* ***a -d (→):*** *Values with the same capital letters in the same rows for each analysis differ significantly (P<0.05)*

**Table 7.** *Changes in the microbiological analysis results of tulum cheese samples during storage*

| Samples | Proteolytic Bacteria | | | | Total Coliform | | | | *Bifidobacterium animalis* spp. *lactis* | | | | *Lactobacillus acidophilus* | | | | Total *Enterobacteriaceae* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Storage Time (Day) | | | | Storage Time (Day) | | | | Storage Time (Day) | | | | Storage Time (Day) | | | | Storage Time (Day) | | | |
| | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 | 2 | 30 | 60 | 90 |
| TCBS | 4.02$^{d}$ | 4.79$^{c}$ | 5.20$^{b}$ | 5.42$^{a}$ | 3.10 | 2.46 | <2 | <2 | 5.39$^{a}$ | 5.34$^{b}$ | 5.16$^{c}$ | 4.99$^{d}$ | 3.27$^{a}$ | 3.18$^{b}$ | 3.05$^{c}$ | 3.02$^{c}$ | <2 | <2 | <2 | <2 |
| TCBC | 4.00$^{d}$ | 4.75$^{c}$ | 5.01$^{b}$ | 5.41$^{a}$ | 2.45 | <2 | <2 | <2 | 6.19$^{a}$ | 6.09$^{b}$ | 6.03$^{c}$ | 5.94$^{d}$ | 3.34$^{a}$ | 3.25$^{b}$ | 3.18$^{c}$ | 3.01$^{d}$ | <2 | <2 | <2 | <2 |
| TCBİ | 3.96$^{d}$ | 4.73$^{c}$ | 5.06$^{b}$ | 5.40$^{a}$ | <2 | <2 | <2 | <2 | 5.72$^{a}$ | 5.65$^{b}$ | 5.42$^{c}$ | 5.06$^{d}$ | 3.23$^{a}$ | 3.07$^{b}$ | 2.95$^{c}$ | 2.88$^{d}$ | <2 | <2 | <2 | <2 |
| TCLS | 3.94$^{d}$ | 4.80$^{c}$ | 5.19$^{b}$ | 5.31$^{a}$ | <2 | <2 | <2 | <2 | 3.55$^{a}$ | 3.31$^{b}$ | 3.07$^{c}$ | 2.91$^{d}$ | 5.42$^{a}$ | 5.25$^{b}$ | 5.11$^{c}$ | 4.94$^{d}$ | <2 | <2 | <2 | <2 |
| TCLC | 4.02$^{d}$ | 4.78$^{c}$ | 5.13$^{b}$ | 5.27$^{a}$ | 2.85 | <2 | <2 | <2 | 3.43$^{a}$ | 3.21$^{b}$ | 3.03$^{c}$ | 2.75$^{d}$ | 5.39$^{a}$ | 5.26$^{b}$ | 5.17$^{c}$ | 5.03$^{d}$ | <2 | <2 | <2 | <2 |
| TCLİ | 3.44$^{d}$ | 4.82$^{c}$ | 5.03$^{b}$ | 5.23$^{a}$ | <2 | <2 | <2 | <2 | 3.41$^{a}$ | 3.19$^{b}$ | 2.75$^{c}$ | 2.55$^{d}$ | 5.42$^{a}$ | 5.26$^{b}$ | 5.14$^{c}$ | 4.94$^{d}$ | <2 | <2 | <2 | <2 |
| TCMS | 4.71$^{d}$ | 4.66$^{c}$ | 5.40$^{b}$ | 5.58$^{a}$ | 2.57 | <2 | <2 | <2 | 5.36$^{a}$ | 5.15$^{b}$ | 5.02$^{c}$ | 4.87$^{d}$ | 5.25$^{a}$ | 5.18$^{b}$ | 5.13$^{c}$ | 4.86$^{d}$ | <2 | <2 | <2 | <2 |
| TCMC | 4.61$^{d}$ | 5.01$^{c}$ | 5.38$^{b}$ | 5.59$^{a}$ | <2 | <2 | <2 | <2 | 5.36$^{a}$ | 5.14$^{b}$ | 5.02$^{c}$ | 4.85$^{d}$ | 5.36$^{a}$ | 5.22$^{b}$ | 5.13$^{c}$ | 4.90$^{d}$ | <2 | <2 | <2 | <2 |
| TCMİ | 4.53$^{d}$ | 4.89$^{c}$ | 5.16$^{b}$ | 5.49$^{a}$ | <2 | <2 | <2 | <2 | 5.25$^{a}$ | 5.18$^{b}$ | 5.05$^{c}$ | 4.73$^{d}$ | 5.34$^{a}$ | 5.25$^{b}$ | 5.12$^{c}$ | 4.90$^{d}$ | <2 | <2 | <2 | <2 |

***TCBS:*** *Tulum Cheese Bifidobacterium animalis spp. lactic Skin Bag,* ***TCBC:*** *Tulum Cheese Bifidobacterium animalis spp. lactic appendix,* ***TCBİ:*** *Tulum Cheese Bifidobacterium animalis spp. lactic intestine,* ***TCLS:*** *Tulum Cheese Lactobacillus acidophilus Skin Bag,* ***TCLC:*** *Tulum Cheese Lactobacillus acidophilus appendix,* ***TCLİ:*** *Tulum Cheese Lactobacillus acidophilus intestine,* ***TCMS:*** *Tulum Cheese Mix Skin Bag,* ***TCMC:*** *Tulum Cheese Mix appendix,* ***TCMİ:*** *Tulum Cheese Mix intestine,* ***a - d (→):*** *Values with the same capital letters in the same rows for each analysis differ significantly (P<0.05)*

# DISCUSSION

Probiotic addition (P<0.05), storage time (P<0.0001), and probiotic x storage period interactions (P<0.05) were statistically significant on the acidity values of Erzincan tulum cheeses produced with probiotic bacteria and stored in different packages (P<0.05) *(Table 3)*. Acidity values of the samples were found to increase during storage (P<0.0001), and the highest acidity value was determined in TCBI samples with 1.36% *(Table 5)* at the end of storage, which could be attributed to the probiotic bacteria added during the production stage. Andiç et al.[39] and Çakır [40] have reported that the acidity values of tulum cheeses increase with an increase in the storage period.

Storage period (P<0.0001), probiotic bacteria x packaging (P<0.05) and probiotic x storage time (P<0.05) interactions influenced the pH values of the samples *(Table 3)*. During the storage period, the pH values of all cheese samples decreased (P<0.05) *(Table 5)*. Because of the 90-day-storage period, the lowest pH value was determined in TCBI with 4.54, and the addition of probiotic bacteria was associated with a decrease in pH values. Keles and Atasever [4] and Morul and İşleyici [41] have similarly reported that the pH values of tulum cheeses decreased during the storage period.

Our results indicate that the increase regarding milk acidity and the decrease in pH values in all the samples during

storage period was due to the lactic acid produced by the activities of starter and non-starter bacteria found in the cheese which could ferment lactose.

Probiotic bacteria x Packaging x Storage Period *(Table 3)* had a significant effect on the water activity of the tulum cheeses. During the storage period, the water activity ($a_w$) of all tulum cheese samples decreased (P<0.05) *(Table 5)*. At the beginning of storage, the highest $a_w$ value was determined in TCMI sample with 0.940, whereas the lowest aw value at the end of the 90-day-storage period was determined in TCLI sample with 0.849. Erdem and Patır [42] have reported that the average $a_w$ values in tulum cheeses range between 0.910 and 0.930. However, and Morul and İşleyici [41] determined these values to range between 0.870 and 0.980. The differences between the studies were associated with the differences in the packing materials and storage period.

The effect of Probiotic bacteria x Packaging x Storage Period interactions had a significant effect on TAMB, TAPB, and yeast/mold counts *(Table 3)* (P<0.05). TAMB counts increased in all tulum cheese samples during the storage period (P<0.05) *(Table 5)*. The highest increase was found to be in TCBC with 1.2 log cfu/g. The results obtained were lower than those reported by Çağlar [43] thus, corroborating the results of Güven and Konar [44], Hayaloglu et al.[45], and Çakır [40].

Similarly, TAPB counts increased in all tulum cheese samples during the storage period (P<0.05) *(Table 5)*. At the beginning of the storage period, the lowest TAPB count was in TCBC with 2.16 log CFU/g whereas the highest value was in TCMC sample with 3.86 log cfu/g. Morul and İşleyici [41] have reported the TAPB counts in Divle tulum cheeses were between 2.70 and 8.48 log cfu/g while Kara and Akkaya [46] have reported the TAPB counts in Afyon tulum cheeses to range between 3.07 and 5.83 log cfu/g.

The total yeast/mold counts in cheese samples increased during the storage period (P<0.05) *(Table 6)*. The highest increase was in the TCLC sample with 2.85 log cfu/g, and the lowest increase was in the TCLS with 1.29 log cfu/g. These results are lower than those reported by Çağlar [43], Çetin et al.[47], Hayaloğlu et al.[45], and similar to those reported by Öksüztepe et al.[48]. The differences regarding TAMB, TAPB, and yeast/mold counts from similar previous studies was associated with the microbial quality of the milk used and the differences in hygiene and sanitation conditions during production.

The effects of adding probiotic bacteria, packaging, storage period, probiotic bacteria x packaging and probiotic bacteria x packaging interaction, probiotic bacteria x packaging x storage period interactions on lactic acid bacteria counts and *Lactococci* counts were statistically significant at P<0.01 level while probiotic bacteria x storage period was statistically significant at P<0.05 level *(Table 4)*.

Lactic acid bacteria count increased by an average of 0.5 log cfu/g during the entire storage period, The highest increase was determined in TCLS with 0.75 log cfu/g (P<0.05) *(Table 6)*. The results obtained in this study are higher those reported by Güven and Konar [44], lower than those of Morul and İşleyici [41] and Kara and Akkaya [46] and similar to those of Çetin et al.[47]. The difference between the results can be associated with the presence of probiotic bacteria used in production, packaging materials, and storage conditions.

There was an increase in the *Lactococci* count during the storage period (P<0.05) *(Table 4)*. The highest increase was determined in the TCMS sample *(Table 6)*. Kara and Akkaya [46], in their study on the determination of microbiological and physicochemical properties and lactic acid bacteria distribution of Afyon tulum cheeses, reported that the average *Lactococci* counts similar to those obtained in the present study.

There was an increase in the *Pseudomonas* count during storage in tulum cheese, and the highest increase was found in TCMS (P<0.05) *(Table 6)*. Morul and İşleyici [41] reported the average *Pseudomonas* counts to be 3.60 log cfu/g, which is similar to the counts reported in the present study.

The lipolytic bacterial counts of the samples increased during storage (P<0.05) *(Table 6)*. At the end of the 90-day-storage period, the highest numbers of lipolytic bacteria were detected in TCMC with 5.30 log cfu/g. Kara and Akkaya [46] have reported that the average lipolytic bacteria counts in Afyon tulum cheeses ranged between 2.55 and 4.60 log cfu/g. Similarly, the proteolytic bacteria count increased by an average of 1 log cfu/g during storage *(Table 6)*. The highest increase was found to be at TCLS with 1.32 log cfu/g. Kara and Akkaya [46] determined the average proteolytic bacterial counts to be 2.55 log cfu/g. The difference between the studies was attributed to the packaging material used and the duration of the storage period.

Bacterial counts of *Bifidobacterium animalis* spp. *lactis* species decreased throughout the entire storage period (P<0.05) *(Table 7)*. The highest bacterial count was determined in the TCBC sample with 5.93 log cfu/g.

Similar to the decrease in the Bifidobacterium bacteria counts, the number of *Lactobacillus acidophilus* were determined to decrease by an average of 0.5 log cfu/g during 90 days of storage (P<0.05) *(Table 7)*. The highest decrease was in the TCLS sample with 0.5 log cfu/g, and the lowest decrease was in TCBS with 0.25 log cfu/g.

The decrease in *Bifidobacterium animalis* spp. *lactis* and *Lactobacillus acidophilus* bacterial counts are associated with a decrease in $a_w$ value and the increase in lactic acid bacteria and *Lactococci* counts and acidity percentage during the storage period.

Erzincan tulum cheese is a local cheese produced traditionally in Turkey. Pasteurization is not commonly used in traditional cheese production methods. This study aimed to produce a pasteurized novel dairy product with a good taste and rich nutritional value, which does not pose a health risk.

Moreover, the addition of probiotic bacteria to the tulum cheese increases the functional properties of the cheese that has beneficial effects on human health, particularly the digestive system.

Some adverse effects such as odor originating from the skin bag used in traditional Erzincan tulum cheese production were avoided by using different packaging materials. The taste and aroma of the product were enhanced to produce a more appealing product with aesthetic and health benefits for the food industry in both Turkey and the world.

## REFERENCES

**1. Akın N:** Temel Peynir Bilimi-I. Damla Ofset, Konya, Turkey, 2010.

**2. Çetinkaya A:** Yöresel Peynirlerimiz. 212, Academic Book Production, Kars, Turkey 2005.

**3. Beresford TP, Fitzsimons NA, Brennan NL, Cogan TM:** Recent advances in cheese microbiology. *Int Dairy J*, 11 (4-7): 259-274, 2001. DOI: 10.1016/S0958-6946(01)00056-5

**4. Demir P, Öksüztepe G, İncili KG, İlhak Oİ:** Vakum paketli Şavak tulum peynirlerinde potasyum sorbatın kullanımı. *Kafkas Univ Vet Fak Derg,* 23 (1): 23-30, 2017. DOI: 10.9775/kvfd.2016.15707

**5. Karaibrahimoğlu Y, Üçüncü M:** Determination of process and product parameters of Erzincan tulum cheese. *E U Muhendislik Fak Derg,* 6 (2): 79-97, 1988

**6. Çakmakçı S, Çakır Y:** Erzincan tulum (Savak) cheese and our similar cheese. *3. Traditional Foods Symposium,* 10-12 May, 218, Konya, Turkey, 2012.

**7. Çakmakçı S, Dağdemir E, Hayaloğlu AA, Gürses M, Gündoğdu E:** Influence of ripening container on the lactic acid bacteria population in tulum cheese. *World J Microbiol Biotechnol*, 24, 293-299, 2008. DOI: 10.1007/s11274-007-9470-z

**8. Şengül M:** Starter culture characteristics of some lactic acid bacteria strains isolated from tulum cheese and determination of some properties of cheese. *PhD Thesis,* Atatürk University, Institute of Natural and Applied Science, Turkey, 2001.

**9. Yilmaz G, Ayar A, Akin N:** The effect of microbial lipase on the lipolysis during the ripening of tulum cheese. *J Food Eng,* 69 (3): 269-274, 2005. DOI: 10.1016/j.jfoodeng.2004.08.017

**10. Öner Z, Şimşek B, Sağdıç O:** Determination of some properties of Turkish tulum cheese. *Milchwissenschaft,* 58 (3-4): 152-154, 2003.

**11. Kurt A, Çakmakçı S, Çağlar A, Akyüz N:** A study on the structure, sensory, physical and chemical properties of Erzincan tulum (Savak) cheese. *Food*, 16 (5): 295-302, 1991.

**12. Şengül M, Çakmakçı S:** Microbial quality of tulum cheeses marketed in eastern part of Turkey. *International Dairy Symposium,* 24-28 May, 96-99, Isparta, Turkey, 2004.

**13. Şengül M, Türkoğlu H, Çakmakçı S, Çon AH:** Effects of casing materials and ripening period on some microbiological properties of tulum cheese. *Pak J Biol Sci*, 4 (7): 854-857, 2001. DOI: 10.3923/pjbs.2001.854.857

**14. Çakmakçı S:** Development of traditional method production and production technology of Erzincan tulum (Savak) cheese. *V. Milk and Dairy Products Symposium,* 21-22 May, Tekirdağ, MPM Publications No: 621, 117-125s, Ankara, Turkey, 1998.

**15. Akarca G, Tomar O, Gök V:** Effect of different packaging methods on the quality of stuffed and sliced mozzarella cheese during storage. *J Food Process Preserv,* 39 (6): 2912-2918, 2015. DOI: 10.1111/jfpp.12542

**16. Çağlar A, Bor Y, Tomar O, Beykaya M, Gök V:** Mechanical and microbiological properties of natural casings using in meat products. *Kafkas Univ Vet Fak Derg,* 24 (3): 327-334, 2018. DOI: 10.9775/kvfd.2017.18885

**17. Çakır İ:** Determination of some probiotic properties in *Lactobacillus* and *Bifidobacter. PhD Thesis,* Ankara University, Institute of Natural and Applied Science, Turkey, 2003.

**18. Salminen S:** Probiotics: Scientific support for use. *Food Technol*, 53 (11): 66-77, 1999.

**19. Gibson GR, Fuller R:** The role of probiotics and prebiotics in the functional food concept. **In,** Sadler MJ, Saltmarsh M (Eds): Functional Foods: The Consumer, The Products and the Evidence. 3-14, Royal Society of Chemistry, Cambridge, 1998.

**20. Dave RI, Shah NP:** Viability of yoghurt and probiotic bacteria in yoghurt made from commercial starter cultures. *Int Dairy J*, 7 (1): 31-41, 1997. DOI: 10.1016/S0958-6946(96)00046-5

**21. Dikici A:** During the production and development of Şavak tulum cheese investigation of life and acid adaptation capability of *Escherichia coli* O157: H7, *Listeria monocytogenes* and *Salmonella*. *PhD Thesis,* Fırat University, Institute of Health Sciences, Turkey, 2008.

**22. AOAC:** Official Methods of Analysis. Official Method 937.05Lactic Acid in Milk and Milk Products. Assoction of Official Analytical Chemists, Washington, 2000.

**23. TSE:** Turkish Standard Institute, TS 6235 EN ISO 6887-1. Microbiology of Food and Animal Feed. General Rules of The Initial Suspension and Decimal Dilutions of Test Samples Preparation, 2001.

**24. Halkman K:** Gıda mikrobiyolojisi uygulamaları. Başak Matbaacılık Ltd. Şti, Ankara, Turkey, 2005.

**25. ISO:** International Standard Organization, 11290-1:2017 Microbiology of the food chain, Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. - Part 1: Detection method, 2017.

**26. ISO:** International Standard Organization. 11290-2:2017 Microbiology of the food chain, Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. - Part 2: Enumeration method, 2017.

**27. Flowers RS, D'aust JY, Andrews WH, Bailey JS:** *Salmonella.* **In**, Vanderzant C, Spilttstoesser DF (Eds): Compendium of the Methods for the Microbiological Examinations of Foods. 371-422, American Public Health Association, 1992.

**28. ISO:** International Standard Organization, 6579-1:2017 Horizontal method for the detection, enumeration and serotyping of *Salmonella*, 2017.

**29. ISO:** International Standard Organization, 4833-2:2013 Horizontal method for the enumeration of microorganisms - Part 2: Colony count at 30 degrees C by the surface plating technique, 2013.

**30. ISO:** International Standard Organization, 4833-1:2013 Microbiology of the food chain - Horizontal method for the enumeration of micro-organisms - Part 1: Colony count at 30 degrees C by the pour plate technique, 2013.

**31. ISO:** International Standard Organization, 6579-1:2017 Horizontal method for the detection, enumeration and serotyping of *Salmonella*, 2017.

**32. ISO:** International Standard Organization, 13720:2010 specifies a method for the enumeration of presumptive *Pseudomonas* spp. present in meat and meat products, including poultry, 2010.

**33. ISO:** International Standard Organization, 16649-1:2001 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* - Part 1: Colony-count technique at 44 degrees C using membranes and 5-bromo-4-chloro-3-indolyl beta-D-glucuronide, 2001.

**34. ISO:** International Standard Organization, 16649-2:2001 Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration

of beta-glucuronidase-positive *Escherichia coli* - Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide, 2001.

**35. ISO:** International Standard Organization, 16649-3:2015 Microbiology of the food chain- Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* - Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide, 2015.

**36. ISO:** International Standard Organization, ISO 21528-2:2004 Microbiology of food and animal feeding stuffs Horizontal methods for the detection and enumeration of *Enterobacteriaceae.* 2004.

**37. ISO:** International Standard Organization, 4832 General Guidance fort the Enumeration of Coliforms Colony Count Technique, 1991.

**38. ISO:** International Standard Organization, 6888-1 Horizontal Method for the Enumeration of Coagulase- positive Staphylococci Technique using Baird Parker Agar Medium, 1999.

**39. Andiç S, Tunçtürk Y, Gençcelep H:** Effect of freezing storage and vacuum packaging on lipolysis and organic acid exchange in motal cheese. *Food*, 35 (6): 423-430, 2010.

**40. Çakır Y:** The influence of some quality characteristics of Erzincan tulum cheese on *(Nigella sativa* L.) Nigella. *Msc Thesis,* Atatürk University, Institute of Natural and Applied Science, Turkey, 2012.

**41. Morul F, İşleyici Ö:** Chemical and microbiological properties of Divle tulum cheese. *Y Y U Vet Fak Derg*, 23 (2): 71-76, 2012.

**42. Erdem G, Patır B:** Elazığ'da tüketime sunulan tulum peynirlerinde histamin düzeyleri ile bazı kimyasal kalite parametreleri üzerine araştırmalar. *F Ü Sağ Bil Vet Derg,* 31 (3): 235-241, 2017.

**43. Çağlar A:** Çiğ sütten üretilen ve farklı ambalajlama materyallerinde olgunlaştırılan Erzincan tulum peynirlerinin mikrobiyolojik özelliklerindeki değişmeler. *Atatürk Üniv Ziraat Fak Derg*, 32 (3): 285-292, 2001.

**44. Güven M, Konar A:** İnek sütlerinden üretilen ve farklı ambalajlarda olgunlaştırılan tulum peynirlerinin mikrobiyolojik özellikleri. *Gıda*, 19 (3): 179-185, 1994.

**45. Hayaloğlu AA, Çakmakçı S, Brenchany EG, Deegan KC, McSweeney PLH:** Microbiology, biochemistry and volatile composition of tulum cheese ripened in goat's skin or plastic bags. *J Dairy Sci,* 90 (3): 1102-1121, 2007. DOI: 10.3168/jds.S0022-0302(07)71597-7

**46. Kara R, Akkaya L:** Afyon tulum peynirinin mikrobiyolojik ve fiziko-kimyasal özellikleri ile laktik asit bakteri dağılımlarının belirlenmesi. A*KU J Sci Eng,* 15 (1): 1-6, 2015. DOI: 10.5578/fmbd.8717

**47. Çetin B, Gürses M, Şengül M:** Effect of relative humidity change on some microbiological properties of tulum cheese. *Turkey 9. Food Congress,* 24-26 May, Bolu, Turkey, 2006.

**48. Öksüztepe G, Patır B, Çalıcıoğlu M:** Identification and distribution of lactic acid bacteria during the ripening of Şavak tulum cheese. *Turk J Vet Anim Sci,* 29 (3): 873-879, 2005.

Kafkas Univ Vet Fak Derg
24 (5): 655-662, 2018
DOI: 10.9775/kvfd.2018.19670

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# The Influence of Carcass Microlocation on the Speed of Postmortem Changes and Carcass Decomposition [1]

Zdravko TOMIĆ [1] Nenad STOJANAC [1] Marko R. CINCOVIĆ [1] Nikolina NOVAKOV [1]
Zorana KOVAČEVIĆ [1] Ognjen STEVANČEVIĆ [1] Jelena ALEKSIĆ [2]

[1] *This research was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project No. TR31034*

[1] Faculty of Agriculture, Department of Veterinary Medicine, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, SERBIA

[2] Faculty of Veterinary Medicine, University of Belgrade, Bulevar Oslobođenja 18, 11000 Belgrade, SERBIA

**Article Code: KVFD-2018-19670 Received: 08.03.2018 Accepted: 20.06.2018 Published Online: 20.06.2018**

**How to Cite This Article**

**Tomić Z, Stojanac N, Cincović MR, Novakov N, Kovačević Z, Stevančević O, Aleksić J:** The influence of carcass microlocation on the speed of postmortem changes and carcass decomposition. *Kafkas Univ Vet Fak Derg,* 24 (5): 655-662, 2018. DOI: 10.9775/kvfd.2018.19670

## Abstract

Determining the post-mortem interval (PMI) is often a very demanding and delicate job which requires a good knowledge of postmortem changes. In this study, 20 domestic pig carcasses *(Sus scrofa)* whose death occurred within 8 h before the start of the study were simultaneously laid at the same geographical location, but in different environments (on the ground surface - S; buried in the ground - G; placed in a crate and buried in the ground - C; submerged in water - W; and hanging in the air - A). One carcass from each group was sampled on days 14, 28, 120 and 190 from the beginning of the experiment, and on that occasion, a detailed analysis of postmortem changes and an autopsy was carried out. The difference in the rate of decomposition among groups was statistically significant. The fastest decomposition occurred in carcasses placed in a crate and buried, because during the winter period the temperature in the air was below 0°C. At that time, the decomposition process and the insect activity were slowed or stopped on carcasses in groups S, A, W, and G to some extent, while the ground and wooden crate were good thermal insulators for group C carcasses and provided better conditions for insect activity.

***Keywords:*** *Forensic veterinary medicine, Postmortem changes, Decomposition, Taphonomy, Pig carcass*

# Karkas Mikrolokasyonunun Postmortem Değişiklikler ve Karkas Dekompozisyonu Üzerine Etkisi

## Öz

Ölüm zamanını belirlemek çoğu zaman zor ve hassas bir iş olup postmortem oluşan değişiklikleri bilmeyi gerektirir. Bu çalışmada ölümleri 8 saat içerisinde gerçekleşmiş 20 adet evcil domuz *(Sus scrofa)* karkası aynı zamanda ve aynı coğrafik lokasyonda fakat farklı ortamlarda (toprak yüzeyinde (S), toprağa gömülü (G), tabut içerisinde toprağa gömülü (C), su içerisinde (W), havada asılı (A)) tutuldu. Her gruptan bir karkas çalışmanın başlandıcından sonra 14, 28, 120 ve 190. günlerde alınarak postmortem değişiklikleri belirlemek amacıyla analiz edildi ve otopsileri gerçekleştirildi. Gruplar arasındaki dekompozisyon oran farkları istatistiksel olarak anlamlı bulundu. En hızlı dekompozisyon, kış süresince hava sıcaklığının 0°C altında olması sebebiyle tabuta yerleştirilerek gömülenlerde oluştu. Bu sürede dekompozisyon süreci ve böcek aktivitesi S, A, W ve G gruplarındaki karkaslarda bir seviyeye kadar yavaşladı veya durdu. Toprak ve tahta tabut iyi bir ısı yalıtımı görevi görerek grup C'deki karkaslarda böcek aktivitesi için daha uygun şartlar sağladı.

***Anahtar sözcükler:*** *Adli veteriner hekimlik, Postmortem değişiklikler, Dekompozisyon, Tafonomi, Domuz karkası*

## INTRODUCTION

Finding, discovering and assessing the postmortem age of a corpse is always a challenge, because in order for a case to be resolved correctly it is necessary for forensic experts to first determine the time of death, i.e., the postmortem interval (PMI). The establishment of PMI is an important part of medical and legal research, aiding the team to include/exclude suspects [1,2]. The science of PMI also underpins the fundamental ability to identify a corpse in the first place [1,2]. This is a demanding task because many biotic and abiotic factors affect the decomposition of a corpse, including processes within the body itself (autolysis, putrefaction, decay). The decomposition of a corpse is a continuous

**İletişim (Correspondence)**
+381 21 4853515
stojanac.n@gmail.com

process that begins from the moment of death and ends when only the skeleton of the corpse remains, which also decomposes, but much more slowly than other tissues. Understanding the decomposition process is the basis for a successful PMI assessment [3]. In corpse decomposition studies, pig carcasses are most often used for determining postmortem changes due to their similarities with the anatomy, physiology, and the microbiota of the human digestive tract [4,5].

In order for postmortem changes on the carcasses to be compared and used for determining PMI, total body score (TBS) is used to quantify changes in carcass decomposition. Many authors have studied the relationship between TBS and accumulated degree days (ADD) with the aim of making a more precise and more reliable determination of PMI [1,6-8]. Many authors have examined environmental influences such as temperature and humidity, the impact of physical-chemical agents, the presence of clothing on the body, and the role of insects on the speed of post-mortem changes to determine PMI [9-11]. Casper's rule states: at a tolerably similar average temperature, the same post-mortem changes develop in suspended carcasses in one week (or a month), in submerged carcasses in two weeks (or months) and in carcasses buried in the ground in eight weeks (months) [12,13]. It is still widely used [14] which illustrates the lack of suitable methods [15]. Characteristics of decomposition can be identified and distinguished, but it is almost impossible to determine the exact time of death on the basis of this data only.

As a significant amount of time has passed since this discovery was made, and every region of the world has its own climatic characteristics, the need has arisen for this research to be conducted in Serbia for the first time. Serbia is characterized by a high suicide rate [16], as well as a large number of migrants who have been passing through the country on the road to EU countries in recent years. Migrants often enter and pass through Serbia illegally, so they are not on any official records and nor are their disappearances reported. Only with the discovery of a corpse does the identification and examination of all the circumstances leading to the death begin.

The aim of this study was to describe and compare the decomposition rate of pig carcasses with the same PMI, in the same geographical location, but placed in different environments (on the ground surface, buried in the ground with and without a crate, submerged in water and hanging in the air). Also, the influence of microlocation (where a body would decompose most rapidly in the climatic conditions present during the study) on the decomposition of the carcass was determined.

## MATERIAL and METHODS

This study was conducted from October 2016 until April 2017 in Serbia, in a region remote from a populated area (45°21'55.68"N 19°47'22.63"E). The climate in this region is continental, characterized by four seasons, with hot summers (temperatures up to 40°C) and cold winters (temperatures down to -20°C), while spring and autumn are mild. Rainfall mainly occurs during spring and autumn months (with average 500 mm of rainfall per annum), and the vegetation is typical for lowland areas with low vegetation.

The animals were selected from 8 farms with a population of 200.000 pigs, which were from the same epizootiological area, which means that they had almost identical living conditions [17] and approximately 50-70 pigs died during every day. Only pigs that died of natural causes, with no external wounds, were used. The criteria for selecting the 20 domestic pig carcasses *(Sus scrofa)* were that the pigs did not have clinical symptoms of disease at the time of their death, that anamnesis stated they were in good health, that they were in good body condition and that not more than 8 h had passed since the moment of death. The age of the pigs before death was 3-4 months and the carcasses weighed between 33 and 38 kg and all were in same gender (female).

Four pig carcasses were placed in each of five situations: on the ground surface (S), buried in the ground (G), in a wooden crate and buried in the ground (C), submerged in water in a barrel (W), or hanging in the air (A). The experiment was simultaneously set up for all five groups. Four pig carcasses were placed on the ground surface; carcasses were under one protective net of wire and sheet metal, in order to be protected from predators. Four pig carcasses were each buried in a separate 120 cm deep hole in the ground ($CaCO_3$ 15.52, N 0.1, $P_2O_2$ 23.63, $K_2O$ 27.59, pH 7.31). The distance between each of the holes where the carcasses were placed was 5 m. Four pig carcasses were placed in four separate wooden crates measuring 120 x 45 x 40 cm. The wooden crates were buried in the ground at a depth of 120 cm, each 5 m apart. Drinking water ($Ca^{2+}$ 75.2, $Mg^{2+}$ 14.5, $K^+$ 1.25, $Na^+$ 10.0, $HCO_3^-$ 307.1, $Cl^-$ 2.3, $SO_4^{2-}$ 7.6, pH 7.6) was placed in four 300 l barrels, and one pig carcass was placed in each barrel. This part of the study was performed in a place protected from direct sunlight and surrounded by trees and overgrown grass. Finally, four pig carcasses were hung with a rope around the neck area on a metal tube placed between two trees. The distance between the carcasses was 80 cm, and the distance between the hind legs of the carcasses and the ground was 1.5 m. Also, hanging carcasses were protected from direct sunlight and surrounded by trees.

The carcasses were examined and measured before the study began. From all the groups (S, G, C, W and A), one carcass was taken on days 14, 28, 120 and 190 from the beginning of the study. The carcasses were transported to an autopsy room where a detailed examination of the carcasses took place with weight measurements and autopsies. All characteristic changes were descriptively

described and photographed. TBS was used to assess the rate of decomposition, and the qualitative descriptions of the stages of decomposition were converted into quantitative scores.

TBS to quantify postmortem changes was expressed in points depending on the decomposition rate, and has been previously described [7,8]. The head and neck (0-12), trunk (0-11) and limbs (0-9) were separately scored. For all three body regions, point 0 represented a fresh carcass without discoloration, and the last point was the stage of skeletonization.

Temperature and precipitation was measured throughout the study and the daily maximum, minimum and average temperatures were recorded. For evaluation of PMI, ADD and TBS were used. To measure the temperature, a data logger located on site was used, as was data from the Republic Hydrometeorological Institute, which has a station 1.8 km from the experimental site. ADD was calculated according to the formula ADD=(Tmax+Tmin)/2-threshold, where Tmax is the maximum daily temperature, Tmin is the minimum daily temperature, and 0°C was taken as the threshold, a value that is accepted in many forensic studies [18-20]. Starting from the first day, the daily ADD obtained was cumulatively added for each day, and when Tmax+Tmin<0, 0 was added for that day.

The two-way ANOVA was conducted, where TBS was selected as the dependent variable, and the body region, microlocation and day were selected as independent factors. Estimated influence of factors and their interaction (body region×microlocation, body region×day and microlocation×day) to TBS.

# RESULTS

### *Meteorological Data*

The geographic location where the research was conducted has a characteristic continental climate with four seasons. The study lasted from mid-autumn to mid-spring. The minimum and maximum temperature, as well as the amount of precipitation, was recorded daily *(Fig. 1)*. The highest temperature during the study was 26.2°C (April) and the lowest was -19.4°C (January). There was a total of 29 days when the temperature did not exceed 0°C within a 24 h period. During the study, a total of 176.3 mm/m$^2$ of rain fell, and snow fell for 18 days while snow cover lasted for 33 days.

### *Weight of Pig Carcasses*

After 14 days, the weight of carcasses in groups S, G, C, and A was reduced while the weight increased in group W. Similarly, on days 28 and 120, the weight of carcasses in groups S, G, C and A continued to decrease almost linearly while the weight of the carcasses in the water remained virtually unchanged compared to their weights on day 14. The weights of carcasses from groups S, G, C and W were not measured at the end of the study, on day 190, because they were in an advanced stage of decomposition (or total skeletonization) and it was not possible to take weight measurements. Only the weight of carcass A was measured on day 190, and it was 35.4% of the carcass weight measured at the beginning of the study *(Fig. 2)*.

### *Decomposition Process of Pig Carcasses*

As pig carcasses in groups G and C were buried, and group W submerged in water, changes were noticed only after carcass exhumation or removal from water. Comparable postmortem changes during the decomposition of carcasses are described in *Table 1*. During each sampling, the decomposition stages of the carcasses was assessed and the TBS for each carcass was determined *(Table 1)*. *Fig. 3* shows decomposition stages of carcasses from the different environments immediately prior to sampling and autopsies.

At the first sampling on day 14, the carcasses from all groups were approximately in the same decomposition

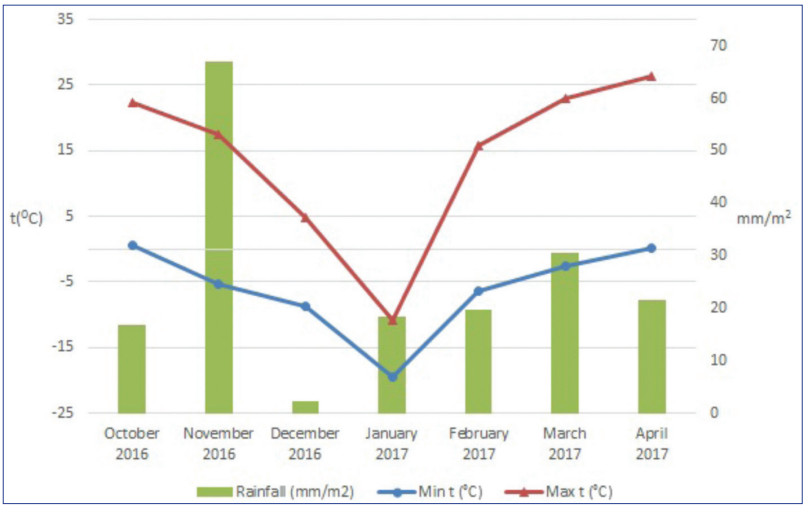



**Fig 1.** The minimum and maximum temperatures with precipitation data during the study period (October 2016 to April 2017)

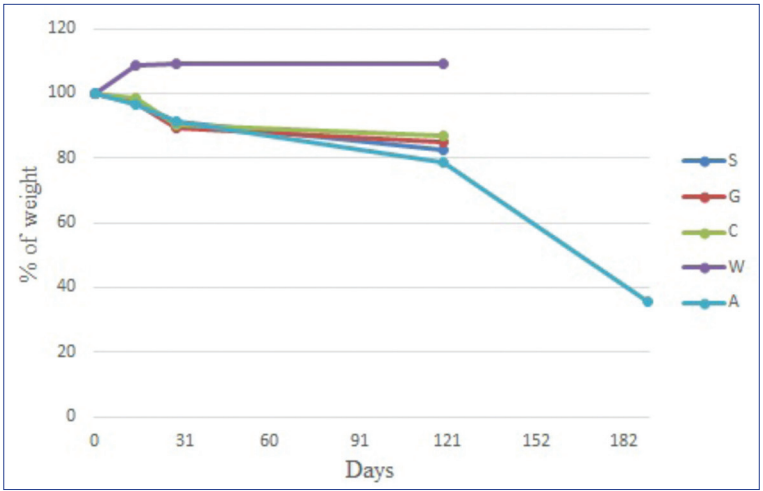



**Fig 2.** Weight of pig carcasses at the beginning of the experiment and the moment of the sampling

***Table 1.*** *Summary of postmortem interval, accumulated degree days and total body score for three body regions in carcasses from different microlocations and decomposition stage at the moment of carcass sampling*

| PMI | ADD | Body Regions | S | G | C | W | A |
|---|---|---|---|---|---|---|---|
| 14 | 127.8 | HN | 1 | 1 | 1 | 1 | 2 |
| | | T | 2 | 1 | 1 | 1 | 2 |
| | | L | 1 | 1 | 1 | 1 | 1 |
| | | TBS | 4 | 3 | 3 | 3 | 5 |
| | | Decomposition stage | Bloat | Bloat | Bloat | Bloat | Bloat |
| 28 | 224.3 | HN | 2 | 2 | 3 | 2 | 8 |
| | | T | 2 | 1 | 3 | 2 | 5 |
| | | L | 2 | 2 | 3 | 2 | 4 |
| | | TBS | 6 | 5 | 9 | 6 | 17 |
| | | Decomposition stage | Bloat | Bloat | Active decay | Bloat | Active decay |
| 120 | 414.1 | HN | 4 | 4 | 7 | 4 | 8 |
| | | T | 4 | 4 | 5 | 4 | 7 |
| | | L | 3 | 4 | 5 | 3 | 5 |
| | | TBS | 11 | 12 | 17 | 11 | 20 |
| | | Decomposition stage | Active decay | Active decay | Advance decay | Active decay | Active decay |
| 190 | 1130.6 | HN | 10 | 8 | 12 | 7 | 10 |
| | | T | 8 | 6 | 11 | 10 | 9 |
| | | L | 8 | 6 | 9 | 7 | 7 |
| | | TBS | 26 | 20 | 32 | 24 | 26 |
| | | Decomposition stage | Advance decay | Advance decay | Skeletoni-zation | Advance decay | Advance decay |

*PMI-postmortem interval; ADD-accumulated degree days; S-on the ground surface; G-buried in the ground; C-in a wooden crate and buried in the ground; W-submerged in water in a barrel; A-hanging in the air; HN-head and neck; T-trunk; L-limbs; TBS- total body score*

stage (TBS scores of 3-5). On day 28, there was a significant difference in the decomposition of carcasses in the experimental groups. Carcasses S, G, and W reached approximately the same decomposition stage (TBS of 5-6), while the carcasses in the crates (C) decomposed much faster reaching TBS of 9, while the hanging carcasses (A) were in the fastest reaching TBS of 17 in the same period. On day 120, the ratio of the decomposition stages was similar, as carcasses in groups S, G and W were still at approximately the same decomposition stage (TBS of 11-12) while carcasses in groups C and A were at a faster reaching decomposition stage (TBS of 17 and 20, respectively). By day 120, decomposition was fastest on hanging carcasses and then, during the period from days 120 to 190, the fastest changes took place on the carcasses buried in the crate. At the end of the study, (after 190 days) total skeletonization had occurred only in the group C carcass (TBS of 32), while the lowest decomposition stage (TBS of 20) was established in the carcass buried in the ground (G), and the remaining three groups (carcasses S,


**Fig 3.** Comparison of decomposition process of the carcasses from groups S, G, C, W and A. 0, 28 and 190 days

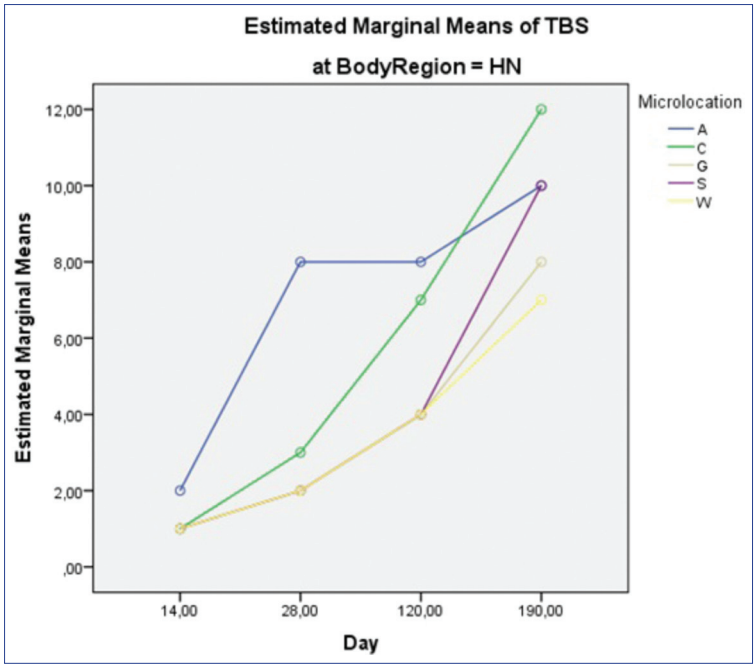



**Fig 4.** Graph showing the influence of head and neck, day, microlocation and their interaction to total body score

W and A) were at equal stages of decomposition (TBS of 24-26) *(Table 1* and *Fig. 3)*.

The speed of postmortem changes and carcass decomposition indicate influence of body region (F=6.364, P<0.05), microlocation (F=4.279, P<0.05) and day (F=45.642, P<0.001) to TBS, and their interaction differences were observed between body region×microlocation (F=2.789, P<0.05) and microlocation×day (F=5.842, P<0.001), while between body region×day no significant difference was observed (F=2.023, P>0.05). Decomposition of body regions, head and neck (HN), trunk (T) and limbs (L) was statistically different among groups (S, G, C, W, A) during the study *(Fig. 4-6)*.

## DISCUSSION

Many authors have examined PMI in relation to various environmental influences such as temperature, freezing, soil, insects, lime, body size, or clothing [3,21-24]. As temperature is the leading external factor for the decomposition of a carcass, when determining PMI, the most common temperature influence is expressed through accumulated degree days (ADD) [25,26]. In this research, all carcasses had the same PMI, which was determined at the beginning of the study, and all carcasses were positioned at the same geographic location at the same time, and therefore, were exposed to the same general atmospheric conditions, but in different environments (on the ground, in the ground, in a crate in the ground, in water or hanging in the air). A comparison of the decomposition rate over the same time interval indicates the influence of the microlocation of the body on the speed of the development of post-mortem changes.

The weight or the size of the carcass affects the rate of postmortem changes and the PMI, and it is known that smaller carcasses decompose faster than larger ones [21]. The carcasses in all experimental groups were of approximately the same size and, therefore, the influence of the size of the carcasses in the different experimental groups on the decomposition rates was negligible. Only the carcass in the water (W) underwent an increase in weight (9%) on day 14 of the study, which was due to the passive entry of water into the respiratory and digestive tract organs. The same increase in weight was found on days 28

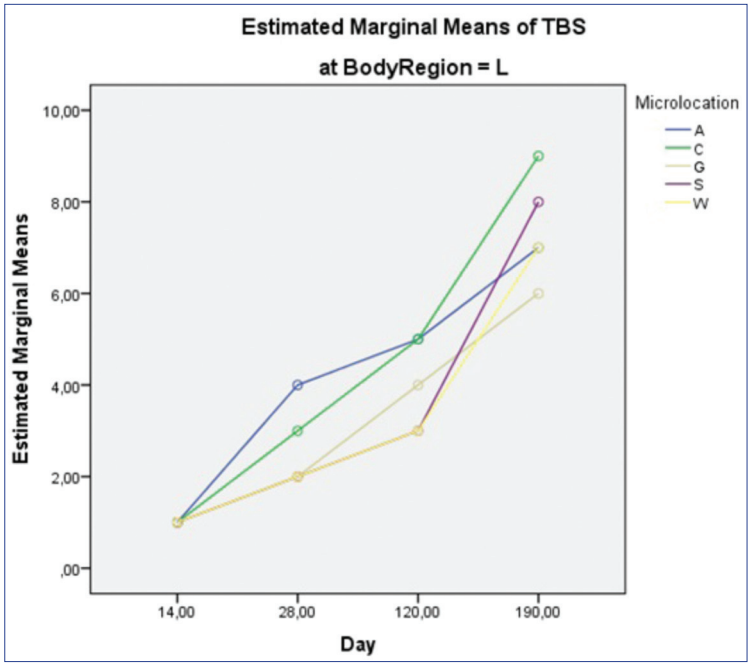



**Fig 5.** Graph showing the influence of trunk, day, microlocation and their interaction to total body score

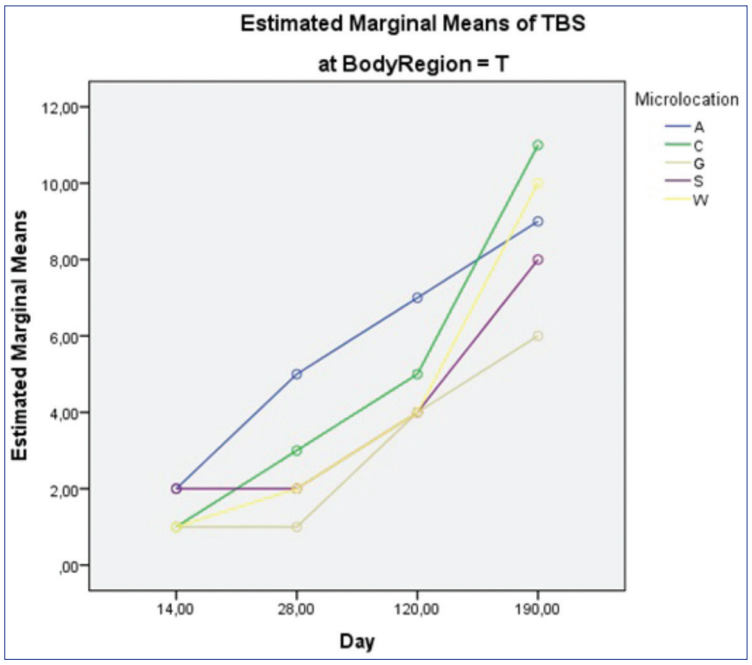



**Fig 6.** Graph showing the nfluence of limbs, day, microlocation and their interaction to total body score

and 120. Group A carcasses underwent the greatest weight loss, and on days 120 and 190 had lost 21.2% and 64.6%, respectively, of the weight measured at the beginning of the study. Although on day 190 only the carcass from group A was compact and could be measured, the small weight observed was due to the high degree of dehydration and mummification. Weight loss which occurred in carcasses from groups S, G, C, and A was accompanied by the decomposition we observed, and our finding the weight decreased has been confirmed by other studies [25,26]. However, proof that this cannot be relied on with any certainty to determine the PMI is the weight of group W carcasses as well as the possibility of losing certain body parts as a result of scavenging or in the case of the hanging pigs, as a result of skeletal element loss via gravity [22].

Since all carcasses were situated at the same geographic location and were exposed to the same general atmospheric conditions at the time of sampling, they would have had the same ADD if only the air temperature were taken into account *(Table 1)*. However, the temperatures to which the carcasses from the groups were actually exposed were different (air, water, earth temperature), and hence, a comparison of changes depending on ADD would have inaccuracies. As the PMI in this research was predetermined and known, with the aim being to compare the same PMI for carcasses set in different environments, the ADD at an internal level for each group was not required, but the ADD values from this study *(Table 1)* can be used for comparison with similar studies by other authors.

Until day 120, carcasses from groups S and G were in very similar decomposition stages, as determined in previous studies [1,7,27], while after day 120, an increase in temperature *(Fig. 1)* quickened the rate of decomposition of carcasses on the ground surface [28,29]. Rainfall did not necessarily cause an increase in decomposition, because the largest quantity of rain fell in the first 28 days of the study *(Fig. 1)*, and there was no difference in the decomposition of the carcasses exposed to the rain (group S) and those sheltered from the rain (group G). After day 120, the slower decomposition of carcasses buried in the ground was the result of lower temperatures in the ground than on the ground surface and in the air and because that reduced exposure to insects. Troutman et al.[30] found that carcass decomposition may be up to eight times slower in the ground compared to on the ground. This could not be confirmed in our study, as carcasses in groups S and G did not reach skeletonization stage on day 190. As in the study carried out by Marais-Werner et al.[7], carcasses in group S and G were in the decomposition stage of TBS <26 on day 190.

During the early stages of decomposition, group A carcasses experienced the highest rate of postmortem changes and decomposition due to the carcasses' exposure to insects, and to disturbances to the position of the abdominal cavity organs as a result of the effects of the ground and

atmospheric phenomena such as wind and humidity. Group S carcasses were exposed to very similar conditions, except that they were on the ground, which resulted in the same degree of decomposition in both groups at the end of this study. Other authors have reached similar results [22,26] in their studies, with the exception that the early stages of decomposition occurred earlier on carcasses on the ground surface.

Between day 28 and day 120, i.e., during the winter period, when the lowest temperature occurred *(Fig. 1)*, the ADD increased very slightly (from 224.3 to 414.1), which resulted in the cessation of insect activity, and thus, in the decomposition of the carcasses in groups S, G, A and W. The greatest degree of decomposition among all the different groups of carcasses occurred in group C as a result of the influence of insect activity and temperature, because ground at the depth of 120 cm and wooden crate were good thermal insulators [15], and there were 29 days when the temperature was 0°C, and then, the decomposition processes in the other groups of carcasses was stopped. Although group C carcasses were in a crate and buried in the ground at a depth of 120 cm, the presence of a large number of larvae was noticed on day 120 and day 190. Although it is not common to find insects on the carcasses in the ground at that depth, it is possible this is a consequence of the contamination of the corpses and/or wooden crates during the period from death to burying, because in that period the daily temperatures were 20°C and higher. Also, larvae presence can be a result of higher temperatures in the wooden crate than in the air during the winter period, and therefore, more favorable conditions for insect activity [11,31]. That and the looseness of the ground could have enabled easy passage of insects to carcasses. After 120 days, temperatures began to rise and ADD increased from 120 to 190 days (from 414.1 to 1130.6), resulting in both insect activity and faster decomposition of the carcasses in the other groups.

On carcasses submerged in water, the fastest changes occurred on the trunk which appeared above water during active decay stages despite suggestions in the literature that the head always decomposes at a faster rate than the body. Previous studies by other authors [32,33] showed that the decomposition of soft body tissues occurred at the same rate both in the water and on the ground surface, which was confirmed in this study. Certainly, the decomposition rate of a carcass in naturally occurring waters will differ from the decomposition rate of carcasses in this study as decomposition is influenced by a series of water characteristics, with its specific flora and fauna, as well as the depth at which the carcass is located [28].

Comparing the decomposition of carcasses in this study with Casper's rule, we have almost the opposite finding, i.e. in this study, the carcass placed in a wooden crate in the ground was the first to decompose, while the times for the decomposition of the carcasses left in the open or in water were almost identical. Some authors [34] have explained Casper's rule by different quantities of oxygen in the air or water, i.e. the differences in the rates of putrefactive changes are based on the dissimilar amount of oxygen in the air, water and underground, which was not confirmed in this study.

The present study has found difference in the rate of decomposition among groups. Decomposition rates were significantly different in the different body regions (HN, T and L) of the carcasses at different microlocations *(Fig. 4-6)*, due to the characteristics of the environment, i.e the microlocation on/in which the carcasses had been laid.

In this study, temperature had the greatest impact on decomposition directly and indirectly through insect activity. In the period from November to March, the average minimum temperature was below 0°C *(Fig. 1)*, which slowed or stopped the decomposition processes for the carcasses that were left outdoors (S, A, W, and G to some extent). Although it was physically harder for insects to reach the carcasses in the ground (G and C), the influence of ground and wooden crate as thermo-insulators led to higher temperatures at these microlocations and better conditions for insect activity, which resulted in faster decomposition of the carcasses. Water also presented a physical barrier for insects, so the decomposition was the fastest on the part of the carcass (trunk) that was out of the water and exposed to a much higher oxygen level [34], and consequently more accessible to insects *(Fig. 3)*.

In this research, after the early stage of decomposition, the fastest development of postmortem changes and decomposition occurred on carcasses buried in a crate underground. All carcasses had the same PMI and were laid at the same geographic location, but in different environments (on the ground, in the ground, in a crate in the ground, in water and hung in the air), which had a crucial impact on the decomposition rate. The discovery of two or more carcasses at the same geographical location does not automatically mean that they must have the same PMI, but rather, it is necessary to pay special attention to an analysis of the microlocation in which the carcass is located. These results should be useful as a key for determining PMI and comparing PMI between corpses found at the same time in the same location, but in different environments. Certainly, this is the first study of this kind to be conducted in Serbia and the region. However, this topic is deserving of further research due to the geographical position of the Balkans and current and historical events related to this area, because of which there is a need to improve existing databases for the determination of PMI.

## REFERENCE

**1. Megyesi MS, Nawrocki SP, Haskell NH:** Using accumulated degree-days to estimate the postmortem interval from decomposed human

remains. *J Forensic Sci,* 50 (3): 618-626, 2005. DOI: 10.1520/JFS2004017

**2. Keough N, Myburgh J, Steyn M:** Scoring of decomposition: A proposed amendment to the method when using a pig model for human studies. *J Forensic Sci,* 62 (4): 986-993, 2017. DOI: 10.1111/1556-4029.13390

**3. Li L, Wang J, Wang Y:** A comparative study of the decomposition of pig carcasses in a methyl methacrylate box and open air conditions. *J Forensic Leg Med,* 42, 92-95, 2016. DOI: 10.1016/j.jflm.2016.06.001

**4. Schoenly KG, Haskell NH, Mills DK, Bieme-Ndi C, Larsen K, LeeY:** Recreating death's acre in the school yard: Using pig carcasses as model corpses. *Am Biol Teach,* 68 (7): 402-410, 2006. DOI: 10.1662/0002-7685(2006)68[402:RDAITS]2.0.CO;2

**5. Notter SJ, Stuart BH, Rowe R, Langlois N:** The initial changes of fat deposits during the decomposition of human and pig remains. *J Forensic Sci*, 54 (1): 195-201, 2009. DOI: 10.1111/j.1556-4029.2008.00911.x

**6. Nawrocka M, Frątczak K, Matuszewski S:** Inter-rater reliability of total body score-a scale for quantification of corpse decomposition. *J Forensic Sci*, 61 (3): 798-802, 2016. DOI: 10.1111/1556-4029.13105

**7. Marais-Werner A, Myburgh J, Becker PJ, Steyn M:** A comparison between decomposition rates of buried and surface remains in a temperate region of South Africa. *Int J Legal Med*, 132 (1): 301-309, 2018. DOI: 10.1007/s00414-017-1618-2

**8. Moffatt C, Simmons T, Lynch-Aird J:** An improved equation for TBS and ADD: Establishing a reliable postmortem interval framework for casework and experimental studies. *J Forensic Sci,* 61 (S1): S201-S207, 2016. DOI: 10.1111/1556-4029.12931

**9. Schotsmans EMJ, Fletcher JN, Denton J, Janaway RC, Wilson AS:** Long-term effects of hydrated lime and quicklime on the decay of human remains using pig cadavers as human body analogues: Field experiments. *Forensic Sci Int*, 238, 141.e1-141.e13, 2014. DOI: 10.1016/j.forsciint.2013.12.046

**10. Matuszewski S, Konwerski S, Frątczak K, Szafałowicz M:** Effect of body mass and clothing on decomposition of pig carcasses. *Int J Legal Med,* 128 (6): 1039-1048, 2014. DOI: 10.1007/s00414-014-0965-5

**11. Lyu Z, Wan LH, Yang YQ, Tang R, Xu LZ:** A checklist of beetles (Insecta, Coleoptera) on pig carcasses in the suburban area of southwestern China: A preliminary study and its forensic relevance. *J Forensic Leg Med,* 41, 42-48, 2016. DOI: 10.1016/j.jflm.2016.04.009

**12. Casper JL:** Handbook of the practice of forensic medicine, based upon personal experience. The New Sydenham Society, London, 1861.

**13. Dix J, Graham M:** Time of death, decomposition and identification: An atlas. CRC Press, Florida, 1999.

**14. Brinkmann B, Madea B:** Handbuch Gerichtliche Medizin. Springer Verlag, Berlin, 2004.

**15. Fiedler S, Graw M:** Decomposition of buried corpses, with special reference to the formation of adipocere. *Naturwissenschaften*, 90, 291-300, 2003. DOI: 10.1007/s00114-003-0437-0

**16. Ilic M, Ilic I:** Suicide in Serbia. *J Affect Disord,* 193, 187-193, 2016. DOI: 10.1016/j.jad.2015.12.063

**17. Stojanac N, Stevančević O, Savić B, Davidov I, Cincović MR, Potkonjak A, Spasojević J:** Forensic findings on acute mortality of piglets after ingestion of aflatoxin. *Kafkas Univ Vet Fak Derg*, 21 (3): 437-440, 2015. DOI: 10.9775/kvfd.2014.12395

**18. Meyer J, Anderson B, Carter DO:** Seasonal variation of carcass decomposition and gravesoil chemistry in a cold (Dfa) climate. *J Forensic Sci,* 58, 1175-1182, 2013. DOI: 10.1111/1556-4029.12169

**19. Smith AC:** The effects of sharp-force thoracic trauma on the rate and pattern of decomposition. *J Forensic Sci,* 59 (2): 319-326, 2014. DOI: 10.1111/1556-4029.12338

**20. Spicka A, Johnson R, Bushing J, Higley LG, Carter DO:** Carcass mass can influence rate of decomposition and release of ninhydrin-reactive nitrogen into gravesoil. *Forensic Sci Int*, 209, 80-85, 2011. DOI: 10.1016/j.forsciint.2011.01.002

**21. Sutherland A, Myburgh J, Steyn M, Becker PJ:** The effect of body size on the rate of decomposition in a temperate region of South Africa. *Forensic Sci Int,* 231 (1-3): 257-262, 2013. DOI: 10.1016/j.forsciint.2013.05.035

**22. Lynch-Aird J, Moffatt C, Simmons T:** Decomposition rate and pattern in hanging pigs. *J Forensic Sci,* 60 (5): 1155-1163, 2015. DOI: 10.1111/1556-4029.12796

**23. Roberts LG, Dabbs GR:** A taphonomic study exploring the differences in decomposition rate and manner between frozen and never frozen domestic pigs *(Sus scrofa)*. *J Forensic Sci,* 60 (3): 588-594, 2015. DOI: 10.1111/1556-4029.12717

**24. Ueland M, Nizio KD, Forbes SL, Stuart BH:** The interactive effect of the degradation of cotton clothing and decomposition fluid production associated with decaying remains. *Forensic Sci Int,* 255, 56-63, 2015. DOI: 10.1016/j.forsciint.2015.05.029

**25. Adlam RE, Simmons T:** The effect of repeated physical disturbance on soft tissue decomposition-are taphonomic studies an accurate reflection of decomposition? *J Forensic Sci*, 52 (5): 1007-1014, 2007. DOI: 10.1111/j.1556-4029.2007.00510.x

**26. Shalaby OA, deCarvalho LM, Goff ML:** Comparison of patterns of decomposition in a hanging carcass and a carcass in contact with soil in a xerophytic habitat on the island of Oahu, Hawaii. *J Forensic Sci,* 45 (6): 1267-1273, 2000. DOI: 10.1520/JFS14877J

**27. Comstock JL, Desaulniers JP, LeBlanc HN, Forbes SL:** New decomposition stages to describe scenarios involving the partial and complete exclusion of insects. *J Can Soc Forensic Sci,* 48, 1-19, 2014. DOI: 10.1080/00085030.2014.929850

**28. Heaton V, Lagden A, Moffatt C, Simmons T:** Predicting the post-mortem submersion interval for human remains recovered from U.K. waterways. *J Forensic Sci,* 55 (2): 302-307, 2010. DOI: 10.1111/j.1556-4029.2009.01291.x

**29. Myburgh J, L'Abbé EN, Steyn M, Becker PJ:** Estimating the post-mortem interval (PMI) using accumulated degree-days (ADD) in a temperate region of South Africa. *Forensic Sci Int,* 229 (1-3): 165.e1-165.e6, 2013. DOI: 10.1016/j.forsciint.2013.03.037

**30. Troutman L, Moffat C, Simmons T:** A preliminary examination of differential decomposition patterns in mass graves. *J Forensic Sci,* 59 (3): 621-626, 2014. DOI: 10.1111/1556-4029.12388

**31. Archer MS:** Rainfall and temperature effects on the decomposition rate of exposed neonatal remains. *Sci Justice*, 44 (1): 35-41, 2004. DOI: 10.1016/S1355-0306(04)71683-4

**32. Haglund W:** Disappearance of soft tissue and the disarticulation of human remains from aqueous environments. *J Forensic Sci,* 38 (4): 806-815, 1993. DOI: 10.1520/JFS13476J

**33. Anderson GS, Hobischak NR:** Decomposition of carrion in the marine environment in British Columbia, Canada. *Int J Legal Med,* 118, 206-209, 2004. DOI: 10.1007/s00414-004-0447-2

**34. Madea B, Dettmeyer R, Schmidt P:** Leichenschau. **In,** Madea B (Ed): Praxis Rechtsmedizin. Springer, Berlin, 2007.

Kafkas Univ Vet Fak Derg
24 (5): 663-671, 2018
DOI: 10.9775/kvfd.2018.19675

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Clinical, Bacteriological, and Histopathological Aspects of Endotoxic Pyometra in Bitches [1]

Mürşide Ayşe DEMİREL [1,a] Sevil ATALAY VURAL [2,b] Rıfat VURAL [3,c]
Osman KUTSAL [2,d] Ziynet GÜNEN [4,e] Şükrü KÜPLÜLÜ [3,f]

[1] *Presented in 91st International Scivac Congress as an oral presentation, Rimini-Italy, 26-29 May 2016 and published in Abstract Book*

[1] Department of Pharmacology, Faculty of Pharmacy, University of Gazi, TR-06330 Ankara - TURKEY
[2] Department of Pathology, Faculty of Veterinary Medicine, Ankara University, TR-06110 Ankara - TURKEY
[3] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Ankara University, TR-06110 Ankara - TURKEY
[4] Depatment of Pathology, Bornova Veterinary Control Institute, TR-35010 İzmir - TURKEY
[a] ORCID: 0000-0002-7082-8976; [b] ORCID: 0000-0003-2111-3381; [c] ORCID: 0000-0001-7252-7977; [d] ORCID: 0000-0003-3599-6867
[e] ORCID: 0000-0002-2878-0307; [f] ORCID: 0000-0001-7488-9736

**Article Code: KVFD-2018-19675 Received: 09.03.2018 Accepted: 27.07.2018 Published Online: 28.07.2018**

**How to Cite This Article**

**Demirel MA, Atalay Vural S, Vural R, Kutsal O, Günen Z, Küplülü Ş:** Clinical, bacteriological, and histopathological aspects of endotoxic pyometra in bitches. *Kafkas Univ Vet Fak Derg,* 24 (5): 663-671, 2018. DOI: 10.9775/kvfd.2018.19675

## Abstract

The object of this study was to compare the histopathologic change in uterus as well as the findings of clinical and bacteriological of twenty-four bitches with pyometra. Uterine lesions were grouped as acute endometritis, subacute endometritis, acute metritis, subacute metritis. Complete blood-count, blood biochemical parameters and toxin levels were determined. Only alkaline phosphatase levels were significantly different between all groups (P<0.01). Blood endotoxin level was different between the acute and subacute endometritis groups (P<0.01), and subacute endometritis and acute metritis groups (P<0.01). All bitches in the subacute endometritis group recovered. Three bitches in the acute endometritis group, two bitches in the acute metritis group and one bitch in the subacute metritis group died. The blood endotoxin level was determined to be quite high in the dead bitches. There was a difference in the levels of blood urea nitrogen (P<0.05), creatinine (P<0.01), and toxin (P<0.0001) between the dead and surviving bitches. In conclusion, the prognosis of pyometra may be recovered in bitches diagnosed with subacute endometritis associated with low levels of circulating endotoxin. The endotoxemic bitches that have blood endotoxin level above 0.96 EU/mL along with significantly increased alkaline phosphatase, blood urea nitrogen, and creatinine concentration would likely end up with a mortality outcome for acute endometritis, acute metritis, or subacute metritis. These parameters may serve as good prognostic markers in bitches with pyometra.

***Keywords:*** *Bacteriology, Bitch, Histopathology, Prognosis, Pyometra*

# Köpeklerde Endotoksik Pyometranın Klinik, Bakteriyolojik ve Histopatolojik Özellikleri

## Öz

Bu çalışmanın amacı, pyometralı yirmi dört köpeğin uteruslarındaki histopatolojik değişikliklerin karşılaştırılmasıdır. Uterus lezyonları akut endometritis, subakut endometritis, akut metritis, subakut metritis olarak gruplandırıldı. Tam kan sayımı, kan biyokimyasal parametreler ve toksin seviyeleri belirlendi. Tüm gruplar arasındaki farklılık sadece alkalen fosfotaz seviyesinde önemliydi (P<0.01). Kan endotoksin seviyesi, akut ve subakut endometritis grupları (P<0.01) ile subakut endometritis ve akut metritis grupları (P<0.01) arasında farklıydı. Subakut endometritis grubundaki tüm köpekler iyileşti. Akut endometritis grubunda üç köpek, akut metritis grubunda iki köpek ve subakut metritis grubunda ise bir köpek öldü. Kan endotoksin seviyesinin ölen köpeklerde oldukça yüksek seviyede olduğu belirlendi. Ölen ve yaşayan köpekler arasında kan üre nitrojen (P<0.05), kreatinin (P<0.01) ve toksin (P<0.0001) seviyelerinin farklı olduğu görüldü. Sonuç olarak, dolaşımdaki endotoksinin düşük seviyesi ile ilişkili olarak subakut endometritis tanısı konulan köpeklerde pyometranın prognozunun iyileştirilebileceği düşünüldü. Yüksek seviyede alkalen fosfotaz, kan üre azotu ve kreatinin konsantrasyonu ile birlikte 0.96 EU/mL'nin üstündeki kan endotoksin düzeyine sahip endotoksemik köpeklerde akut endometritis, akut metritis veya subakut metritis sonucu ölüm görülmesinin muhtemel olabileceği kanısına varıldı. Bu parametreler, pyometralı köpeklerde iyi bir prognostik belirteçler olarak kullanılabilir.

***Anahtar sözcükler:*** *Bakteriyoloji, Köpek, Histopatoloji, Prognoz, Pyometra*

**İletişim (Correspondence)**
+90 312 2023356
aysedemirel@gazi.edu.tr

# INTRODUCTION

Pyometra is a common inflammatory disease of the uterus and a hormone-related condition in adult bitches. It is characterized by genital and systemic illness with various clinical and pathological findings [1-3]. Prolonged or repeated progesterone exposure causes cystic endometrial hyperplasia, which is a thickening of the endometrial wall. When a bacterial infection and cystic endometrial hyperplasia occur in the uterus, pyometra becomes inevitable [1,4]. Most bacteria isolated from uterine content are gram negative bacteria that release lipopolysaccharide-structured endotoxin from their cell walls [1,3,5]. Multiple organ failure due to sepsis [6] and death may be observed in this disease [7-9]. Previous studies have shown that the mortality rate caused by pyometra is about 4-26% [7,9,10]. Renal dysfunction and hepatocellular damage caused by septicemia and/or diminished hepatic circulation and cellular hypoxia in a dehydrated bitch can lead to alteration in variety of serum biochemical parameters. In addition, suppression in bone marrow activity which leads to anemia and remarkably increased leukocytes can occur due to the toxin effect in bitches with pyometra related to endotoxemia [11,12]. Besides, the prognosis of pyometra may vary according to the absorption of bacterial toxin and the histomorphological alterations of the uterus [2,13-16]. Therefore, the determination of the blood endotoxin level and histopathological changes in the uterus are more important for estimating the prognosis of the disease in bitches with pyometra [2,14,17]. The prognosis and mortality prediction of pyometra remain the subject of research. The aim of this study is to evaluate the relationship between the blood endotoxin level and histopathological aspects according to the type of bacteria isolated from the uterine content and to investigate the risk status, prognosis, and possible death reasons in canine pyometra cases.

# MATERIAL and METHODS

### *Animals*

The present study was maintained in accordance with the directions of Guide for the Care and Use of Animal in Research. This research was performed in twenty-four bitches with pyometra from different breeds [Terrier (n=17), Collie (n=3), Cocker spaniel (n=1), Pekingese (n=1), Labrador retriever (n=1), Rottweiler (n=1)] and of varied ages (9.95±0.79 years; the range was 2-18 years) that were brought to the Gynecology Clinic of the Faculty of Veterinary Medicine, Ankara University. Pyometra was diagnosed by anamnesis and clinical examination including the evaluation of ultrasonographic findings (6-8 mHz with multifrequency linear and sector probes, B mode real time, Pie Medical, 100 Falco). In present study, the bitches were included with observation of at least one of sign such as vaginal discharge, anorexia, polyuria/polydipsia, lethargy, vomiting, dehydration, or weakness in the rear legs. Ovariohysterectomy was decided following the diagnosis of pyometra. Stabilizing the animals' conditions was attempted prior to surgery by giving intravenous fluid and antibiotics. Supportive therapy was maintained for at least seven days in the postoperative stage.

### *Serum Biochemical Parameters and Endotoxin Levels*

Blood samples were preoperatively collected from the *vena cephalica antebrachii* into evacuated heparinized (Vacutest®; Arzergrande, Italy) tubes for complete blood-count-analysis and into plain tubes (Venoject®; Leuven, Belgium) for the determination of biochemical parameters and toxin levels. All samples were analyzed at The Laboratory of the Veterinary Faculty, Ankara University. The biochemical functions and blood toxin level were evaluated by centrifuging blood samples at 3000 rpm for 15 min and sera were stored at -80°C in a freezer until analysis. The serum biochemical parameters were evaluated using a chemistry analyzer (Reflotron® Plus, Roche, Istanbul): Asparateaminotransferase (AST), alanineaminotransferase (ALT), alkalinephosphatase (ALP), creatinine, and blood urea nitrogen (BUN) levels.

The blood endotoxin level was measured by utilizing an E-Toxate™ assay test kit (Sigma-Aldrich, Germany). The E-Toxate™ reagent working solution containing a Limulus Amebocyte Lysate (LAL) gel formulation assay was prepared according to the manufacturer's instructions.

### *Bacteriological Examination*

Swab samples (Cultiplast®; LP Italiana SPA, Italy) for bacteriological examination were immediately taken under sterile conditions from uterine lumen following ovariohysterectomy. The all samples were cultured in an accredited laboratory (Accreditation No: AB-0031-T). The identification of isolates was performed with the conventional and Vitex automated method (Biomerieux, France). The isolates' susceptibility was assigned to post-operative antibiotic treatment. The minimum inhibitory concentrations were determined according to the standards of the National Committee of Clinical Laboratory Standards (NCCLS, 2000 and 2002), utilizing Mueller Hinton broth (Oxoid, UK).

### *The Evaluation of Histopathologic Aspect*

After the ovariohysterectomy, the removed ovary and uterine tissues were submitted to the Laboratory of the Pathology Department at the Faculty of Veterinary, Ankara University for histopathological examination. The tissues were fixed in a 10% formaline buffer and embedded in paraffin. Paraffin blocks were sectioned at 5 µm, stained with Hematoxylin-Eosin (HδE), and examined under a light microscope. Microscopically, samples were classified as acute endometritis, subacute endometritis, acute metritis, and subacute metritis according to the inflammatory cell

infiltrations, hemorrhage in uterine layers, mucosal and vascular changes and glandular hyperplasia.

### *Statistical Analysis*

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad, USA) and one-way ANOVA was performed to determine the differences between groups. *t-test* was used to compare two groups, and Fisher's exact test was used to evaluate the statistical significance of the difference between all blood parameters in the dead and surviving bitches. The results were expressed as mean±S.E.M. The level of significance for all statistical tests was $P<0.05$.

## RESULTS

Gram negative bacteria were isolated in 18 cases and gram positive bacteria were isolated in five cases from uterine content; one case presented no growth. It was observed that 13 of the 23 cases were *Escherichia coli*. Open pyometra was recorded in 16 cases and closed pyometra was recorded in eight cases in the present study. The results of anamnesis, viability, and clinical findings were presented according to histopathological classification in *Table 1*. The differences between the groups for these results were not statistically significant.

The hematological and blood biochemical parameters of all groups are given in *Table 2*. Only the difference in ALP level was significant ($P<0.01$) between the groups. However, when the differences between the two groups were analyzed, ALT, ALP, BUN, creatinine, and lymphocyte values were found be significant *(Table 2)*. Although, there was no significant difference in the blood endotoxin level between the all groups ($P>0.05$), it was found significant for the difference between acute and subacute endometritis groups ($P<0.01$), and subacute endometritis and acute metritis groups ($P<0.05$). Besides, there was a difference in the levels of BUN ($P<0.05$), creatinine ($P<0.01$), and toxin ($P<0.0001$) between the dead and surviving bitches.

### *Uterine Pathological Alterations of the Cystic Endometrial Hyperplasia-Pyometra Complex*

#### Macroscopic Findings

Generally, it was apparent that the subserosal veins associated with segmental expansion and thickening of the uterine mucosa were quite significant. Necrotic and/or hemorrhagic areas were observed in the uterine mucosa for seven cases and it was determined that six of these cases were dead after the ovariohysterectomy. In two of the dead cases in which Coagulase-positive Staphylococcus (CPS) was isolated, both necrotic and hemorrhagic areas were found. In four of the dead cases, gram negative bacteria were isolated and hemorrhagic areas were detected.

#### Microscopic Findings

*Acute Endometritis:* Microscopically, acute endometritis and glandular hyperplasia were observed in eight cases. Three bitches were dead after the ovariohysterectomy in this group. Gram negative bacteria (three *E. coli*, two *Pseudomonas aeruginosa)* were isolated in five cases and gram positive bacteria (two CPS, one Enterococcus) were isolated in three cases. Furthermore, CPS was isolated from another and *E. coli* was isolated from the uterine content in one of the dead bitches. The blood endotoxin level was significantly higher in the death cases in which *E. coli* was isolated *(Table 3)*. In this case, the degenerated surface epithelial cells, leukocytosis in the stratum vasculare, and cystic degeneration *(Fig. 1-a)* on the glandula uteri were markedly observed. In dead cases, isolated CPS, congestion, and hemorrhage on the stromal connective

***Table 1.*** *The result of anamnesis, clinical examination, and viability findings according to groups*

| Parameters and Features | Acute Endometritis (n=8) | Subacute Endometritis (n=5) | Acute Metritis (n=8) | Subacute Metritis (n=3) |
|---|---|---|---|---|
| MeanAge (year) | 11.50±1.26 | 6.40±2.31 | 10.13±1.09 | 11.33±0.33 |
| Breed | 5 Terrier; 1 Cocker<br>1 Collie; 1 Pekignese | 4 Terrier<br>1 Labrodor | 5 Terrier; 2 Collie<br>1 Rotweiler | 3 Terrier |
| Mean duration of disease (range day) | 34.13±21.05<br>(7-180 days) | 43.40±26.77<br>(20-210 days) | 11.50±1.79<br>(7-21 days) | 9.66±2.66<br>(7, 7 and 15 days) |
| Mean time of prostrus onset (day) | 105.60±39.04 | 145.00±67.49 | 132.50±51.50 | 151.70±107.00 |
| Previous litters | Yes (n=1)<br>No (n=7) | Yes (n=2)<br>No (n=3) | Yes (n=2)<br>No (n=6) | No (n=3) |
| Cervix closure | Opened (n=7)<br>Closed (n=1) | Opened (n=5) | Closed (n=8) | Opened (n=3) |
| Mean body temperature (°C) | 39.79±0.07 | 39.42±0.03 | 39.24±0.1 | 39.44±0.05 |
| Mean heart rate (beat/min) | 123.50±0.43 | 125.44±0.12 | 128.32±0.23 | 124.65±0.14 |
| Mean respiratory rate (breaths/min) | 23.13±0.26 | 24.18±0.13 | 24.42±0.22 | 23.66±0.26 |
| Viability | Survival (n=5)<br>Death (n=3) | Survival (n=5) | Survival (n=6)<br>Death (n=2) | Survival (n=2)<br>Death (n=1) |

***Table 2.*** *The values of haematological and blood biochemical parameters between groups*

| Cases | AST (IU/L) | ALT (IU/L) | ALP ( IU/L) | BUN (mg/dL) | Creatinin (mg/dL) | Erytrocyte (x10$^6$µl) | Leucocyte (x10$^3$µL) | Hct (%) | Band neut. (%) | Lymphocyte (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| **Acute Endometritis** | | | | | | | | | | |
| Case 1 | 70 | 10.7 | 249 | 18.59 | 1.18 | 6.5 | 60 | 35 | 27 | 1 |
| Case 2* | 70.4 | 18 | 212 | 121 | 3.07 | 5.5 | 61 | 29 | 14 | 4 |
| Case 3 | 30.8 | 38.9 | 90 | 14.9 | 0.745 | 6.710 | 11.4 | 31 | - | 17 |
| Case 4 | 57.6 | 41.2 | 149 | 27.33 | 0.889 | 3.26 | 54.5 | 25 | 9 | 5 |
| Case 5 | 69 | 23.4 | 102 | 51.86 | 1.04 | 5.64 | 35 | 35 | 12 | 5 |
| Case 6 | 36.6 | 21.9 | 135 | 18.83 | 0.529 | 4.1 | 44 | 25 | 5 | 4 |
| Case 7* | 61 | 60 | 130 | 42.05 | 2.8 | 5.2 | 28.2 | 34 | 27 | 25 |
| Case 8* | 108 | 90.5 | 399 | 134.11 | 0.959 | 7.25 | 59 | 30 | 11 | 2 |
| Mean±SEM | 62.93±8.37 | 38.08±9.32$^{a}$ | 183.30±36.19$^{c}$ | 53.58±16.79$^{f,g}$ | 1.40±0.34$^{i,j}$ | 5.52±0.47 | 44.14±6.37 | 30.50±1.43 | 13.13±3.39 | 7.87±3.00$^{l}$ |
| **Subacute Endometritis** | | | | | | | | | | |
| Case 1 | 45.6 | 61.1 | 170 | 14.43 | 0.625 | 6.64 | 16.4 | 37 | 30 | 9 |
| Case 2 | 34.7 | 17.6 | 93.2 | 29.34 | 1.2 | 6.78 | 16.2 | 37 | 33 | 8 |
| Case 3 | 72 | 29 | 121 | 9.70 | 0.85 | 4.7 | 90 | 28 | 8 | 7 |
| Case 4 | 55.8 | 19 | 97 | 9.81 | 0.87 | 5.9 | 17.4 | 36 | 23 | 10 |
| Case 5 | 61.3 | 27.5 | 226 | 18.92 | 0.866 | 3.55 | 60 | 24 | 19 | 11 |
| Mean±SEM | 53.88±6.41 | 30.84±7.89$^{b}$ | 141.40±25.19$^{d}$ | 16.44±3.64 | 0.88±0.09$^{i,k}$ | 5.51±0.61 | 40.00±15.06 | 32.40±2.69 | 22.60±4.41 | 9.00±0.70$^{l,m}$ |
| **Acute Metritis** | | | | | | | | | | |
| Case 1* | 34.9 | 18.8 | 255 | 85.98 | 1.45 | 4.9 | 55.3 | 22 | 18 | **9** |
| Case 2 | 33.7 | 20.4 | 458 | 20.04 | 1.29 | 5.02 | 26.1 | 35 | 13 | 7 |
| Case 3 | 89.3 | 23.5 | 208 | 42.99 | 1.9 | 5.0 | 5.0 | 31 | 7 | 3 |
| Case 4 | 73 | 10.1 | 204 | 19.62 | 0.865 | 5.9 | 29.8 | 31 | 27 | 4 |
| Case 5 | 64.7 | 23.5 | 212 | 19.62 | 0.856 | 3.66 | 60.0 | 24 | 19 | 11 |
| Case 6* | 70.2 | 12.2 | 305 | 54.67 | 3.3 | 4.8 | 47.4 | 27 | 18 | 7 |
| Case 7 | 55.6 | 27.4 | 633 | 18.64 | 0.871 | 7.6 | 16.8 | 40 | 4 | 10 |
| Case 8 | 85.4 | 19.8 | 256 | 41.12 | 1.8 | 5.4 | 82.5 | 21 | 38 | - |
| Mean±SEM | 63.35±7.38 | 19.46±2.05$^{a,b}$ | 316.40±53.97$^{c,d,e}$ | 37.84±8.43$^{h}$ | 1.54±0.29$^{k}$ | 5.28±0.39 | 40.36±9.01 | 28.88±2.34 | 18.00±3.83 | 6.37±1.33 |
| **Subacute Metritis** | | | | | | | | | | |
| Case 1 | 45.9 | 5 | 96 | 11.02 | 0.826 | 4.1 | 63.2 | 24 | 22 | 4 |
| Case 2* | 55 | 12.8 | 20 | 140.18 | 8.1 | 5.92 | 58.3 | 21 | 24 | 7 |
| Case 3 | 56.8 | 19.8 | 149 | 40.18 | 2.34 | 4.95 | 13 | 36 | 4 | 18 |
| Mean±SEM | 52.57±3.37 | 12.53±4.27 | 88.33±37.43$^{e}$ | 63.79±39.11$^{g,h}$ | 3.75±2.21$^{j}$ | 4.99±0.52 | 44.83±15.98 | 27.00±4.58 | 16.67±6.36 | 9.66±4.25$^{m}$ |

** dead cases*
*There is a statistical importance between the values shown with different letters in the same column (b. k. i. k. l. m P< 0.01) (d. e. h. P<0.05) (a. c. f. g. j P<0.001)*

tissue and degenerative hyperplasia and atrophy on the glandula uteri were determined *(Table 3)*.

*Subacute Endometritis:* Subacute endometritis was microscopically detected in five cases. *E. coli* (n=2), *K. oxytoca* (n=1) and *ß-hemolytic streptococcus* (n=2) were isolated in this group. All of these bitches recovered after the ovariohysterectomy. Glandular hyperplasia was found in the uterus of a case in which *E. coli* and *K. oxytoca* were isolated *(Fig.1-b)*. Squamous metaplasia on the surface epithelial cells was determined in the case of isolated *K. oxytoca*. An atrophic uterine gland was detected in two cases of isolated *ß-hemolytic streptococcus*. Squamous metaplasia was observed on the surface epithelial cells in one of these cases *(Table 4)*.

*Acute Metritis:* Acute metritis was determined in eight cases. Two bitches died after the ovariohysterectomy in the group. While no bacterial growth was observed in one case, gram negative bacteria (five of *E. coli*, one of *Ps. aeruginosa*, and one of *K. pneumonia*) were isolated in other cases. Papillary hyperplasia and cyst-like honeycomb were detected in the case with no bacteria and with isolated *K. pneumonia*. There was inflammation in both

***Table 3.*** *The histopathological view of cases with acute endometritis*

| Cases | Surface Epithelial Cells | Inflammation | | T. Muscularis | Str. Vasculare | Stromal Connective Tissue | Gl.Uteri | Isolated Bacteria | Time to Onset of the Disease (day) | Toxin Level (EU/mL) | Cervix | Viability |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Endomet. | Myomet. | | | | | | | | | |
| 1 | Normal | + | - | Hyperemia | Leukocytosis, swelling in endothelial cells | - | Large cysts | E coli | 20 | 0.24 | Opened | Survival |
| 2 | Degenerated | ++ | - | Hyperemia | Leukocytosis | Congestion | Cystic degenaration | E coli | 30 | 3.84 | Opened | Death |
| 3 | Normal | - | - | Congestion | - | Congestion, Hemorrhage | Uniform | E coli | 7 | 0.48 | Opened | Survival |
| 4 | Normal | + | - | Hyperemia | - | Hemorrhage | Papillary hyperplasia, Large cysts | Ps aeruginosa | 180 | 0.48 | Opened | Survival |
| 5 | Degenerated | + | + | Hyperemia | - | Oedemma | Large cysts | Ps aeruginosa | 7 | 0.96 | Closed | Survival |
| 6 | - | - | - | - | - | - | Papillary hyperplasia, Large cysts | Enterococ | 15 | - | Opened | Survival |
| 7 | - | + | - | - | - | Oedemma | Atrophic | CPS | 7 | - | Opened | Death |
| 8 | Degenerated | + | - | - | - | Oedemma, Congestion, Hemorrhage | Degenerated hyperplasia | CPS | 7 | - | Opened | Death |

*Endomet.: Endometrium; Myomet.: Myometrium; T. muscularis: Tunica muscularis; Str. vasculare: Stratum vasculare; Gl. uteri: Glandula uteri; CPS: Coagulase positive Staphylococ*

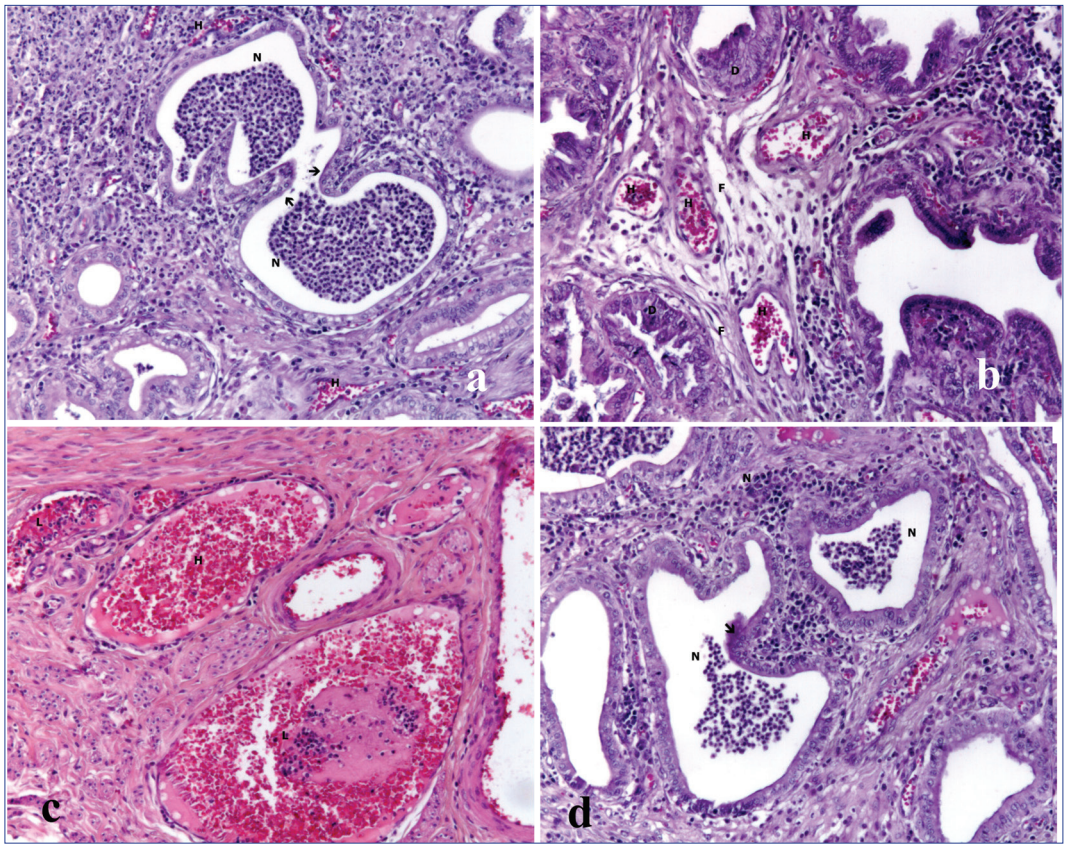



**Fig 1.** The view of histopathological changes in uterine tissue. **(a)** Acute endometritis; neutrophile leucocyte in lamina propria and tunica muscularis (N) and few mononuclear cell infiltration, papillar cystic dilatations in glands *(arrows)* and hyperemia (H), **(b)** Subacute endometritis; hyperplasie in gland epithelial cells (D), connective tissue in lamina propria (F) and other mononuclear cell infiltrations, hyperemia (H), **(c)** Acute metritis; leukostasis in tunica muscularis veins (L) and hyperemia (H) **(d)** Subacute metritis; mononuclear inflammatory cells aand connective tissue in lamina propria with neutrophile leukocyte infiltration in gland lumen (N), papillar hyperplasie *(arrow)* and cystic dilatations in glands and hyperemia (H), HE, x100

**Table 4.** *The histopathological view of cases with subacute endometritis*

| Cases | Surface Epithelial Cells | Inflammation | | T. Muscularis | Str. Vasculare | Stromal Connective Tissue | Gl.Uteri | Isolated Bacteria | Time to Onset of the Disease (day) | Toxin Level (EU/mL) | Cervix | Viability |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Endomet. | Myomet. | | | | | | | | | |
| 1 | Squamous metaplasia in some area | + | - | Hyperemia | - | Oedemma, Congestion, Hemorrhage, fibroblast proliferation | Large cysts | *K oxytoca* | 210 | 0.24 | Opened | Survival |
| 2 | Degenerated | + | - | Hyperemia | - | Oedemma, Congestion | Large cysts | *E coli* | 20 | 0.48 | Opened | Survival |
| 3 | Degenerated | + | + | Hyperemia | Leukocytosis | Oedemma, Congestion | Hyperplasia, Large cysts | *E coli* | 20 | 0.48 | Opened | Survival |
| 4 | Normal | ++ | - | Hyperemia, Intense hemorrhage | - | Congestion, Intense hemorrhage | Intense atrophic | β HemolyticStrep | 150 | - | Opened | Survival |
| 5 | Metaplasia | - | - | - | - | Hemorrhage | Atrophic | β HemolyticStrep | 20 | - | Opened | Survival |

*Endomet.: Endometrium; Myomet.: Myometrium; T. muscularis: Tunica muscularis; Str. vasculare: Stratum vasculare; Gl. uteri: Glandula uteri; β HemolyticStrep: ß hemolytic Streptococus*

**Table 5.** *The histopathological view of cases with acute metritis*

| Cases | Surface Epithelial Cells | Inflammation | | T. Muscularis | Str.Vasculare | Stromal Connective Tissue | Gl.Uteri | Isolated Bacteria | Time to Onset of the Disease (day) | Toxin Level (EU/mL) | Cervix | Viability |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Endomet. | Myomet. | | | | | | | | | |
| 1 | Squamous metaplasia | + | + | Hemorrhage | Leukocytosis, swelling in endothelial cells | Congestion, Intense hemorrhage | Irregular stroma, Small cysts | *E coli* | 10 | 1.92 | Closed | Death |
| 2 | Degenerated | + | - | - | Hyperemia | Oedemma, Congestion, Hemorrhage | Cystic degenaration | *E coli* | 7 | 0.96 | Closed | Survival |
| 3 | Degenerated | + | - | - | Leukocytosis, swelling in endothelial cells | Oedemma, Congestion, Hemorrhage | Papillary hyperplasia, Large cysts | *E coli* | 15 | 0.96 | Closed | Survival |
| 4 | Degenerated | + | - | Hyperemia | Leukocytosis | Oedemma, Congestion, Hemorrhage, Fibroblast proliferation | Irregular stroma, Small cysts | *E coli* | 10 | 0.96 | Closed | Survival |
| 5 | Degenerated | ++ | - | Hyperemia | - | Fibroblast proliferation | Papillary hyperplasia, Large cysts | *E coli* | 21 | 0.48 | Closed | Survival |
| 6 | Squamous metaplasia | + | + | Oedemma | Leukocytosis, Hyperemia | Oedemma, Congestion | Large cyst | *Ps aeruginosa* | 7 | 3.84 | Closed | Death |
| 7 | Degenerated | + | - | Hyperemia | Leukocytosis, swelling in endothelial cells | Congestion, Hemorrhage | Papillary hyperplasia, Cysts-like honeycomb | *K pneumonia* | 7 | 0.48 | Closed | Survival |
| 8 | Degenerated | + | - | Hyperemia | Leukocytosis | Oedemma, Congestion | Papillary hyperplasia, Cysts-like honeycomb | No growth bacteria | 15 | - | Closed | Survival |

*Endomet.: Endometrium; Myomet.: Myometrium; T. muscularis: Tunica muscularis; Str. vasculare: Stratum vasculare; Gl. uteri: Glandula uteri*

the endometrium and myometrium, and squamous metaplasia on the surface epithelial cells was found in dead bitches compared to recovered bitches. In the dead cases in which *E. coli* was isolated, the blood endotoxin level was 1.92 EU/mL. Intensive hemorrhage on the stromal connective tissue was discovered in this case *(Fig. 1-c)* and the blood endotoxin level in the dead case with isolated *Ps. aeruginosa* was 3.84 EU/mL; unlike the other cases, edema was seen in the tunica muscularis *(Table 5)*.

*Subacute Metritis:* Subacute metritis was seen in three cases, one case died following the operation. *E. coli* was isolated in all three cases, and inflammation was found in both the endometrium and myometrium in these cases.

**Table 6.** *The histopathological view of cases with subacute endometritis*

| Cases | Surface Epithelial Cells | Inflammation | | T. Muscularis | Str. Vasculare | Stromal Connective Tissue | Gl. Uteri | Isolated Bacteria | Time to Onset of the Disease (day) | Toxin Level (EU/mL) | Cervix | Viability |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Endomet. | Myomet. | | | | | | | | | |
| 1 | Normal | + | + | Oedemma, Hyperemia | Leukocytosis, swelling in endothelial cells | - | Hyperplasia, Large cysts | *E coli* | 7 | 0.48 | Opened | Survival |
| 2 | Degenerated | +++ | +++ | Oedemma, Hemorrhage | Leukocytosis, Hyperemia | Congestion | Large cysts lining to papillary extension | *E coli* | 15 | 0.96 | Opened | Death |
| 3 | Normal | + | + | - | - | Hemorrhage, Congestion | Uniform | *E coli* | 7 | 0.96 | Opened | Survival |

*Endomet.: Endometrium; Myomet.: Myometrium; T. muscularis: Tunica muscularis; Str. vasculare: Stratum vasculare; Gl. uteri: Glandula uteri*

The inflammation was severe in the dead bitch *(Fig. 1-d)* and the degeneration of the surface epithelial cells was established in this bitch *(Table 6)*.

## DISCUSSION

Pyometra, which is also known as cystic endometrial hyperplasia-pyometra complex, is a uterine bacterial infection associated with the thickening of the endometrium due to an increase in the size and number of endometrial glands [4,10,17]. If pyometra is allowed to continue untreated, death may occur due to bacterial toxic shock and multiple organ failure [4,9,11]. However, bacterial endotoxins damage the vascular endothelial structure and cause a prothrombin-antithrombin imbalance; this situation could lead to disseminated intravascular coagulation (DIC) and clinical findings may be severe in bitches with endotoxic pyometra. The prognosis of the disease is more important and the surgical approach may carry a high risk of death [18-20]. It was determined that gram negative bacteria (three *E. coli* and one *Ps. aeruginosa*) were isolated from four out of the six bitches that died. We conceived that the death could be observed due to DIC or/and multiple organ dysfunction in these bitches with poor prognosis.

The disease is generally caused by a Gram negative bacteria, typically *E. coli* [21], but other bacterial species such as staphylococci, streptococci, *Enterococcus* spp., *Klebsiella* spp. and *Ps. aeruginosa*, *Proteus* spp., *Pasteurella* spp. could also been detected [11,22,23]. It has been established that if *E. coli* migrates to the uterus, it binds with higher affinity to the endometrium and myometrium layers and constitutes a resistance to the uterus' defense mechanisms such as lactoferrin and mucin-1 [24-27]. As reported in previous studies [4,11,23,28], gram negative bacteria (75%) such as *E. coli*, Klebsiella spp. and *Ps. aeruginosa* were dominantly isolated and *E. coli* (54.16%) was the most isolated bacteria in this study.

It is well known that changes in levels of liver and kidney enzymes could be caused by the effects of gram negative bacterial endotoxin [2,11,29,30]. Furthermore, bacterial endotoxins induce a marked increase in the number of circulating polymorphonuclear phagocytes (regenerative left shift). In addition, anemia could be observed due to the bone marrow suppression by bacterial endotoxins [2,24,30-32]. In the present study, the changes in the different degrees were observed in the blood biochemical and hematological parameters in bitches with isolated gram negative bacteria. The levels of ALP, BUN, and creatinine showed a positive correlation (r=0.87) with the blood endotoxin level. The increased levels of ALP, renal deficiency, and high toxin levels in the bitches with isolated gram negative bacteria were associated with each other in pyometra, as indicated in previous studies [2,33,34]. The bitches in which significant deviations in the levels of BUN ($P<0.01$), creatinine ($P<0.01$) and toxin ($P<0.0001$) were observed showed severe clinical signs and poor prognosis, and were dead within two days following the ovariohysterectomy. We thought that the high toxin amount in these bacteria lead to damage in the liver and kidney; in particular, irreversible acute renal failure caused death, as reported in previous studies [4,9,11,35,36].

A uterine content swab obtained from one bitch in our study was bacteriologically sterile. Bacteria caused by this disease may have been killed by uterine defense mechanisms or antibiotic treatment [37]. The leucocyte count was found to be significantly elevated (82.5 x 103 µL) in this bitch. In previous studies, neutrophils consisting of high concentration lactoferrin, which has a critical role in the uterine defense mechanism, infiltrated the uterus and glandular epithelial cells might express this protein to kill bacteria or inhibit bacterial growth [24,38-40]. In the present study, CPS was isolated in two bitches. Severe clinical symptoms including increased blood biochemical and hematological parameters were observed, and the time to onset of the disease was seven days in these bitches. These cases were dead within two days following the ovariohysterectomy. Staphylococci species can cause a variety of life-threatening infections such as sepsis and toxic shock syndrome. These species released harmful proteins and enzymes, which can cause tissue and

organ damage [41,42]. We thought that these bitches were irreversibly affected by coagulase-positive staphylococci in a short time period and death was observed.

Beta hemolytic streptococci, like staphylococci, can cause life-threatening acute infections and deaths due to their secreted virulence factors such as streptolysin. In addition, these organisms stimulate the production and activities of superantigens. These superantigens are important in the development of toxic shock syndrome [42,43]. In this study, beta hemolytic streptococci were isolated in two bitches; clinical and laboratory findings were not severe, and these cases survived after the operation. We noted that systemic antibiotics had been occasionally administered to these bitches with open cervix pyometra at another clinic, but the bitches had shown no improvement. It was considered that antibiotics could slow the disease's progression. Enterococcal infections have long been associated with a higher risk of treatment failure because of their resistance to many antibiotics (penicillin by beta-lactamases, chloramphenicol, tetracyclines, rifampin, fluoroquinolones, and aminoglycosides) and mortality [44]. In the present study, Enterococcus was isolated in one case. Blood biochemical parameters within the reference values and changes in the mid-level hematological parameters were determined. The clinical symptoms were not severe and this case recovered after the operation. It is thought that this bitch survived due to appropriate antibiotic administration and there were no severe clinical findings.

In conclusion, the results of the present study revealed that the prognosis of the disease may improve in bitches diagnosed with subacute endometritis associated with low levels of blood endotoxin. However, for acute endometritis, acute metritis, and subacute metritis, the endotoxemic bitches with a blood endotoxin level above 0.96 EU/mL and significantly increased ALP, BUN, and creatinine concentration would likely end up with mortality outcome following ovariohysterectomy. These parameters may serve as good prognostic markers in bitches with pyometra.

## REFERENCES

**1. Baithalu RK, Maharana BR, Mishra C, Sarangi L, Samal L:** Canine pyometra. *Vet World*, 3 (7): 340-342, 2010

**2. Uçmak M, Tek Ç, Gündüz MC, Sabuncu A, Şenünver A, Bağcigil FA, Bakırel T:** Optimum timing for operation in bitches with pyometra related to endotoxemia. *Turk J Vet Anim Sci*, 36 (1): 35-42, 2012. DOI: 10.3906/vet-1102-762

**3. Karlsson I, Hagman R, Guo Y, Humblot P, Wang L, Wernersson S:** Pathogenic *Escherichia coli* and lipopolysaccharide enhance the expression of IL-8, CXCL5, and CXCL10 in canine endometrial stromal cells. *Theriogenology,* 84, 34-42, 2015. DOI: 10.1016/j.theriogenology.2015.02.008

**4. Kempisty B, Bukowska D, Wozna M, Piotrowska H, Jackowska M, Zuraw A, Ciesiolka S, Antosik P, Maryniak H, Ociepa E, Porowski Sz, Brussow KP, Jaskowski JM, Nowicki M:** Endometritis and pyometra in bitches: A review. *Vet Med*, 58 (6): 289-297, 2013

**5. Sykes JE:** Gram negative bacterial infections. **In,** Sykes JE (Ed): Canine and Feline Infectious Diseases. Part II Major infectious diseases and their etiologic agents. Section 2 Bacterial diseases, 355-364, Saunders Elsevier, 2013.

**6. Ramachandran G:** Gram-positive and gram-negative bacterial toxins in sepsis. *Virulence,* 5, 213-218, 2014. DOI: 10.4161/viru.27024

**7. Küplülü S, Vural MR, Demirel A, Polat M, Akcay A:** The comparative evaluation of serum biochemical, haematological, bacteriological and clinical findings of dead and recovered bitches with pyometra in the postoperative process. *Acta Vet-Beograd*, 59 (2-3): 193-204, 2009. DOI: 10.2298/AVB0903193K

**8. Jena B, Sadasiva Rao K, Reddy KCS, Raghavend KBP:** Physiological and haematological parameters of bitches affected with pyometra. *Vet World,* 6 (7): 409-412, 2013. DOI: 10.5455/vetworld.2013.409-412

**9. Jitpean S, Ström-Holst B, Emanuelson U, Höglund O, Pettersson A, Alneryd-Bull C, Hagman R:** Outcome of pyometra in female dogs and predictors of peritonitis and prolonged postoperative hospitalization in surgically treated cases. *BMC Vet Res,* 10: 6, 2014. DOI: 10.1186/1746-6148-10-6

**10. Gibson A, Dean R, Yates D, Stavisky J:** A retrospective study of pyometra at five RSPCA hospitals in the UK: 1728 cases from 2006 to 2011. *Vet Rec*, 173, 396, 2013. DOI: 10.1136/vr.101514

**11. Sant'Anna MC, Giordano LGP, Flaiban KKMC, Muller EE, Martins MIM:** Prognostic markers of canine pyometra. *Arq Bras Med Vet Zootec,* 66 (6): 1711-1717, 2014. DOI: 10.1590/1678-6859

**12. Bronwyn Crane M:** Pyometra. **In,** Silverstein D, Hopper K (Eds): Small Animal Critical Care Medicine Part XIII: Urogenital Disorders Chapter 126, 2nd edn., 667-671, Saunders Elsevier, 2015.

**13. Van Cruchten S, Van den Broeck W, Simoens P, Lauwers H:** Scanning electron microscopic changes of the canine uterine luminal surface during oestrus and late metoestrus. *Reprod Domest Anim*, 37 (3):121-126, 2002. DOI: 10.1046/j.1439-0531.2002.00347.x

**14. Hagman R, Kindahl H, Fransson BA, Bergström A, Ström Holst B, Lagerstedt AS:** Differentiation between pyometra and cystic endometrial hyperplasia/mucometra in bitches by prostaglandin $F_{2\alpha}$ metabolite analysis. *Theriogenology*, 66, 198-206, 2006. DOI: 10.1016/j.theriogenology.2005.11.002

**15. Ambrósio CE, Brolio MP, Martins DS, Morini JC, Carvalho AF, Miglino MA:** Endometrial alterations, early placentation and maternal fetal interaction in carnivores. *Rev Bras Reprod Anim*, 35 (2): 217-228, 2011

**16. Bartel C, Tichy A, Schoenkypl S, Aurich C, Walter I:** Effects of steroid hormones on differentiated glandular epithelial and stromal cells in a three dimensional cell culture model of the canine endometrium. *BMC Vet Res*, 9:86, 2013. DOI: 10.1186/1746-6148-9-86

**17. De Bosschere H, Ducatelle R, Vermeirsch H, Simoens, P, Coryn M:** Estrogen-alpha and progesterone receptor expression in cystic endometrial hyperplasia and pyometra in the bitch. *Anim Reprod Sci*, 70, 251-259, 2002. DOI: 10.1016/S0378-4320(02)00013-1

**18. Smith FO:** Canine pyometra. *Theriogenology*, 66, 610-612, 2006. DOI: 10.1016/j.theriogenology.2006.04.023

**19. Tanja P, Barbara C, Kristina D, Pečar J, Alenka N, Butinar J:** Haemostasis impairment in bitches with pyometra. *Acta Vet-Beograd*, 56: (5-6), 529-540, 2006. DOI: 10.2298/AVB0606529P

**20. Radwińska J, Domosławska A, Pomianowski A, Żarczyńska K, Jurczak A:** Implications of blood coagulation and fibrinolytic disorders in severe endometritis-pyometra complex in bitches. *Bull Vet Inst Pulawy*, 56, 293-297, 2012. DOI: 10.2478/v10213-012-0053-x

**21. Siqueira AK, Ribeiro MG, Leite D da S, Tiba MR, de Moura C, Lopes MD, Prestes NC, Salerno T, da Silva AV:** Virulence factors in *Escherichia coli* strains isolated from urinary tract infection and pyometra cases and from feces of healthy dogs. *Res Vet Sci*, 86 (2): 206-210, 2009. DOI: 10.1016/j.rvsc.2008.07.018

**22. Arora A, Sandford J, Browning GF, Sandy JR, Wright PJ:** A model for cystic endometrial hyperplasia/pyometra complex in the bitch. *Theriogenology*, 66, 1530-1536, 2006. DOI: 10.1016/j.theriogenology.2006.02.019

**23. Coggan JA, Melville PA, Motos de Oliveira C, Faustino M, Moreno AM, Benites NR:** Microbiological and histopathological aspects of canine

pyometra. *Braz J Microbiol*, 39 (3): 477-483, 2008. DOI: 10.1590/S1517-83822008000300012

**24. Kida K, Baba E, Torri R, Kawate N, Hatoya S, Wijewardana V, Sugiura K, Sawada T, Tamada H, Inaba T:** Lactoferrin expression in the canine uterus during the estrous cycle and with pyometra. *Theriogenology*, 66, 1325-1333, 2006. DOI: 10.1016/j.theriogenology.2006.04.028

**25. Ishiguro K, Baba E, Torii R, Tamada H, Kawate N, Hatoya S, Wijewardana V, Kumagai D, Sugiura K, Sawada T, Inaba T:** Reduction of mucin-l gene expression associated with increased *Escherichia coli* adherence in the canine uterus in the early stage of dioestrus. *Vet J*, 173, 325-332, 2007. DOI: 10.1016/j.tvjl.2005.11.009

**26. Dovc P, Kunej T, Williams GA:** Genetics and Genomics of Reproductive Disorders. **In,** Jiang Z, Ott TL (Eds): Reproductive Genomics in Domestic Animals, Chapter 4, 67-97, Wiley-Blackwell, USA, 2010

**27. Voorwald FA, Marchi FA, Villacis RAR, Alves CEF, Toniollo GH, Amorim RL, Drigo SA, Rogatto SR:** Molecular expression profile reveals potential biomarkers and therapeutic targets in canine endometrial lesions. *PLoS One,* 10 (7): e0133894, 2015. DOI: 10.1371/journal.pone.0133894

**28. Bassessar V, Verma Y, Swamy M:** Antibiogram of bacterial species isolated from canine pyometra. *Vet World,* 6 (8): 546-549, 2013. DOI: 10.5455/vetworld.2013.546-549

**29. Fransson BA:** Ovaries and uterus. **In,** Tobias KM, Johnston SA (Eds): Veterinary Surgery: Small Animal: 2-Volume Set. Urogenital System, Section XI, Chapter 109, 1871-1891, Saunders Elsevier, 2012

**30. Jena B, Rao KS, Reddy KCS, Raghavender KBP:** Comparative efficacy of various therapeutic protocols in the treatment of pyometra in bitches. *Vet Med*, 58 (5): 271-276, 2013

**31. Hagman R, Kindahl H, Lagerstedt AS:** Pyometra in bitches induces elevated plasma endotoxin and prostaglandin $F_{2\alpha}$ metabolite levels. *Acta Vet Scand*, 47 (1): 55-68, 2006. DOI: 10.1186/1751-0147-47-55

**32. Chandra R, Villanueva E, Feketova E, Machiedo GW, Haskò G, Deitch EA, Spolarics Z:** Endotoxemia down-regulates bone marrow lymphopoiesis but stimulates myelopoiesis: The effect of G6PD deficiency. *J Leukoc Biol*, 83, 1541-1550, 2008. DOI: 10.1189/jlb.1207838

**33. Feldman EC, Nelson RW:** Cystic endometrial hyperplasiapyometra complex. **In,** Feldman EC, Nelson RW (Eds): Canine and Feline Endocrinology and Reproduction, 852-866, W.B. Saunders Co, USA, 2004.

**34. Maddens B, Daminet S, Smets P, Meyer E:** *Escherichia coli* pyometra induces transient glomerular and tubular dysfunction in dogs. *J Vet Intern Med,* 24, 1263-1270, 2010. DOI: 10.1111/j.1939-1676.2010.0603.x

**35. Heiene R, Kristiansen V, Teige J, Jansen JH:** Renal histomorphology in dogs with pyometra and control dogs, and long term clinical outcome with respect to signs of kidney disease. *Acta Vet Scand,* 49: 13, 2007. DOI: 10.1186/1751-0147-49-13

**36. Demirel MA, Küplülü Ş:** Investigation on the antiendotoxic effect of the combination of polymyxin E and ampicillin in dogs with endotoxic pyometra. *Kafkas Univ Vet Fak Derg*, 16, 313-318, 2010. DOI: 10.9775/kvfd.2009.730

**37. Bigliardi E, Parmigiani E, Cavirani S, Luppi A, Bonati L, Corradi A:** Ultrasonography and cystic hyperplasia-pyometra complex in the bitch. *Reprod Domest Anim*, 39, 136-140, 2004. DOI: 10.1111/j.1439-0531.2004.00489.x

**38. Hagman R, Kühn I:** *Escherichia coli* strains isolated from the uterus and urinary bladder of bitches suffering from pyometra: Comparison by restriction enzyme digestion and pulsed-field gel electrophoresis. *Vet Microbiol*, 84, 143-153, 2002. DOI: 10.1016/S0378-1135(01)00449-7

**39. Wernicki A, Krzyzanowski J, Puchalski A:** Characterization of Escherichia coli strains associated with canine pyometra. *Pol J Vet Sci,* 5, 51-56, 2002.

**40. Toydemir Karabulut TSF, Inal Gültekin G, Ateş A, Turna Yilmaz Ö, Enginler SÖ, Kirşan I, Erdoğan Bamaç Ö, Arun SS, Demirtaş B:** Effects of pyometra on some oxidative stress parameters, inflammatory mediators and neutrophil segmentation in bitches. *Kafkas Univ Vet Fak Derg*, 22, 837-846, 2016. DOI: 10.9775/kvfd.2016.15443

**41. Vehmas-Ali T, Sandholm M:** Balance between bacteria and host - the bacteria's point of view. **In,** Sandholm M, Honkanen-Buzalski T, Kaartinen L, Pyörälä S (Eds): The Bovine Udder and Mastitis, 49-58, Finland: Gummerus Kirjapaino Oy, Jyväskylä, 1995.

**42. Spaulding AR, Salgado-Pabón W, Kohler PL, Horswill AR, Leung DYM, Schlieverta PM:** Staphylococcal and streptococcal superantigen exotoxins. *Clin Microbiol Rev,* 26, 422-447, 2013. DOI: 10.1128/CMR.00104-12

**43. Sierig G, Cywes C, Wessels MR, Ashbaugh CD:** Cytotoxic effects of streptolysin o and streptolysin s enhance the virulence of poorly encapsulated group a streptococci. *Infect Immun,* 71 (1): 446-455, 2003. DOI: 10.1128/IAI.71.1.446-455.2003

**44. Chatterjee I, Iredell JR, Woods M, Lipman J:** The implications of enterococci for the intensive care unit. *Crit Care Resusc*, 9 (1): 69-75, 2007.

Kafkas Univ Vet Fak Derg
24 (5): 673-679, 2018
DOI: 10.9775/kvfd.2018.19713

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Evaluation of Dietary Synbiotic Supplementation on Growth Performance, Muscle Antioxidant Ability and Mineral Accumulations, and Meat Quality in Late-finishing Pigs

Ye Fei CHENG [1] Yue Ping CHEN [1] Ming Fang DU [1] Chao WEN [1] Yan Min ZHOU [1]

[1] College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, P. R. CHINA

**Article Code: KVFD-2018-19713 Received: 06.03.2018 Accepted: 09.07.2018 Published Online: 10.07.2018**

**How to Cite This Article**

**Cheng YF, Chen YP, Du MF, Wen C, Zhou YM:** Evaluation of dietary synbiotic supplementation on growth performance, muscle antioxidant ability and mineral accumulations, and meat quality in late-finishing pigs. *Kafkas Univ Vet Fak Derg,* 24 (5): 673-679, 2018. DOI: 10.9775/kvfd.2018.19713

## Abstract

The present study aimed to investigate the effects of synbiotic supplementation on growth performance, muscle antioxidant capacity and mineral contents, and meat quality in late-finishing pigs. Fifty barrow pigs were randomly allocated into two treatments with five replicates each and fed a basal diet supplemented with or without 1 g/kg synbiotic consisted of prebiotics (yeast cell wall and xylooligosaccharide) and probiotics *(Clostridium butyricum, Bacillus licheniformis,* and *Bacillus subtilis)* for 21 days, respectively. Treatment did not affect growth performance in late-finishing pigs (P>0.05). Compared with the control group, pigs in the synbiotic group exhibited a higher superoxide dismutase activity in the *Longissimus dorsi* (LD) muscle, whereas a lower malondialdehyde concentration in the gluteus muscle (P<0.05). Additionally, dietary synbiotic inclusion decreased drip loss in the LD and gluteus muscles at 48 h post-mortem, and cooking loss in the LD muscle compared with the control group (P<0.05). In contrast, dietary synbiotic supplementation numerically reduced total lead retention in the gluteus muscle (P<0.1). The results suggested that dietary synbiotic supplementation to the diet of late-finishing pigs would enhance muscle antioxidant capacity, improve meat quality, whereas numerically reduce muscle lead retention.

***Keywords:*** *Synbiotic, Muscle antioxidant capacity, Meat quality, Mineral accumulations, Late-finishing pigs*

# Domuzların Geç-Son Yetiştirme Döneminde Sinbiyotik İlavesinin Büyüme Performansı, Kas Antioksidan Kapasitesi, Mineral Birikimi ve Et Kalitesi Üzerine Etkilerinin Değerlendirilmesi

## Öz

Bu çalışmada domuzların geç-son yetiştirme döneminde sinbiyotik ilavesinin büyüme performansı, kas antioksidan kapasitesi, mineral birikimi ve et kalitesi üzerine etlilerinin araştırılması amaçlanmıştır. Çalışmada elli adet hadım edilmiş domuz 5 tekrar, iki uygulama grubu olarak rastgele ayrılmış, probiyotik (Mantar hücre duvarı ve Ksilooligosakkarit) ve probiyotikten *(Clostridium butyricum, Bacillus licheniformis* ve *Bacillus subtilis)* oluşan 1 g/kg sinbiyotik katılmış veya katılmamış bazal diyet ile 21 gün süresince beslendi. Sinbiyotik uygulaması geç-son yetiştirme dönemindeki domuzlarda büyüme performansına etki etmedi (P>0.05). Kontrol grubu ile karşılaştırıldığında, sinbiyotik grubundaki domuzların *Longissimus dorsi* (LD) kasında süperoksit dismutaz aktivitesi daha yüksek gluteal kasında da malondialdehit konsantrasyonu daha düşüktü (P<0.05). Diyette sinbiyotik verilmesi, kontrol grubu ile karşılaştırıldığında LD ve gluteal kaslarında postmortem 48. saatte damla kaybında ve LD kasında pişirme kaybında azalmaya neden oldu (P<0.05). Diyette sinbiyotik verilmesi gluteal kasında sayısal olarak azalmış kurşun tutulmasına neden oldu (P<0.1). Elde edilen sonuçlar domuzların geç-son yetiştirme döneminde diyete sinbiyotik ilavesinin kas antioksidan kapasitesinde iyileşmeye ve et kalitesinde gelişmeye neden olurken sayısal olarak kas kurşun tutulumunda azalmaya neden olduğunu göstermiştir.

***Anahtar sözcükler:*** *Sinbiyotik, Kas antioksidan kapasitesi, Et kalitesi, Mineral birikimi, Geç-son yetiştirme dönemi domuzu*

## INTRODUCTION

Sub-therapeutic dosages of antibiotics were usually used in commercial feed for the purpose of growth promotion in livestock production, which subsequently resulted in the reduction of their therapeutic effectiveness against pathogenic microorganisms and development of anti-microbial resistance [1]. As a consequence, the European Union has forbidden the addition of antibiotics as growth promoters in the feed since 2006 due to the public

**İletişim (Correspondence)**
+86 25 84396067 Fax: +86 25 84395314
zhouym6308@163.com

concern about the antibiotics resistance and food safety [2]. Accordingly, the search for alternatives to antibiotics has become more essential and emergent. Probiotic, prebiotic and/or their combination may represent potential alternatives approaches to replace antibiotics in animal production, and have received increasing attention due to their beneficial effects on livestock in recent years [3]. The term of synbiotic is defined as a combination of both probiotic and prebiotic, which could confer benefits on host animal by encouraging a better balance of gastrointestinal tract where living microorganisms are improved, and by selectively stimulating the favorable growth and/or activity of one or a limited number of health-promoting bacteria [3]. Previously published studies conducted on pigs have demonstrated that the supplementation of synbiotic with different components could replace antibiotics [4], promote body weight gain and feed conversion ratio [5], improve intestinal morphology [6], modulate intestinal microflora composition [5], regulate lipid metabolism [7], and enhance immune function [8]. Additionally, dietary synbiotic inclusion would also be an effective method to alleviate immune suppression in pigs induced by pathogenic microbe [9].

In addition to the regulatory function on animals, the effect of various synbiotics supplementation on meat quality in animals especially poultry has also been reported [10,11]. As active components of synbiotic, published papers have indicated that either probiotic *(Clostridium butyricum*, *Bacillus licheniformis*, and *Bacillus subtilis)* [11,12] or prebiotic (xylooligosaccharide and yeast cell wall) [12,13] could improve meat quality in animals including pigs and broilers. We recently manufactured a synbiotic that was composed of prebiotics (yeast cell wall and xylooligosaccharide) and probiotics *(Clostridium butyricum*, *Bacillus licheniformis*, and *Bacillus subtilis*), and demonstrated that the supplementation of which could promote growth performance, improve meat quality, whereas reduce muscle chromium (Cr) accumulation in broilers during a 42-day experiment [14]. We currently made a new synbiotic containing similar components to those used in our previous trial [14], and hypothesized that this type of synbiotic could exert beneficial consequences on late-finishing pigs. The aim of the present study was therefore conducted to evaluate the effects of dietary synbiotic supplementation on growth performance, muscle antioxidant ability and mineral accumulations, and meat quality in late-finishing pigs.

## MATERIAL and METHODS

All procedures describing management and care of animals in this experiment were conducted under approval by Institutional Animal Care and Use Committee of Nanjing Agricultural University (Protocol No. NJAU-CAST-2014-179).

### *Animals, Diets, and Experimental Design*

A total of fifty late-finishing barrow pigs [(Landrace × Yorkshire) × Duroc] with similar initial body weight (P>0.05) were randomly allocated into two treatments of five pens each, and fed a basal diet supplemented without or with 1 g/kg synbiotic for 21 days, respectively. The basal diet is formulated according to NRC (2012), and the ingredient and nutrient levels of which are presented in *Table 1*. The detailed components of synbiotic (1 g) were shown as following: yeast cell wall (500 mg), xylooligosaccharide (100 mg), *Clostridium butyricum* ($3\times10^9$ CFU), *Bacillus licheniformis* ($2\times10^9$ CFU), and *Bacillus subtilis* ($3\times10^9$ CFU). Additionally, mineral contents in the diets were detected and showed in *Table 2*. Pigs were housed in the naturally ventilated shelter of 16 pens per shelter. Each shelter was designed with the solid concrete floor and large windows controlled by curtains. Additionally, relative humidity and temperature in the environment were recorded using data loggers. Pigs in each pen had *ad libitum* access to feed by the separate feeder and clean water by nipple waterer. At the end of the experiment, pigs were weighed after a 12-h fasting period, and feed consumption was recorded on a basis of the pen. All data were collected to calculate average daily gain, average daily feed intake, and feed/gain ratio.

***Table 1.*** *Composition and nutrient contents of the basal diet (g/kg, as feed basis)*

| Items | Content |
|---|---|
| **Ingredients** | |
| Corn | 792.5 |
| Soybean meal | 171.0 |
| Soybean oil | 13.0 |
| L-lysine | 1.20 |
| Choline chloride | 1.40 |
| Salt | 4.20 |
| Limestone | 8.80 |
| Monocalcium phosphate | 4.60 |
| Vitamin premix[1] | 0. 30 |
| Mineral premix[2] | 3.00 |
| Total | 1000 |
| **Calculated chemical composition[3]** | |
| Metabolizable energy, MJ/kg | 14.23 |
| Crude protein | 137.6 |
| Calcium | 4.80 |
| Available phosphorus | 2.00 |
| Lysine | 6.80 |

*[1] Provided per kg of complete diet: Vitamin A: 5.512 IU; Vitamin $D_3$: 2.250 IU; Vitamin E 24 IU; Vitamin $K_3$: 3 mg; Thiamin: 3 mg; Riboflavin 6 mg; Vitamin $B_6$: 3 mg; Vitamin $B_{12}$: 0.024 mg; Pantothenate: 15 mg; Folic acid: 1.2 mg; biotin: 0.15 mg*
*[2] Provided per kg of complete diet: 8 mg Cu (as copper sulfate); 60 mg Fe (as ferrous sulfate); 10 mg Mn (as manganese sulfate); 60 mg Zn (as zinc sulfate); 0.14 mg I (as potassium iodide); and 0.3 mg Se (as sodium selenite).*
*[3] Calculated according to tables of feed composition and nutritive values in China (2012)*

**Table 2.** *Mineral contents in the diets (mg/kg)*

| Item[1,2] | Control | Synbiotic |
|---|---|---|
| Zn | 99.5±5.3 | 103±6 |
| Fe | 436±14 | 442±13 |
| Mg | 2504±110 | 2549±65 |
| Mn | 51.9±2.5 | 52.5±4.2 |
| Cu | 53.3±6.3 | 57.6±8.0 |
| Cr | 2.32±0.15 | 2.67±0.18 |
| Pb | 4.27±0.52 | 4.58±0.41 |
| Cd | -- | -- |

*[1] Zn, zinc; Fe, iron; Mg, magnesium; Mn, manganese; Cu, copper; Cr, chromium; Pb, lead; Cd, cadmium*
*[2] Values represent means and standard error of triplicate samples*
*"--" indicates that mineral below the detection limit*

### Samples Collection

At the end of the experiment, 20 late-finishing pigs (2 pigs per pen) were selected for the collection of muscle samples. In detail, pigs were transported to a commercial pork packing plant, electrically stunned (225 to 380 V, 0.5 A, for 5 to 6 s), exsanguinated and eviscerated according to standard commercial procedures, and split down the center of the vertebral column. Part of the left *Longissimus dorsi* (LD) and gluteus muscles were collected into self-sealing bags and immediately refrigerated at 4°C for subsequent determination of pH value, meat color, drip loss, and cooking loss. Meanwhile, part of the right LD and gluteus muscles were taken and quickly frozen at -20°C for the determination of antioxidant-related parameters and mineral contents.

### Determination of Antioxidant-Related Indexes

*Longissimus dorsi* and gluteus muscles samples were homogenized in 5 volumes (w/v) of 154 mmol/L ice-cold sterile sodium chloride solution using an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, OH, USA) at a high speed for 10 s. The homogenate was spun at 4450 × *g* for 15 min at 4°C. The collected supernatant was used to determine concentrations of malondialdehyde (MDA) and reduced glutathione (GSH), and activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). The related parameters were measured according to the manufacturer's recommended procedures using commercial chemistry assay kits (Nanjing Jiancheng Institute of Bioengineering, Jiangsu, P. R. China). Total protein content in the muscles was assayed following the description by our previous experiment [14]. Results for MDA and GSH contents were expressed as nmol per milligram protein, and mg per gram protein, respectively, and for GSH-Px and SOD activities were expressed as specific activity units per milligram protein of muscles in late-finishing pigs

### Meat Quality Assay

The pH value in muscles was measured (3 different places around each muscle sample and averaged) both at 45 min and 24 h post-mortem using a portable pH meter (HI9125, HANNA Instruments, Italy), which was calibrated by 2-point method against standard buffer solutions with pH values of 4.0 and 7.0. Meat color in the muscles was estimated at 45 min after slaughter by three variables in triplicate using a hand-held colorimeter (Minolta CR-400, Konica Minolta, Tokyo, Japan) based on the CIELAB system (L*=Lightness; a*=Redness; b*=Yellowness). The drip loss in the muscles at 24 h and 48 h post-mortem was assessed by calculating the difference between the initial and final weight of muscles. Briefly, LD and gluteus muscles were cut and suspended in sealed place at 4°C for 24 h and 48 h, respectively. The weight loss during 24- and 48-h hang time for each sample was recorded for the calculation of drip loss, which was expressed as weight loss (g)/initial weight (kg). The cooking loss in the muscles was determined in accordance with the methodology by our previous study [15]. In brief, roughly 15-mm-thick LD and gluteus muscles were weighed, placed into a plastic bag and cooked in a water bath until its internal temperature reached to 75°C, which was maintained for 20 min. The internal temperature was monitored during cooking using a hand-held temperature probe. Cooked muscle samples were allowed to cool to room temperature, blotted dry, and weighed. The difference of between pre- and post-cooking weight was recorded for cooking loss calculation, and it was expressed as weight loss (g)/initial weight (kg).

### Measurement of Muscle Mineral Contents

The mineral contents including Zn, Fe, Mn, Mg, Cu, Pb, and Cr in the muscles and diets were determined following the description as described by our previous studies [14,15]. In detail, each feed (around 0.5 g) and fresh muscle sample (approximately 2.0 g) was weighed and put in a digestion tube, added with 10 mL volume of mixture acid containing perchloric acid and nitric acid at a ratio of 1:4, which were stand for 12 h at room temperature for subsequent digestion. The digestion procedure was the same to that described by our previous study [15] using a heating block. Delaying digestion time if the digestive solution was not clear and adding appropriate mixture acid carefully if the digestive solution was rare during the digestion process. The mineral contents in the final solution were analyzed with an inductively coupled plasma mass spectrometry, which was selected, operated, and optimized in accordance with the methodology by Yang et al.[15].

### Statistical Analysis

All data were performed using SPSS 18.0 statistical software with pen as the experimental unit, and were analysed by independent samples t test. Statistical difference and trend between the two groups were considered if $P<0.05$ and $0.05<P<0.1$, respectively. The means and standard errors of mean are presented.

# RESULTS

## Growth Performance

Compared with the control group *(Table 3)*, dietary synbiotic supplementation did not affect growth performance in late-finishing pigs (P>0.05).

## Muscle Antioxidant Ability

Pigs in the synbiotic group exhibited a higher SOD activity in the LD muscle *(Table 4*, P<0.05), whereas a lower MDA concentration in the gluteus muscle (P<0.05) compared with those in the control group. However, treatment did not alter remaining antioxidant parameters in the muscles (P>0.05).

## Meat Quality

Dietary synbiotic supplementation did not affect meat color and pH value in the muscles *(Table 5*, P>0.05). Drip loss at 48 h post-mortem in the LD and gluteus muscles (P<0.05) and cooking loss in the LD muscle (P<0.05) in the synbiotic group were lower than the control group.

***Table 3.*** *Dietary synbiotic supplementation on growth performance in late-finishing pigs*

| Items | Control | Synbiotic | SEM[1] | P-value |
|---|---|---|---|---|
| Initial body weight (kg) | 98.6 | 100 | 2.6 | 0.771 |
| Final body weight (kg) | 119 | 120 | 2 | 0.786 |
| Average daily gain (kg/d) | 0.95 | 0.95 | 0.03 | 0.894 |
| Average daily feed intake (kg/d) | 3.67 | 3.61 | 0.08 | 0.772 |
| Feed/gain ratio (kg/kg) | 3.85 | 3.82 | 0.04 | 0.765 |

[1] *SEM, standard errors of mean*

***Table 4.*** *Dietary synbiotic supplementation on muscle antioxidant capacity in late-finishing pigs*

| Items[1] | Control | Synbiotic | SEM[2] | P-value |
|---|---|---|---|---|
| ***Longissimus dorsi muscle*** | | | | |
| MDA (nmol/mg protein) | 0.53 | 0.48 | 0.04 | 0.575 |
| SOD (U/mg protein) | 59.2 | 70.4 | 2.6 | 0.016 |
| GSH (mg/g protein) | 7.42 | 7.83 | 0.53 | 0.724 |
| GSH-Px (U/mg protein) | 23.6 | 28.8 | 2.5 | 0.328 |
| ***Gluteus muscle*** | | | | |
| MDA (nmol/mg protein) | 0.64 | 0.50 | 0.03 | 0.004 |
| SOD (U/mg protein) | 64.1 | 69.7 | 3.1 | 0.387 |
| GSH (mg/g protein) | 9.00 | 8.90 | 0.73 | 0.950 |
| GSH-Px (U/mg protein) | 23.2 | 25.2 | 1.4 | 0.537 |

[1] *MDA, malondialdehyde; SOD, superoxide dismutase; GSH, reduced glutathione; GSH-Px, glutathione peroxidase*
[2] *SEM, standard errors of mean*

***Table 5.*** *Dietary synbiotic supplementation on meat quality in late-finishing pigs*

| Items | Control | Synbiotic | SEM[1] | P-value |
|---|---|---|---|---|
| ***Longissimus dorsi muscle*** | | | | |
| $Lightness_{45\ min}$ | 41.3 | 41.9 | 1.0 | 0.757 |
| $Redness_{45\ min}$ | 6.93 | 8.20 | 0.36 | 0.128 |
| $Yellowness_{45\ min}$ | 6.11 | 6.59 | 0.19 | 0.224 |
| $pH_{45\ min}$ | 6.55 | 6.38 | 0.07 | 0.268 |
| $pH_{24\ h}$ | 5.66 | 5.70 | 0.03 | 0.481 |
| Drip $loss_{24\ h}$ (g/kg) | 47.0 | 41.5 | 2.8 | 0.353 |
| Drip $loss_{48\ h}$ (g/kg) | 76.8 | 60.8 | 3.8 | 0.019 |
| Cooking loss (g/kg) | 274 | 224 | 11 | 0.014 |
| ***Gluteus muscle*** | | | | |
| $Lightness_{45\ min}$ | 36.1 | 38.2 | 1.0 | 0.336 |
| $Redness_{45\ min}$ | 13.7 | 13.7 | 1.0 | 0.976 |
| $Yellowness_{45\ min}$ | 6.19 | 6.71 | 0.33 | 0.465 |
| $pH_{45\ min}$ | 6.57 | 6.57 | 0.05 | 0.967 |
| $pH_{24\ h}$ | 5.79 | 5.72 | 0.04 | 0.414 |
| Drip $loss_{24\ h}$ (g/kg) | 32.3 | 29.2 | 1.0 | 0.123 |
| Drip $loss_{48\ h}$ (g/kg) | 63.2 | 52.8 | 2.3 | 0.044 |
| Cooking loss (g/kg) | 306 | 290 | 10 | 0.437 |

[1] *SEM, standard errors of mean*

## Muscle Mineral Contents

Total Pb accumulation in the gluteus muscle was numerically reduced with the dietary synbiotic inclusion when compared with the control group *(Table 6*, P<0.1). However, the concentrations of muscle Zn, Fe, Mn, Mg, Cu, and Cr were similar between the two groups (P>0.05).

# DISCUSSION

Probiotic, prebiotic and/or synbiotic represent potential alternatives to antibiotic that can promote growth performance in livestock. Shim et al.[5] reported that the supplementation of synbiotic *(Lactobacillus acidophilus*, *Lactobacillus bulgaricus, Bacillus subtilis*, *Saccharomyces cerevisiae*, and oligofructose) would increase average daily gain in suckling piglets. Meanwhile, as one component of synbiotic prepared in this study, yeast cell wall mainly consisted of β-glucans and manno oligosaccharides were demonstrated that it could promote growth performance in weanling pigs [16,17]. Inconsistent with those results above-mentioned, the inclusion of dietary synbiotic comprising prebiotics (yeast cell wall and xylooligosaccharide) and probiotics *(Clostridium butyricum*, *Bacillus licheniformis* and *Bacillus subtilis)* did not alter growth performance in late-finishing pigs in the present study, which was in agreement with the results by Liong et al.[7], who found that the supplementation of synbiotic containing *Lactobacillus acidophilus* and fructooligosaccharide exerted no effect on

**Table 6.** *Dietary synbiotic supplementation on muscle mineral accumulations in late-finishing pigs (mg/kg)*

| Items | Control | Synbiotic | SEM[1] | P-value |
|---|---|---|---|---|
| ***Longissimus dorsi muscle*** | | | | |
| Zn | 13.1 | 13.5 | 0.5 | 0.749 |
| Fe | 8.12 | 9.15 | 0.6 | 0.389 |
| Mn | 0.238 | 0.239 | 0.015 | 0.942 |
| Mg | 329 | 321 | 7 | 0.610 |
| Cu | 0.67 | 0.65 | 0.01 | 0.542 |
| Pb | 0.130 | 0.140 | 0.009 | 0.608 |
| Cr | 0.28 | 0.27 | 0.01 | 0.760 |
| ***Gluteus muscle*** | | | | |
| Zn | 15.0 | 15.3 | 0.5 | 0.777 |
| Fe | 9.82 | 8.72 | 0.6 | 0.440 |
| Mn | 0.28 | 0.22 | 0.02 | 0.150 |
| Mg | 303 | 302 | 7 | 0.922 |
| Cu | 0.80 | 0.68 | 0.05 | 0.291 |
| Pb | 0.168 | 0.152 | 0.005 | 0.088 |
| Cr | 0.49 | 0.39 | 0.04 | 0.247 |

[1] *SEM, standard errors of mean*

growth rate, feed intake, and feed/gain ratio in growing-finishing pig. The unaffected growth performance by synbiotic supplementation in this study may result from the development of digestive system, immunity, and capacity as pigs becoming older, and it was supported by the findings of Alexopoulos et al.[12]. Further studies need to be conducted to evaluate the positive effect of this type of synbiotic on growth performance in pigs during early period. However, it has also been reported that probiotics (*Bacillus subtilis* and *Clostridium butyricum* endospores) inclusion could enhance growth performance in growing-finishing pigs [18]. Therefore, breed, management, and synbiotic variation and its dosage would be nonnegligible factors for growth discrepancy in pigs.

Enzymatic antioxidant system as a main antioxidant defence system plays a vital role in maintenance of redox balance. SOD is one of important components in enzymatic antioxidant system that can catalyze the dismutation of superoxide anions. Additionally, lipid peroxidation is a process that carbon-carbon double bond (s) of lipid is attacked by oxygen free radicals. MDA is decomposed product of lipid peroxidation, and the accumulation of which is an essential indicator for lipid peroxidation. In this study, dietary synbiotic enhanced SOD activity in the LD muscle, whereas reduced MDA concentration in the gluteus muscle. Similarly, decreased MDA accumulation in the thigh muscle of broilers has been observed with similar synbiotic inclusion in our previous trial [14]. Reduced MDA content in the muscle by the supplementation of synbiotic suggests a better oxidative stability of muscle, which may result from regulatory effect of probiotic, prebiotic and/or synbiotic on lipid metabolism [18,19]. As active component of synbiotic used in the present study, it has been reported that *Clostridium butyricum* [19], *Bacillus licheniformis* [20], *Bacillus subtilis* [21], yeast cell wall [22] or xylooligosaccharide [23] supplementation could improve antioxidant capacity (elevated antioxidant enzymes activities and/or reduced MDA content) in the tissues in animals and aquatic organism. Accordingly, a higher SOD activity in the LD muscle, but a lower MDA accumulation in the gluteus muscle with synbiotic inclusion in the present study may be related to the effect of synbiotic on improvement of body antioxidant capacity as well.

Previously published papers have demonstrated that the inclusion of probiotic or prebiotic, similar to the components of synbiotic appeared in the current study, could improve meat quality in broilers [13,24,25]. However, few literatures are available about the effect of this kind of probiotics, prebiotics and/or synbiotic on meat quality in finishing pigs. In this study, the supplementation of dietary synbiotic reduced drip loss at 48 h post-mortem in the muscles and cooking loss in gluteus muscle in late-finishing pigs, and it was consistent with the results by Yang et al.[25], Park and Kim [24], and Cho et al.[13] who reported that probiotic *(Clostridium butyricum* or *Bacillus subtilis)* or prebiotic (β-glucan) supplementation could decrease drip loss and/or cooking loss of raw muscle in broilers. Lipid peroxidation in meat usually results in the overproduction of free radicals and reduces water reservation among myofibrils, which would subsequently increase juice loss of meat [26]. Meat has endogenous antioxidant enzymes such as SOD and GSH-Px that play an important role in protecting against the damages induced by free radical including superoxide anion radical. Therefore, reduced drip loss in the both LD and gluteus muscles and cooking loss in the gluteus muscle in the synbiotic group in the current study may be related to the simultaneously increased SOD activity in LD muscle but decreased MDA accumulation in gluteus muscle.

Food safety such as heavy metal residue resulting from human activities has threatened human beings via food chain, and has also raised public attention due to the damage induced by heavy metal accumulation. The reduction of heavy metal accumulation from feed to animal meat mainly including pork and chicken meat would improve food safety in term of heavy metal retention. *In vitro* studies have indicated that *Bacillus* and fungal cell wall structure such as yeast cell wall used as biosorbents could effectively adsorb heavy metals as Pb, Cu, Cd and/or Cr from wastewater and/or soil contaminated with heavy metals [27,28]. In our recently published paper, synbiotic supplementation that was composed of yeast cell wall, xylo-oligosaccharide, *Clostridium butyricum*, *Bacillus licheniformis*, and *Bacillus subtilis* could significantly reduce total Cr retention in the thigh muscle of broilers [14]. In this study, significant decrease of total Pb and/or Cr accumulations

were not observed in the muscle, but numerical reduction of total Pb accumulation in the gluteus muscle was observed by the supplementation of dietary synbiotic. The discrepancy on muscle mineral accumulations and especially heavy metal retentions between the two experiments may be related to species, duration of synbiotic inclusion, management, and diets. Numerically reduction of total Pb retention in the gluteus muscle in the present study suggests a possibility that dietary synbiotic supplementation could reduce the risk of heavy metal accumulation from animals to human and its negative consequences on public health at some extent, which may attributed to the strong adsorption ability of some components in synbiotic including yeast cell wall and *Bacillus* to heavy metals *in vitro*.

In conclusion, dietary synbiotic supplementation at the level of 1 g/kg to the diet of late-finishing pigs that was consisted of prebiotics (yeast cell wall and xylooligosaccharide) and probiotics *(Clostridium butyricum*, *Bacillus licheniformis*, and *Bacillus subtilis)* could enhance oxidative stability (increased SOD activity in the LD muscle, whereas decreased MDA concentration in the gluteus muscle), improve meat quality (reduced drip loss at 48 h post-mortem in the muscles and cooking loss in the LD muscle), and numerically lower total Pb retention in the gluteus muscle in late-finishing pigs.

## Acknowledgements

We are very grateful to our lab-mates for their technical assistance.

## Conflicts of Interest

The authors declared that they have no conflict of interest.

## REFERENCES

**1. Tien YC, Li B, Zhang T, Scott A, Murray R, Sabourin L, Marti R, Topp E:** Impact of dairy manure pre-application treatment on manure composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. *Sci Total Environ,* 581-582, 32-39, 2017. DOI: 10.1016/j.scitotenv.2016.12.138

**2.Lammie SL, Hughes JM:** Antimicrobial resistance, food safety, and one health: The need for convergence. *Annu Rev Food Sci Technol,* 7 (1): 287-312, 2016. DOI: 10.1146/annurev-food-041715-033251

**3.Hume ME:** Historic perspective: Prebiotics, probiotics, and other alternatives to antibiotics. *Poult Sci,* 90 (11): 2663-2669, 2011. DOI: 10.3382/ps.2010-01030

**4.Chu GM, Lee SJ, Jeong HS, Lee SS:** Efficacy of probiotics from anaerobic microflora with prebiotics on growth performance and noxious gas emission in growing pigs. *Anim Sci J,* 82 (2): 282-290, 2011. DOI: 10.1111/j.1740-0929.2010.00828.x

**5.Shim SB, Verstegen MWA, Kim IH, Kwon OS, Verdonk JMAJ:** Effects of feeding antibiotic-free creep feed supplemented with oligofructose, probiotics or synbiotics to suckling piglets increases the preweaning weight gain and composition of intestinal microbiota. *Arch Anim Nutr,* 59 (6): 419-427, 2005. DOI: 10.1080/17450390500353234

**6.Marinho MC, Pinho MA, Mascarenhas RD, Silva FC, Lordelo MM, Cunha LF, Freire JPB:** Effect of prebiotic or probiotic supplementation and ileo rectal anastomosis on intestinal morphology of weaned piglets. *Livest Sci,* 108 (1-3): 240-243, 2007. DOI: 10.1016/j.livsci.2007.01.084

**7.Liong MT, Dunshea FR, Shah NP:** Effects of a synbiotic containing *Lactobacillus acidophilus* ATCC 4962 on plasma lipid profiles and morphology of erythrocytes in hypercholesterolaemic pigs on high-and low-fat diets. *Br J Nutr,* 98 (4): 736-744, 2007. DOI: 10.1017/S0007114507747803

**8.Andrejčáková Z, Sopková D, Vlčková R, Kulichová L, Gancarčíková S, Almášiová V, Holovská K, Petrilla V, Krešáková L:** Synbiotics suppress the release of lactate dehydrogenase, promote non-specific immunity and integrity of jejunum mucosa in piglets. *Anim Sci J,* 87 (9): 1157-1166, 2016. DOI: 10.1111/asj.12558

**9.Naqid IA, Owen JP, Maddison BC, Gardner DS, Foster N, Tchórzewska MA, La Ragione RM, Gough KC:** Prebiotic and probiotic agents enhance antibody-based immune responses to *Salmonella Typhimurium* infection in pigs. *Anim Feed Sci Technol,* 201, 57-65, 2015. DOI: 10.1016/j.anifeedsci.2014.12.005

**10.Maiorano G, Sobolewska A, Cianciullo D, Walasik K, Elminowska-Wenda G, Sławińska A, Tavaniello S, Żylińska J, Bardowski J, Bednarczyk M:** Influence of *in ovo* prebiotic and synbiotic administration on meat quality of broiler chickens. *Poult Sci,* 91 (11): 2963-2969, 2012. DOI: 10.3382/ps.2012-02208

**11.Zhang ZF, Zhou TX, Ao X, Kim IH:** Effects of β-glucan and *Bacillus subtilis* on growth performance, blood profiles, relative organ weight and meat quality in broilers fed maize-soybean meal based diets. *Livest Sci,* 150 (1-3): 419-424, 2012. DOI: 10.1016/j.livsci.2012.10.003

**12.Alexopoulos C, Georgoulakis IE, Tzivara A, Kyriakis CS, Govaris A, Kyriakis SC:** Field evaluation of the effect of a probiotic-containing *Bacillus licheniformis* and *Bacillus subtilis* spores on the health status, performance, and carcass quality of grower and finisher pigs. *J Vet Med A Physiol Pathol Clin Med,* 51 (6): 306-312, 2004. DOI: 10.1111/j.1439-0442.2004.00637.x

**13.Cho JH, Zhang ZF, Kim IH:** Effects of single or combined dietary supplementation of β-glucan and kefir on growth performance, blood characteristics and meat quality in broilers. *Br Poult Sci,* 54 (2): 216-221, 2013. DOI: 10.1080/00071668.2013.777691

**14.Cheng YF, Chen YP, Li XH, Yang WL, Wen C, Kang YR, Wang AQ, Zhou YM:** Effects of synbiotic supplementation on growth performance, carcass characteristics, meat quality and muscular antioxidant capacity and mineral contents in broilers. *J Sci Food Agric,* 97 (11): 3699-3705, 2017. DOI: 10.1002/jsfa.8230

**15.Yang WL, Chen YP, Cheng YF, Li XH, Zhang RQ, Wen C, Zhou YM:** An evaluation of zinc bearing palygorskite inclusion on the growth performance, mineral content, meat quality, and antioxidant status of broilers. *Poult Sci,* 95 (4): 878-885, 2016. DOI: 10.3382/ps/pev445

**16.Hahn TW, Lohakare JD, Lee SL, Moon WK, Chae BJ:** Effects of supplementation of β-glucans on growth performance, nutrient digestibility, and immunity in weanling pigs. *J Anim Sci,* 84 (6): 1422-1428, 2006. DOI: 10.2527/2006.8461422x

**17.Che TM, Johnson RW, Kelley KW, Van Alstine WG, Dawson KA, Moran CA, Pettigrew JE:** Mannan oligosaccharide improves immune responses and growth efficiency of nursery pigs experimentally infected with porcine reproductive and respiratory syndrome virus. *J Anim Sci*, 89 (8): 2592-2602, 2011. DOI: 10.2527/jas.2010-3208

**18.Meng QW, Yan L, Ao X, Zhou TX, Wang JP, Lee JH, Kim IH:** Influence of probiotics in different energy and nutrient density diets on growth performance, nutrient digestibility, meat quality, and blood characteristics in growing-finishing pigs. *J Anim Sci,* 88 (10): 3320-3326, 2010. DOI: 10.2527/jas.2009-2308

**19.Liao XD, Wu RJ, Ma G, Zhao LM, Zheng ZJ, Zhang RJ:** Effects of *Clostridium butyricum* on antioxidant properties, meat quality and fatty acid composition of broiler birds. *Lipids Health Dis,* 14, 36, 2015. DOI: 10.1186/s12944-015-0035-0

**20.Lei K, Li YL, Yu DY, Rajput IR, Li WF:** Influence of dietary inclusion of *Bacillus licheniformis* on laying performance, egg quality, antioxidant enzyme activities, and intestinal barrier function of laying hens. *Poult Sci,* 92 (9): 2389-2395, 2013. DOI: 10.3382/ps.2012-02686

**21.Shen WY, Fu LL, Li WF, Zhu YR:** Effect of dietary supplementation with *Bacillus subtilis* on the growth, performance, immune response and antioxidant activities of the shrimp *(Litopenaeus vannamei). Aquac Res,* 41 (11): 1691-1698, 2010. DOI: 10.1111/j.1365-2109.2010.02554.x

**22.Li XH, Chen YP, Cheng YF, Yang WL, Wen C, Zhou YM:** Effect of yeast cell wall powder with different particle sizes on the growth performance, serum metabolites, immunity and oxidative status of broilers. *Anim Feed Sci Technol,* 212, 81-89, 2016. DOI: 10.1016/j.anifeedsci.2015.12.011

**23.Wang J, Cao Y, Wang C, Sun B:** Wheat bran xylooligosaccharides improve blood lipid metabolism and antioxidant status in rats fed a high-fat diet. *Carbohydr Polym,* 86 (3): 1192-1197, 2011. DOI: 10.1016/j.carbpol.2011.06.014

**24.Park JH, Kim IH:** Supplemental effect of probiotic *Bacillus subtilis* B2A on productivity, organ weight, intestinal *Salmonella* microflora, and breast meat quality of growing broiler chicks. *Poult Sci,* 93 (8): 2054-2059, 2014. DOI: 10.3382/ps.2013-03818

**25.Yang X, Zhang B, Guo Y, Jiao P, Long F:** Effects of dietary lipids and *Clostridium butyricum* on fat deposition and meat quality of broiler chickens. *Poult Sci,* 89 (2): 254-260, 2010. DOI: 10.3382/ps.2009-00234

**26.Mercier Y, Gatellier P, Renerre M:** Lipid and protein oxidation *in vitro*, and antioxidant potential in meat from *Charolais* cows finished on pasture or mixed diet. *Meat Sci,* 66 (2): 467-473, 2004. DOI: 10.1016/S0309-1740(03)00135-9

**27.Brady D, Stoll AD, Starke L, Duncan JR:** Chemical and enzymatic extraction of heavy metal binding polymers from isolated cell walls of *Saccharomyces cerevisiae*. *Biotechnol Bioeng,* 44 (3): 297-302, 1994. DOI: 10.1002/bit.260440307

**28.Brunetti G, Farrag K, Soler-Rovira P, Ferrara M, Nigro F, Senesi N:** The effect of compost and *Bacillus licheniformis* on the phytoextraction of Cr, Cu, Pb and Zn by three brassicaceae species from contaminated soils in the Apulia region, Southern Italy. *Geoderma,* 170, 322-330, 2012. DOI: 10.1016/j.geoderma.2011.11.029

Kafkas Univ Vet Fak Derg
24 (5): 681-689, 2018
DOI: 10.9775/kvfd.2018.19720

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Economic Evaluation in Traditional and Industrial Livestock with Different Levels of Milk Production in Ardebil Province with Emphasis on Risk Criteria

Reza SEYEDSHARIFI [1] Mohammad GHADIMI [1] Nemat HEDAYAT EVRIGH [1]
Jamal SEIFDAVATI [1] Azadeh BOUSTAN [1] Hossein ABDI BENAMAR [1]

[1] Department of Animal Science, University of Mohaghegh Ardabili, Ardabil, IRAN

**Article Code: KVFD-2018-19720 Received: 09.03.2018 Accepted: 10.06.2018 Published Online: 11.06.2018**

**How to Cite This Article**

**Seyedsharifi R, Ghadimi M, Hedayat Evrigh N, Seifdavati J, Boustan A, Abdi Benamar H:** Economic evaluation in traditional and industrial livestock with different levels of milk production in Ardebil province with emphasis on risk criteria. *Kafkas Univ Vet Fak Derg,* 24 (5): 681-689, 2018. DOI: 10.9775/kvfd.2018.19720

## Abstract

The main objective of the livestock industry, as an economic production system, is to increase production efficiency through changes in performance and to increase economic productivity. Therefore, in designing genetic improvement programs for domestic animals, it is necessary to pay attention to recognizing the system of production and the factors affecting its performance and the profitability of systems, that is, revenues and costs. For estimation of market liquidity flow and economic returns, using a bio economic model, data on the revenues and costs was used of traditional and industrial cattle in Ardebil province during the years 2012-2016. The nourishment method based on the type of management was divided into two methods: traditional nourishment (in pasture) and industrial nourishment. The results of this study showed that the highest share of revenue and costs of nourishment units was related to milk sales and nutritional costs in both systems respectively. The investment risk level for industrial systems with different levels of milk production (high production, average production and low production) and the traditional system were estimated to be 0.032, 0.078, 0.030 and 0.013, respectively using standard deviation that these numbers represent the degree of deviation of the real result from the average result with medium returns which shows the high risk of investment in industrial dairy cattle compared to traditional dairy cattle. In both systems, the highest estimated relative significance was related to production traits, followed by survival and growth traits, respectively and the least value was related to reproductive traits.

***Keywords:*** *Bio-economic model, Economic value, Risk, Traditional and industrial system*

# Erdebil Bölgesinde Farklı Ölçeklerde Süt Üretimi Olan Geleneksel ve Endüstriyel Hayvancılık İşletmelirindeki Risk Faktörlerinin Ekonomik Değerlendirmesi

## Öz

Hayvancılık endüstrisinin temel amacı, ekonomik bir üretim sistemi olarak, performanstaki değişimler vasıtasıyla üretim verimliliğini artırmak ve ekonomik verimliliği geliştirmektir. Bu nedenle, evcil hayvanlar için genetik iyileştirme programlarının tasarlanmasında, üretim sisteminin ve performansını etkileyen faktörlerin ve sistemlerin karlılığının, yani gelirlerin ve maliyetlerin tanınmasına dikkat edilmesi gerekmektedir. Piyasa likidite akışı ve ekonomik getirilerin tahmininde, biyoekonomik bir model kullanılarak, 2012-2016 yılları arasında Ardebil ilindeki geleneksel ve endüstriyel sığırların gelir ve maliyet verileri kullanılmıştır. Yönetim tipine göre beslenme metodu iki yönteme ayrıldı: geleneksel besleme (otlakta) ve endüstriyel besleme. Çalışmanın sonuçları, her iki sistemde de en yüksek gelir ve beslenme birim maliyetlerinin sırasıyla süt satışları ve beslenme maliyetleri ile ilişkili olduğunu göstermiştir. Farklı seviyelerde süt üretimi (yüksek üretim, ortalama üretim ve düşük üretim) ve geleneksel sisteme sahip endüstriyel sistemler için yatırım riski seviyesi standart sapma kullanılarak sırasıyla 0.032, 0.078, 0.030 ve 0.013 olarak tahmin edilmiştir. Bu rakamlar, geleneksel süt sığırcılığına kıyasla endüstriyel süt sığırlarında yüksek yatırım riskini gösteren orta getirilerle elde edilen ortalama sonuçlardan gerçek sonuçların sapma derecisini temsil etmektedir. Her iki sistemde de, en yüksek tahmini kısmi önem, üretim özellikleri ile ilişkiliydi, bunu sırasıyla hayatta kalma ve büyüme özellikleri takip ediyordu ve en düşük değer, üreme özellikleriyle ilişkiliydi.

***Anahtar sözcükler:*** *Biyoekonomik model, Ekonomik değer, Risk, Geleneksel ve endüstriyel sistem*

**İletişim (Correspondence)**
+98 914 3540078
reza_seyedsharifi@yahoo.com

# INTRODUCTION

Livestock has a special status as the development axis in the country's development programs. The capacity in this section as one of the important sub-sectors of the agricultural industry in the country requires more planning and more appropriate use of these capacities [1]. Nowadays, breeding science has been considered along with other sciences related to livestock industry as one of the important tools to provide a part of deficiencies and increase the quality of livestock production. The first step in the formulation and implementation of management and breeding programs to improve the performance of the traits and the profitability of breeding each breed is to determine the breeding goals and the relative importance of each trait in profitability that should be commensurate with the conditions of local breeding and also should have the sustainability of production [2,3]. One of the most important goals of dairy cattle is to increase profitability through raising revenues and reducing production costs [4]. The economic value of a trait is defined as the variation in profit for a unit of variation in the average of that trait while the other traits remain within the average range [5]. The economic value is influenced by the price of products and production inputs. So that the improvement level of a trait will affect future prices. Therefore, determination of economic values requires knowledge of the level of genetic enhancement in the future and their effect on prices [6]. Using the bio economic modelsis one of the important tools in calculating the economic value and profits of the production system. The bio economic model has three basic components: the design of the herd structure, the calculation of the profit function details for the defined production systems (inputs and outputs) and mathematical description of the processes existing in each production system. Using such models, costs and revenues are obtained based on the actual phenotype function, which depends not only on the potential genetic function, but also on the availability of food sources and feed intake capacity [7,8]. Any kind of investment in livestock faces uncertainties that make risky the return on investment in the future. Since production of livestock products are always exposed to unpredictable competitive markets for inputs and outputs, the risk of pricing may increase over time [9]. Kulak et al.[10] defined the risk as standard deviation or profit variance which can create great differences in the economic values of traits. But its impact on relative economic values, the amount and direction of genetic variation may be small [10]. Economists have defined the risk of investment as a possible deviation from the average return. They also define the risk of investing in conditions of uncertainty as potential losses. So, they have reported in their studies that investors should measure the risk only on the basis of the probability of losses [11].

The purpose of this study was to investigate the effect of risk on the trend of estimating liquidity flows in traditional and industrial livestock with different levels of milk production in Ardebil province.

# MATERIAL and METHODS

In this research, data were used about revenues and costs of the years 2012-2016 on traditional and industrial dairy cattle of Ardabil province based on market conditions to estimate the liquidity flow of these units. Based on the type of management, the method of nourishment was divided into two methods: traditional and industrial nourishment. The industrial system was classified into three levels of low production (up to 25 kg), average production (up to 30 kg) and full production (35 kg and more) based on different levels of milk production.

### *The Traditional Method of Livestock Nourishment in Rangeland*

In this method, native cattle are nourished and livestock is in grazing land over the year (except winter). And when using rangelands, they are fed with supplementary feed (including concentrates such as barley flour, wheat bran, and forage material such as straw). In the cold seasons, livestock is kept in the village in a closed position. In the closed position, the livestock is fed by a mixture of straw, bran, and concentrate.

### *Industrial Nourishment Method*

In the Holstein cattle industry, cattle feeding are done entirely manually and in a closed position. In feeding these livestock, concentrates, straw, alfalfa and corn silage are used. Calves are taken from milk at three months old and are fed with hand feeds from two months old. In this research, the economic system of the cattle herd (in both production systems) was decomposed into revenue and cost components using the system analysis method, and each of these components was subdivided into other subsections. Then, simulation of a bio economic model was performed using a mathematical model and using MATLAB 8.0 programming language [12]. The revenue component included the sale of milk, the sale of surplus heifers, the sale of calves and eliminated cows and the costs included feeding, marketing, heifers nourishment and other costs (management costs) and fixed costs. Management costs included health, human power and reproductive costs, which were used as input parameters of the model. In this study, the annual profit for each breeder cattle was derived from the difference between revenues and costs and according to the following equation: P=R-C

In this equation, P is the annual profit, R is the annual revenue and C is the annual cost of each breeder cattle. The annual revenue per a cow was calculated according to the following formula:

$$R = R_{milk} + R_{(male\text{-}calves)} + R_{(culled\text{-}cows)} + R_{(culled\ -\ heifers)}$$

In this equation, $R_{milk}$ the revenue from the sale of milk, $R_{calves}$ the revenue from sales of calves, $R_{culled\text{-}cows}$ the revenue from the sale of eliminated cows and $R_{culled\text{-}heifers}$ the revenue from the sale of surplus heifers. Each of the above parameters is expressed as follows [3,4]:

$$NCY = \frac{365}{CI}$$

$$PLTy = \frac{PLT}{365}$$

$$NmcCy = 0.5 \times NCY \times cr \times S24$$

$$R_{male-\ calves} = NmcCy \times Pc$$

$$NfcrCy = 0.5 \times NCY \times cr \times S24 \times SR \times PSR$$

$$NfcCy_{cull} = NfcrCy - \left(\frac{1}{PLTy}\right)$$

$$W_{heifer} = bw + (DG \times wa) + (PDG \times dwm)$$

$$R_{culled-\ heifers} = NfcCy_{cull} \times Wheifer \times P_{lw}$$

$$R_{culled-cows} = \frac{LW}{PLTy} \times p_{lw}$$

$$R_{milk} = (MY \times P_m) + \left(FY - (MY \times 0.035)\right) \times P_f$$

Respectively: NCY the number of calves per year, CI the calving interval (day), PLTy production lifetime (years), PLT production lifetime (day), NmcCy number of calves, cr calving rate (percent), S24 survival rate 24 h after birth (percentage), Pc price of calf (Rials), NfcrCy number of heifers, SR survival rates after ablactate (percentage), $NfcCy_{cull}$ number of surplus fattening heifers (percentage), $W_{heifer}$ heifers weight (kg), bw weight of heifer at birth (kg), DG daily weight gain before ablactate (kg/day), PDG daily weight gain after ablactate (kg/day), wa days of birth to ablactate, dwm days of ablactate up to 18 months, $P_{lW}$ the price per kilogram of live cow (Rials), LW live weight of eliminated cow (kg), MY and FY milk and fat production in a course (kg), $P_m$ price of milk per kilogram with 3.5% fat (Rials), $P_f$ price of fat per kilogram (Rials).

Also, the annual cost per a cow was calculated by the following equation:

$$C = C_{(Feedh\text{-}birth\text{-}w)} + C_{(Feedh\text{-}w\text{-}ma)} + C_{(Feedh\text{-}ma\text{-}afc)} + C_{(Feed\text{-}\ cows)} + C_{(Healthh\text{-}birth\text{-}w)} + C_{(Healthh\text{-}w\text{-}ma)} + C_{(Healthh\text{-}ma\text{-}afc)} + C_{(Health\text{-}cows)} + C_{(Laborh\text{-}birth\text{-}w)} + C_{(Laborh\text{-}w\text{-}ma)} + C_{(Laborh\text{-}ma\text{-}afc)} + C_{(Labor\text{-}cows)} + C_{(Reproduction\text{-}heifers)} + C_{(Reproduction\text{-}cows)} + C_{Fix}$$

The variables used in the above relationships are defined as follows:

$C_{feedh\text{-}birth\text{-}w}$: The cost of feeding the heifers from birth to ablactate, $C_{feedh\text{-}w\text{-}ma}$: the cost of feeding heifers from ablactate to 18 months old, $C_{feedh\text{-}ma\text{-}afc}$: the cost of feeding heifers from 18 months old to the first childbirth, $C_{feed\text{-}cows}$: the cost of feeding the milchcows, $C_{healthh\text{-}birth\text{-}w}$: the health costs of the heifers from birth to ablactate, $C_{healthh\text{-}w\text{-}ma}$: health cost of the heifers from ablactate to 18 months old, $C_{healthh\text{-}ma\text{-}afc}$: health costs of the heifers from 18 months old to the first childbirth, $C_{health\text{-}cow}$: health costs for each cattle, $C_{laborh\text{-}birth\text{-}w}$: the cost of manpower from birth to ablactate, $C_{laborh\text{-}w\text{-}ma}$: the cost of man power from ablactate, $C_{laborh\text{-}ma\text{-}afc}$: man power costs from 18 months old to the first childbirth, $C_{labor\text{-}cows}$: the human cost per each cattle, $C_{Reproduction\text{-}heifers}$: the cost of reproduction of the heifers, $C_{Reproduction\text{-}cows}$: the cost of reproduction of the cattle, $C_{Fix}$: fixed costs.

In this research, for calculating the economic coefficients of traits, the average of the trait was increased by one unit and the difference of profit with the base state was considered as the economic coefficient of the trait, while other traits were within the average of the community. The economic coefficient of each trait was estimated using the following equation:

$$V_i = \frac{P_{\mu_i+\Delta} - P_{\mu_i}}{\Delta}$$

In this equation, $V_i$ the economic coefficient, $P_{(\mu i+\Delta)}$ the average profit of each animal after one increase in the trait $P_{(\mu i)}$, the average profit of each animal before changing the average and Δ the average increase rate of the trait I [12,13].

$$RE = \frac{(EVi \times GSDi)}{\sum_{i=1}^{t}(EVi \times GSDi)} \times 100$$

In which: RE, EV, GSD respectively indicate the relative emphasis, absolute economic coefficient and standard genetic deviation for the $i^{th}$ trait and t is the number of traits in the breeding goals.

### *Risk Measurement*

One of the known methods for measuring risk is variance or standard deviation of expected returns. This statistical method measures the distribution of returns around their expected value. It is believed that the greater the dispersion of expected returns, the greater the uncertainty about the occurrence of these returns in the future.

The risk was calculated as [14]:

$$\sigma = \sqrt{\frac{\sum(r_i - \bar{r})^2}{n}}$$

In this equation, according to financial concepts, σ the deviation of actual returns or risk, $r_i$ real returns, $\bar{r}$ average of returns, and n the number of courses. As the standard deviation is lower than the average, the risk will be less.

## RESULTS

### *Analysis of Costs and Revenues of the Production System*

The results of the revenues, costs, and profitability of this research for production systems by breeding methods for a period of 5 years (from 2012 to 2016) are shown

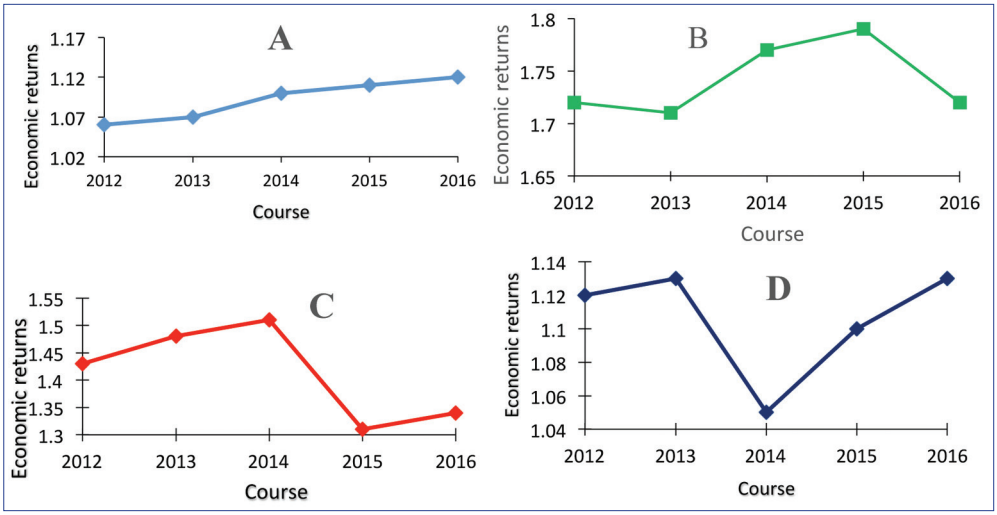



**Fig 1. A:** Economic returns of the traditional system, **B:** Full production in industrial system **C:** Medium production in industrial system, **D:** Low production in industrial system

***Table 1.*** *Revenue, cost and primary profit from the weighted average of traits and system profit change after a unit increase in mean of traits for different levels of milk production in the industrial production system and in traditional breeding system*

| Breeding System | Initial values | MY | FY | SR | PSR | DG | PDG | LW | CI | PLT |
|---|---|---|---|---|---|---|---|---|---|---|
| **Industrial System** | | | | | | | | | | |
| **High production** | | | | | | | | | | |
| Revenue | 145161612.72 | 145167522.72 | 145335612.72 | 145280764.59 | 145277117.08 | 145162550.76 | 145164895.87 | 145180862.72 | 145129732.98 | 145159217.34 |
| Cost | 83255829.06 | 83258409.15 | 83281333.92 | 83314198.98 | 83277691.61 | 83255858.99 | 83255930.27 | 83264856.94 | 83240028.07 | 83252525.31 |
| Profit | 61905783.66 | 61909113.57 | 62054278.80 | 61966565.61 | 61999425.47 | 61906691.77 | 61908965.60 | 61916005.78 | 61889704.91 | 61906692.03 |
| **Medium production** | | | | | | | | | | |
| Revenue | 113481939.39 | 113487767.39 | 113641139.39 | 113591806.70 | 113588443.41 | 113482804.34 | 113484966.71 | 113499689.39 | 113452015.38 | 113479730.66 |
| Cost | 81045595.68 | 81048257.22 | 81074155.13 | 81109653.22 | 81070053.41 | 81045626.51 | 81045699.59 | 81055722.28 | 81028534.83 | 81041813.27 |
| Profit | 32436343.71 | 32439510.17 | 32566984.26 | 32482153.48 | 32518390 | 32437177.83 | 32439267.12 | 32443967.11 | 32423480.55 | 32437917.39 |
| **Low production** | | | | | | | | | | |
| Revenue | 85665904.40 | 85671454.40 | 85823904.40 | 85768653.54 | 85765508.16 | 85666713.30 | 85668735.58 | 85682504.40 | 85637647.21 | 85663838.77 |
| Cost | 77494060.97 | 77496779.78 | 77524768.15 | 77561451.18 | 77520028 | 77494092.57 | 77494167.20 | 77504996.17 | 77476256.65 | 77489963.28 |
| Profit | 8171843.43 | 8174674.62 | 8299136.25 | 8207202.36 | 8245480.16 | 8172620.73 | 8174568.38 | 8177508.23 | 8161390.56 | 8173875.49 |
| **Traditional System** | | | | | | | | | | |
| Revenue | 78577146.79 | 78582690.79 | 78722746.79 | 78668892.46 | 78666083.93 | 78577904.05 | 78579634.92 | 78591946.79 | 78551542.29 | 78575311.53 |
| Cost | 71845765.95 | 71848927.68 | 71876582.46 | 71908079.64 | 71868060.90 | 71845777.42 | 71845799.49 | 71856339.77 | 71829039.10 | 71841984.10 |
| Profit | 6731380.84 | 6733763.11 | 6846164.33 | 6760812.82 | 6798023.03 | 6732126.63 | 6733835.43 | 6735607.02 | 6722503.19 | 6733327.43 |

*MY, milk yield; FY, fat yield; CI, calving interval; DG, preweaning daily gain; PDG, postweaning daily gain; LW, mature live weight; SR, preweaningsurvival rate; PSR, postweaning survival rate (to 18 months); PLT, productive lifetime*

in *Table 1*. Also, the economic returns obtained from this research for the traditional system and the various levels of the industrial system are presented in *Fig. 1*. According to the results: Among the revenue sources in the traditional system the revenue from sales of milk 81.90%, revenue from sale of culled cow 11.30%, revenue from sale of surplus heifers 3.20%, revenue from sale of male calves 3.60% accounted for the most relative share. In the industrial system, the revenue from milk sales with 85.64% in full production, 85.16% in medium production and 84.37% in low production, the revenue from sales of culled cow with 9.42% in full production, 9.44% in medium production and 9.53% in low production, revenue from sales of male calves with 2.11% in full production, 2.66% in medium production and 2.83%in low production and the revenue from sales of surplus heifers with 2.83% in full production, 2.74% in medium production and 3.27% in low production accounted for the most relative share. By increasing the level of milk production, the relative share of milk sales revenue has increased in comparison with other sales of other sources of revenue. Cost sources are divided into two categories: constant and variable costs. In this research, the variable costs were 69475765.96 Rials of the traditional system costs and 78255829.06,

***Table 2.*** *Economic values, economic weights and relative emphasis of traits in industrial production system with different levels of milk production and in traditional breeding system*

| Breeding System | Milk Production | GSD | Economic Value (Rial) | Economic Weight (Rial) | Relative Emphasis (%) |
|---|---|---|---|---|---|
| Industrial system | **High production** | | | | |
| | Milk yield | 561.7 | 3329.91 | 1870410 | 27.12 |
| | Fat yield | 14.9 | 148495.14 | 2212578 | 32.08 |
| | Calving interval | 13.5 | -16528.39 | -223133 | 3.23 |
| | Pre-weaning survival rate | 13.1 | 60781.95 | 796243.6 | 11.54 |
| | Post-weaning survival rate | 16.2 | 96054.4 | 1556081 | 22.56 |
| | Pre-weaning daily gain | 26.15 | 908.12 | 23747.34 | 0.34 |
| | Post-weaning daily gain | 24.95 | 3181.94 | 79389.4 | 1.15 |
| | Mature live weigh | 13.32 | 10222.12 | 136158.6 | 1.97 |
| | Productive lifetime | 0.29 | 908.37 | 263.43 | 0.004 |
| | **Medium production** | | | | |
| | Milk yield | 561.7 | 3166.47 | 1778606.2 | 29.52 |
| | Fat yield | 14.9 | 130640.55 | 1946544.2 | 32.31 |
| | Calving interval | 13.5 | -12863.17 | -173652.79 | 2.88 |
| | Pre-weaning survival rate | 13.1 | 45809.77 | 600107.99 | 9.96 |
| | Post-weaning survival rate | 16.2 | 82046.29 | 1329149.9 | 22.06 |
| | Pre-weaning daily gain | 26.15 | 834.11 | 21811.98 | 0.36 |
| | Post-weaning daily gain | 24.95 | 2923.4 | 72938.83 | 1.21 |
| | Mature live weigh | 13.32 | 7623.4 | 101543.69 | 1.69 |
| | Productive lifetime | 0.29 | 1573.69 | 456.37 | 0.008 |
| | **Low production** | | | | |
| | Milk yield | 561.7 | 2831.20 | 1590285.04 | 29.19 |
| | Fat yield | 14.9 | 127292.82 | 1896663.02 | 34.81 |
| | Calving interval | 13.5 | -10482.61 | -141515.24 | 2.6 |
| | Pre-weaning survival rate | 13.1 | 35358.93 | 463201.98 | 8.5 |
| | Post-weaning survival rate | 16.2 | 73636.74 | 1192915.19 | 21.89 |
| | Pre-weaning daily gain | 26.15 | 777.31 | 20326.66 | 0.37 |
| | Post-weaning daily gain | 24.95 | 2724.95 | 67987.5 | 1.25 |
| | Mature live weigh | 13.32 | 5664.80 | 75455.14 | 1.38 |
| | Productive lifetime | 0.29 | 2032.06 | 589.3 | 0.01 |
| Traditional system | Milk yield | 561.7 | 2382.28 | 1338126.68 | 27.97 |
| | Fat yield | 14.9 | 114783.50 | 1710274.15 | 35.75 |
| | Calving interval | 13.5 | 8877.65- | 119848.28- | 2.5 |
| | Pre-weaning survival rate | 13.1 | 30471.11 | 399171.54 | 8.34 |
| | Post-weaning survival rate | 16.2 | 66642.19 | 1079603.48 | 22.56 |
| | Pre-weaning daily gain | 26.15 | 745.79 | 19502.41 | 0.41 |
| | Post-weaning daily gain | 24.95 | 2454.60 | 61242.27 | 1.28 |
| | Mature live weigh | 13.32 | 4226.18 | 56292.72 | 1.18 |
| | Productive lifetime | 0.29 | 1946.60 | 564.51 | 0.01 |

76045595.68 and 72494060.97 of the industrial system costs with different levels of milk (full production, medium production and low production) respectively and what remains is the share of fixed costs. The reason for the greater share of variable costs in the traditional system is the low share of fixed costs in rural areas.

Among the variable costs, the highest relative contribution in both systems was related to feeding costs (40989063.44 Rialsin the traditional system and 46895611.97, 45885149.42 and 44063297.75 Rials respectively, in three levels of full production, medium production and low production) Production in the industrial system. After feeding, breeding heifers costs (13021199.10 in the traditional system and for the three full, medium and low levels of the industrial system were 12586634.15 and 12854163.32 and 12500080.28 Rials respectively), marketing (15373303.42 in the traditional system and for full production, medium

***Table 3.*** *Estimated risk of the industrial system with different levels of milk production and traditional breeding system*

| Breeding System | Period | $r_i$ | $r_i - \bar{r}$ | $\Sigma(r_i - \bar{r})^2$ | σ |
|---|---|---|---|---|---|
| Industrial system | **High production** | | | | |
| | 2012 | 1.72 | -0.022 | 0.000484 | 0.032 |
| | 2013 | 1.71 | -0.032 | 0.001024 | |
| | 2014 | 1.77 | 0.028 | 0.000784 | |
| | 2015 | 1.79 | 0.048 | 0.002304 | |
| | 2016 | 1.72 | -0.022 | 0.000484 | |
| | **Medium production** | | | | |
| | 2012 | 1.43 | 0.016 | 0.000256 | 0.078 |
| | 2013 | 1.48 | 0.066 | 0.004356 | |
| | 2014 | 1.51 | 0.096 | 0.009216 | |
| | 2015 | 1.31 | -0.104 | 0.010816 | |
| | 2016 | 1.34 | -0.074 | 0.005476 | |
| | **Low production** | | | | |
| | 2012 | 1.12 | 0.014 | 0.000196 | 0.030 |
| | 2013 | 1.13 | 0.024 | 0.000576 | |
| | 2014 | 1.05 | -0.056 | 0.003136 | |
| | 2015 | 1.1 | -0.006 | 0.000036 | |
| | 2016 | 1.13 | 0.024 | 0.000576 | |
| Traditional system | 2012 | 1.06 | -0.032 | 0.001024 | 0.013 |
| | 2013 | 1.07 | -0.022 | 0.000484 | |
| | 2014 | 1.1 | 0.008 | 0.000064 | |
| | 2015 | 1.11 | 0.018 | 0.000324 | |
| | 2016 | 1.12 | 0.028 | 0.000784 | |

production and low production levels in the industrial system were 18713382.94, 17232882.94 and 15850282.94 Rials respectively), and the share of other costs including medical, human power and reproductive costs in the traditional breeding system and industrial breeding system were reported equal to: 92200 Rials (for the traditional system), 60200 Rials (for full production), 73400 Rials (for the medium production) and 80400 Rials (for low production), respectively.

On the one hand, increasing milk production will increase milk sales and, on the other hand, will increase the energy needs for lactation, after that, food costs will increase. However, the outcome of these changes is that revenue will overcome the cost. But when milk production decreases, milk revenue and feed costs for lactation are reduced. In this case, lower revenue will be the dominant cost reduction. Simm [14] reported that in any growing system, more than half of the total cost is due to feeding costs that are consistent with the results of this study. This ratio has been reported 63.3% in Iran by [15]. In the study of milk and meat revenues were 90.3% and 9.7% of total revenues, respectively. Among the costs, the variable costs were 98.8% of the total costs, and feeding costs with 51.3% have the largest share in variable costs. After that, breeding heifers (26.5%), marketing (11.6%) and management costs (including cow health, labor and reproductive costs) accounted for 10.6% of total costs [15].

The ratio of revenue to cost (economic efficiency) [16] in the industrial system, with increasing milk production per a cattle over a period of 5 years (from 2012 to 2016) were estimated 1.72, 1.71, 1.77, 1.79, and 1.72 for full production cows, 1.43, 1.48, 1.51, 1.31, 1.34 for medium production cows and 1.12, 1.13, 1.05, 1.1 and 1.13 for low production cows *(Fig. 1)*. This means that the economic return of each cow (for example, in the medium- production industrial system) has decreased from 43% to 34% during 2012 to 2016, respectively. In other words, the economic return of traditional dairy farms has increased from 6% in 2012 to 12% in 2016.

Zahmatkesh and Amin Afshar [17] reported the total revenue, costs and total profits earned per a cow in a year for Holstein cows in Fars province with an average daily production of 24.5 kg of milk, 44314832 and 35370239 and 8944593 Rials, respectively that ratio of revenue to the cost is 1/25 and the economic efficiency is 25%. Using the numbers presented in the report of this researchers, founded that feeding costs accounted for 77% of the total cost and also sales of milk account for 62% of total revenues. In a

study of [18] the average annual profit per a cow varied from 15206843 to 25921834 Rials and the revenue/cost ratio varied from 1.18 to 1.27% and all these parameters increased with increasing milk production. In general, sales of milk and feed costs accounted for the largest share of revenue and costs, and the share of both of these cases increased with an increase in milk production.

### *The Effect of Traits on the Costs and Revenues of the Production System*

The studied traits in this study can be divided into three groups in terms of the type of their effect on revenue sand costs. This classification is distinguished by the breeding system type and the different levels of milk production in *Table 1*. The first group includes a set of traits that, by increasing their average, increase the revenue and cost of the production system as well as the profits relative to the base state (Initial values). Milk production, milk fat content, the weight gain before and after ablactate, adult live weight, survival rate, pre and post ablactate traits were included in this group. The second group consisted of the longevity of the production which, by increasing its average, the revenue and cost of the production system decreased and the profit of the system increased. The third group included the trait of the interval between two births, which, by increasing its average, revenue and cost of the production system as well as the profit of the system reduced. The effect of increasing one unit in average of traits on revenues, costs and profitability of production systems is shown in *Table 1*. Also, the coefficients and economic weights along with the relative importance of the effective traits on profitability are presented in *Table 2*.

### *Results of Ranking of Traits*

Improvement purposes in breeding cows under studied systems include production traits (milk and fat production), reproductive trait (calf interval), growth traits (the weight gain before and after ablactate, adult body weight) and survival traits (survival rate, before and after ablactate and production's lifetime). According to *Table 2*, in both systems, the highest relative importance of production traits (63.72%) in the traditional system and for full production, medium production and low production levels in the industrial system was 59.2 (full production), 61.83% (medium production) and 64% (low production), after that, the survival traits (30.91% in the traditional system and for full production, medium production and low production in the industrial system respectively were 34.1, 32.03, 30.4), growth traits (2.87% in the traditional method and 3.46%, 3.26% and 3% for full production, medium production, low production respectively in the industrial production system). The lowest amount was related to the reproductive trait (2.5% in the traditional system and for full production levels, medium production and low production of the industrial system 3.24, 2.88 and 2.6%, respectively).

## DISCUSSION

The economic value of milk production was estimated for both systems positively. The positive economic value for milk shows that the genetic improvement of milk production trait has a positive effect on the profitability of the system. According to the results of this study, for each unit increase in milk production in the industrial breeding method, the amount of feed intake during breastfeeding increases due to increased energy needed for livestock breastfeeding. Increasing feeding costs and marketing costs are offset by an increase in milk sales revenue, which produces a positive coefficient for milk. In traditional breeding methods, for each unit increase in the average milk production, the amount of feed intake during lactation is higher than the price of manual feed and the cost of feeding calves increases. But in the industrial process, as milk production increases, calf feeding costs reduced, which is due to the fact that the calf's milk consumption is independently defined again the mother milk production. In general, any factor that reduces the cost of milk production will increase the economic efficiency of milk production. Some researchers have estimated the economic value of this trait as positive and some others have estimated it as negative [18-21].

The economic value of milk fat content was estimated for both production systems positively. Due to the fact that the same rewards are traits to surplus fat from the base level, the major factor that causes the difference in the economic value of this trait in different herds is the nutritional costs associated with the production of fat, which is influenced by the quality and composition of diet. As it is used in herds that use cheaper food, because of the lower costs of fat production, the economic value of this trait is larger. In general, an increase in the average production of fat leads to an increase in the price of milk and its sales revenue. Increasing one unit to the average of this trait leads to an increase in energy requirements and, consequently, an increase in the nutritional cost of lactating cows. Also, the cost of feeding male calves and heifers during infancy is increased due to the use of milk with higher fat percentages. But the total revenue from an increase in a kilogram of average fat production is much higher than the cost of it. Therefore, the economic value of this trait is positive and increases the profits of the production system. Some researchers have reported the economic value of milk fat positive, which is consistent with the results of this study Vatankhah and Faraji [22] have reported economic value of this trait negative.

The economic value of the interval between the two births was negative for both breeding methods. By increasing the average of interval of the two births trait, the annual milk sales revenue and male calves and surplus heifer reduced, consequently, annual revenue declined. Also, the nutritional costs of milk production for lactating cows

declined due to the reduction in milk production, and the nutritional, health and reproductive costs, due to the reduction in the number of calves born per year, resulting in a decrease in total annual costs. It should be noted that by increasing average of this trait, the average annual revenue will be higher than the annual cost. The number of births and milk production per year is inversely proportional to the interval between births. Some researchers also found that the economic value of the interval between the two births was negative, and they explained increasing nurture costs the reason for it. Kahi and Nitter [4] also reported positive the economic value of this trait. Because annual reduced milk production was not considered by their model due to an increase in the interval between two births.

The economic value of daily weight gain pre-weaning was estimated positively. Weight in sales age and weight at birth is a function of daily weight gain pre-weaning and after ablactate. Since sales of male calves in the industrial system are constant at a constant age of alive cow and based on each kilogram, but the sale of surplus heifers is based on the numbers, as a result, for a unit increase in daily gain, the revenue from livestock sales increases and this increase is far greater than the increase in the cost of feeding heifers and male calves. Therefore, the economic value of this trait is positive in the industrial system. It should be noted that, as in a system, the selling price of bull calves and surplus heifers do not depend on the weight of the animal (sales are not based on numbers), the increase in the average of weight gain traits makes a decrease in profits, which is why the economic value of this trait in that system will be negative. Kahi and Nitter [4] have reported a positive economic value of daily weight gain pre-weaning but Sahragard Ahmadi [22] estimated the economic value of weight gain pre-weaning negatively. The economic value of the weight gain trait post-weaning was estimated positively for both systems. With the average increase of this trait, nutritional costs increase in the afterbirth period. However, due to the longer period of post-weaning by increasing the average of this trait, the sales weight will be increased and, as a result, total revenue increases to a greater extent than the total cost. Thus, by increasing one unit in the average of this trait, the annual profit of the production system also increases. Kahi and Nitter [4], Sahragard Ahmadi [22] reported the economic value of weight gain after ablactate positively, which is consistent with the results of this research.

The economic value of live mature cattle was estimated positively for both traditional and industrial nourishment methods. So that the cost of one kilogram to body weight is lower than the revenue earned. Increasing the cost of feeding cows by increasing the average adult body weight is compensated by increasing the revenue from the sale of eliminated cattle, which produces a positive factor for adult body weight. Kahi and Nitter [4] estimated the economic value of this trait positively. The reason for this is that in their research, the revenue from live weight heavier, covered more food costs of the heavier heifers and the maintenance of heavier lactating cows. Some scholars also estimated the economic value of this trait negatively.

Economic value of the survival trait was estimated for both nourishment methods positively. By increasing the survival rate, the number of calves and, consequently, their nourishment costs increases, which is compensated by increasing livestock sales. For this reason, the economic value of the survival rate was estimated pre-weaning and post-weaning positively. The economic value of survival post-weaning was greater than the economic value of survival pre-weaning. Because by increasing the survival rates pre-weaning will increase the feeding costs of calves during infancy. By increasing the survival rate pre-weaning, the number of male calves and surplus heifer for sales increased annually. This leads to an increase in annual revenue. On the other hand, an increase in the average survival rate trait leads to an increase in calves and heifers. Therefore, by increasing the average of this trait, total revenue increases to a greater extent than the total cost, and consequently the annual profit increases .Rogers et al.[23] reported that the use of survival trait in breeding programs due to an increase in the number of adult cattle in the flock, a reduction in the costs associated with buying alternative heifers and increasing the chance of optional removal of livestock in the herd, has led to an increase in the profitability of breeding cattle breeding units [24-27].

The economic value of the production life time was positive for both breeding methods. Due to increasing a unit of production life span, the revenue from the sale of surplus heifers are increased, because of the lower replacement rate. This also reduces the cost of heifer nourishment. Vatankhah and Faraji [21] have estimated economic value the life-time trait positively.

### *Risk of Examined Systems*

In this study, the investment risk rate in traditional and industrial animal farms of Ardabil province was obtained based on standard deviation which it results are presented in *Table 3*, broken down by breeding methods and different levels of milk production. According to the following table, the standard deviation (risk) obtained for industrial production system with different levels of milk production (full production, medium production and low production), and the traditional breeding system of livestock in Ardebil province was estimated 0.032, 0.078, 0.03, 0.013, respectively. The standard deviation indicates the risk or deviation value of the actual result from the average with middle return. This means that although is expected that an average return be obtained, but with regard to the standard deviation, it is possible that the actual return (for example, in the traditional breeding system) be expected 0.013% greater or less than the average return. Obviously,

the greater the scope of the aforesaid change, the more risk the investment will be. According to the results, the average economic return of industrial dairies in Ardebil province is higher than traditional dairy farms and along with this increase in returns; the risk of investment in these units is also higher.

The results showed that the ratio of revenue/cost and economic return in traditional livestock farms is increasing trend and in industrial livestock the trend is almost constant or decreasing. Also, the estimated investment risk in both production systems indicates the high risk of industrial livestock compared to traditional livestock farms. Because inthe investingrisk of traditional farms is only on the livestock. On the other hand, fixed costs in this sector are lower than industrial farms, because native cows need little space, facilities and equipment (due to the greater resistance of these races to environmental conditions). If in industrial farm animals, given that livestock is kept centrally and for a particular purpose, the vulnerability is higher and the incidence of each complication may cause serious damage.

## REFERENCES

**1. Albera A, Carnier P, Groen AF:** Definition of a breeding goal for the Piemontese breed: Economic and biological values and their sensitivity to production circumstances. *Livest Prod Sci*, 89 (1): 66-77, 2004. DOI: 10.1016/j.livprodsci.2003.12.004

**2. Ponzoni RW:** The derivation of economic values combining income and expense indifferent ways: An example with Australian Merino Sheep. *J Anim Breed Genet,* 105 (1-6): 143-153, 1988. DOI: 10.1111/j.1439-0388.1988.tb00284.x

**3. Groen AF:** Cattle breeding goals and production circumstances. PhD Thesis Wageningen Agricultural University, Wageningen, Netherlands, 1989.

**4. Kahi AK, Nitter G:** Developing breeding schemes for pasture based dairy production systems in Kenya I. Derivation of economic values using profit functions. *Livest Prod Sci*, 88 (1-2): 161-177, 2004. DOI: 10.1016/j.livprodsci.2003.10.008

**5. Hazel LN, Lush JL:** The efficiency of three methods selection. *J Hered,* 33 (11): 393-399, 1942. DOI: 10.1093/oxfordjournals.jhered.a105102

**6. Groen AF, Steine T, Colleau JJ, Pederson J, Pribyl J, Reinsch N:** Economic values in dairy cattle breeding with special reference to functional traits. Report of EAAP Working Group. *Livest Prod Sci*, 49 (1): 1-21, 1997. DOI: 10.1016/S0301-6226(97)00041-9

**7. Cartwright TC:** The use of system analysis in animal science whit emphasis on animal breeding. *J Anim Sci*, 49 (3): 817-825, 1979. DOI: 10.2527/jas1979.493817x

**8. Dekkers JCM, Ten Hang JH, Weersink A:** Economic aspects of persistency of lactation in dairy cattle. *Livest Prod Sci*, 53 (3): 237-252, 1998. DOI: 10.1016/S0301-6226(97)00124-3

**9. Hirooka H, Sasaki Y:** Derivation of economic weight for carcass traits in Japanese black cattle from field records. *J Anim Breed Genet*, 115 (1-6): 27-37, 1998. DOI: 10.1111/j.1439-0388.1998.tb00325.x

**10. Kulak K, Wilton J, Fox G, Dekkers J:** Comparisons of genetic value with and without risk for livestock trait improvement. *Livest Prod Sci*, 79 (2-3): 183-191, 2003. DOI: 10.1016/S0301-6226(02)00155-0

**11. Levy H, Sarnat M:** Portfolio and Investment Selection. xvii + 747 pp. Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 1984.

**12. Demuth H, Beale M:** Neural network toolbox user's guide. Matlab User's Guide, Natick, MA. The Math Works Inc. 2001.

**13. Wolfova M, Wolf J, Kvapilik J, Kica J:** Selection for profit in cattle: I. Economic weights for purebred dairy cattle in the Czech Republic. *J Dairy Sci*, 90 (5): 2442-2455, 2007. DOI: 10.3168/jds.2006-614

**14. Simm G:** Genetic improvement of cattle and sheep. Publisher: CABI, 1998. ISBN-13: 978-0851996424

**15. Rafiei F, Mottaghi M, Shadparvar A, Najafi S:** Investigating the effects of production system factors on economic productivity of Holstein herds using a simulation model. *J Agri Sci Iran*, 37 (5): 875-888, 2006.

**16. Krupová Z, Motyčka J, Krupa E, Michaličková M:** Economic importance of milk traits in Czech Holstein cattle under various milk payment systems. *Acta Agric Slov*, Supplement 5, 154-159, 2016.

**17. Zahmatkesh R, Amin Afshar M:** Designing a bioeconomic system for holstein milk cows in fars province. *J Anim Sci Res*, 5 (1): 91-99, 2009.

**18. Vatankhah M, Nafchi MF:** Cost-benefit analysis, economic and biological return in holstein cows in charmahal and bakhtiari province with different levels of milk production. *Anim Prod Res*, 2 (3): 1-9, 2013.

**19. Gibson JP:** Economic weights and index selection of milk production traits when multiple production quotas apply. *Anim Sci*, 49 (2): 171-181, 1989. DOI: 10.1017/S0003356100032293

**20. Athari Mortazavi B, Shadparvar A:** The effect of management, economical and biological factors of different traits in traditional and industrial methods of breeding bovine cows on the economic value. *J Rum Res*, 2 (2): 157-172, 2014.

**21. Vatankhah M, Faraji Nafchi M:** Determination of economic value of important traits in holstein cows in Charmahal and Bakhtiari province with different levels of milk production. *J Anim Sci*, 110 (1): 17-30, 2016.

**22.Sahragard Ahmadi S:** Estimation of the economic coefficients of milk production, fat percentage and Lifetime of mixed and Holstein herds in Lorestan province. *Msc Thesis,* University of Gilan, Iran, 2010.

**23. Rogers PL, Gaskins CT, Johnson KA, MacNeil MD:** Evaluating longevity of composite beef females using survival analysis techniques. *J Anim Sci,* 82 (3): 860-866, 2004. DOI: 10.2527/2004.823860x

**24. Cardoso VL, Pereira Lima ML, Nogueira JR, de Carneiro RLR, Sesana RC, Oliveira EJ, El Faro L:** Economic values for milk production and quality traits in south and southeast regions of Brazil. *R Bras Zootec*, 43 (12): 636-642, 2014. DOI: 10.1590/S1516-35982014001200002

**25. van Arendonk JAM:** Use of profit equations to determine relative economic value of dairy cattle herd life and production from field data. *J Dairy Sci,* 74 (3): 1101-1107, 1991. DOI: 10.3168/jds.S0022-0302(91)78261-1

**26. Vargas B, Groen AF, Herrero M, Van Arendonk JAM:** Economic values for production and functional traits in Holstein cattle of Costa Rica. *Livest Prod Sci*, 75 (2): 101-116, 2002. DOI: 10.1016/S0301-6226(01)00305-0

**27. Seyedsharifi R, Esmailzadeh A, Hedayat Evrigh N, Savarsofla S, Seifdavati J:** Determining the priority selection emphasis on characteristics in terms of optimized and non-optimized conditions of production system in dairy cows. *Kafkas Univ Vet Fak Derg,* 23 (6): 981-988, 2017. DOI: 10.9775/kvfd.2017.18097

Kafkas Univ Vet Fak Derg
24 (5): 691-700, 2018
DOI: 10.9775/kvfd.2018.19734

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Differential Expression of Proteins in Datong Yak and Chaidamu Yellow Cattle *Longissimus lumborum* Muscles and Relation to Meat Water Holding Capacity

Jianlei JIA [1,2] Qinwen ZHANG [2] Qian CHEN [1] Hongjian ZHANG [2] Fangming LIN [2] Jing ZHAO [2]

[1] State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, Xining, Qinghai, 810016, PR CHINA
[2] Agriculture and Animal Husbandry College of Qinghai University, Xining, Qinghai, 810016, PR CHINA

**Article Code: KVFD-2018-19734 Received: 09.03.2018 Accepted: 29.06.2018 Published Online: 29.06.2018**

**How to Cite This Article**

**Jia J, Zhang Q, Chen Q, Zhang H, Lin F, Lear G, Zhao J:** Differential expression of proteins in Datong Yak and Chaidamu Yellow Cattle *Longissimus lumborum* muscles and relation to meat water holding capacity. *Kafkas Univ Vet Fak Derg,* 24 (5): 691-700, 2018. DOI: 10.9775/kvfd.2018.19734

## Abstract

We investigated that proteins differently expressed in Datong Yak and Chaidamu Yellow Cattle *Longissimus longurum* muscles and their relation to tissue water-holding capacity. Samples were classified according to breed and postmortem aging into Yak0h, Cattle0h, Yak24h and Cattle24h groups. Fifty seven differentially expressed proteins were confirmed by MALDI-TOF/TOF-MS. Twenty eight proteins could be identified and were divided into five main categories: structural proteins, metabolic enzymes, stress related proteins, transporter proteins and binding proteins. Myosin light chain (MLC), Heat Shock 27kDa (HSP 27) and Keratin 10 (KRT 10) proteins showed significant differences in expression between yak and cattle meat and may have the potential to be used as biological markers of tissue WHC. Bioinformatics analysis showed differentially these proteins included both metabolic enzymes and structural proteins. The functions of the identified proteins contribute to a more detailed molecular view of the processes behind WHC and are a valuable resource for future investigations.

***Keywords:*** *Bos taurus, LL muscles,* Water-holding capacity, *Meat quality, Proteomics, Bioinformatics*

# Datong Yak ve Chaidamu Sarı Sığırı *Longissimus lumborum* Kasında Proteinlerin Farklı Ekspresyonu ve Etin Su Tutma Kapasitesi İle İlişkisi

## Öz

Bu çalışmada Datong Yak ve Chaidamu Sarı Sığırı *Longissimus lumborum* kasında proteinlerin farklı ekspresyonu ve bu proteinlerin etin su tutma kapasitesi ile ilişkisi araştırılmıştır. Örnekler ırka ve postmortem yaşlanmaya göre sınıflandırıldı (Yak0h, Cattle0h, Yak24h ve Cattle24h). Elli yedi farklı eksprese edilen protein MALDI-TOF/TOF-MS ile onaylandı. Yirmi sekiz protein tanımlanarak yapısal proteinler, metabolik enzimler, stres ilişkili proteinler, taşıyıcı proteinler ve bağlayıcı proteinler olmak üzere beş ana kategoriye ayrıldı. Myosin hafif zincir, Isı şok 27kDa (HSP 27) ve Keratin 10 proteinlerinin yak ve sığır etleri arasında ekspresyon bakımından önemli farklar göstermesi sebebiyle bu proteinlerin doku su tutma kapasitesi için biyolojik marker olarak kullanılabilecekleri kanısına varıldı. Biyoinformatik analizi bu proteinlerin farklı metabolik enzimler ve yapısal proteinleri içerdiğini gösterdi. Tespit edilen proteinlerin fonksiyonları su tutma kapasitesinin arkasında yatan daha derinlemesine moleküler ilişkiye ışık tutmakta ve gelecek araştırmalar için değerli bir kaynak oluşturmaktadır.

***Anahtar sözcükler:*** *Bos taurus, LL kası, Su tutma kapasitesi, Et kalitesi, Proteomiks, Biyoinformatik*

## INTRODUCTION

Yak *(Bos grunniens)* mainly live above an altitude of 3000 meters in the Tibetan plateau. The yak meat has a low fat content, however, the commercial potential of yak meat is limited, in part due to a lack of data regarding meat quality [1]. The physical traits of meat (e.g., tenderness, water holding capacity (WHC) and chromatic features) are widely used for the evaluation of red meat quality during processing and storage [2]. Muscle pH and protein denaturation are used as the main determinants of WHC, informing on meat quality [3]. The rapid degradation of proteins lead to the net charges of the muscle proteins being reduced, which forces more water out of the meat. In addition to protein denaturation, this influences its appearance, palatability and processability [4].

**İletişim (Correspondence)**
+86 1389 7519588 Fax: +86 0971 5318423
zhaojing67926@126.com

The degree of protein denaturation, particularly cytoskeletal proteins in carcass meat, often depends on its WHC and pH [5]. High degrees of myofibrillar and sarcoplasmic protein denaturation could promote myofibril shrinkage and force water out. Some reports show that meat with low WHC and low pH was caused by cellular water loss from the myofibrillar matrix [6,7]. However, others consider that WHC may also increase with ageing due to the breakdown in meat structure and the creation of a "sponge effect", which disrupts the channels through which moisture is lost, physically entrapping free water in meat [8]. Changes in meat during postmortem aging are suggested to be highly coordinated, genetically programmed and an irreversible phenomenon involving a series of physiological, biochemical, and proteomic changes [9]. These varied reports make WHC a promising target for continued studies of meat quality mechanism, though at present little is known about the relationship between WHC and protein changes.

Proteomics has been widely used to predict the meat quality [10]. Numerous two-dimensional electrophoresis (2-DE) gel based studies have already reported the relationships between factors such as tenderness, color, pre-slaughter stress and WHC [11-14]. However, while WHC characteristics play an important role in the dynamic nature of meat postmortem muscle qualities, the biological mechanisms involved in the structural and biochemical changes are not fully understood during the ageing process.

In the present study, we wanted to confirm the relationships between changes in physical meat properties (pH, color, water holding capacity) and related protein changes in the *Longissimus lumborum* (LL) muscle both in Datong Yak and Chaidamu Yellow Cattle as determined by proteomic and bioinformatic methods. Our research objectives were therefore to screen the differential expression of proteins in yak and cattle muscles related to the WHC of meat and to explore the potential molecular mechanisms underlying the proteomic data.

## MATERIAL and METHODS

The study protocol was approved by the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in 2004) and approved by the Institutional Animal Care and Use Committee (State key of laboratory of Plateau Ecology and Agriculture, Qinghai University, China, 2015).

### *Animals and Sample Preparation*

Five Datong yak bulls and five Chaidamu yellow cattle bulls of the same age (4±0.5 years old) were stunned by captive bolt pistol and their blood was drained. Animal slaughtering was approved by the National Administration of Cattle Slaughtering and Quarantine regulations (Qinghai, China). The Datong Yak and Chaidamu Yellow Cattle were randomly choosen from experimental base of Qinghai University (Lvcaoyuan Food Ltd., Datong City, Qinghai Province, China) and the LL muscles were excised from the left half of each carcass at the 11$^{th}$ rib. *M. longissimus thoracis* (100 g) from each carcass was divided into two parts, the first one was analyzed at 0 h after slaughter in 18°C; the second one was aged for 24 h in a refrigerated chamber at 4°C and relative humidity of 98%. Any blood or other contaminants on the LL muscle surface were removed by swabbing with phosphate buffered saline solution, then frozen in liquid nitrogen, and stored at -80°C until the extraction of muscle proteins.

### *Measurement of Meat Qualities*

Intramuscular fat content was analyzed using methods of the Association of Official Analytical Chemists [15]. pH was measured using a portable pH meter (SenvenGo, Mettler-Toledo, Switzerland) at 45 min ($pH_{0h}$) and 24 h ($pH_{24h}$) [16] after slaughter. The electrode was first calibrated at a temperature of 22°C which and used a two-point calibration, and the pH of the calibration buffer used was 7.000 and 4.005 at 25°C.

ACR-400 Minolta colorimeter (Konica Minolta Sensing Americas Inc., Ramsey, NJ, USA) was applied to determine the lightness (L*), redness (a*) and yellowness (b*) of the meat samples at 0 h (exposed to air directly for 30 min at 18°C). For each parameter, values were measured on 5 sites of each sample, respectively [17].

Water holding capacity (WHC) was determined following compression by the filter paper press method [4]. To measure the strength of the meat tissue, Warner-Bratzler (WB) shear force was measured on cooked meat (2.54 cm thick) per muscle according to the protocol of Wheeler [18]. A transversal section of the LL muscle for each animal was cooked to a core temperature of 70°C in a pre-heated water bath, subsequently cooled in running water for 30 min to reach a core temperature below 30°C. The cores (1.27 cm, parallel to longitudinal orientation of fibres) were then taken from each sample and peak force was determined using a V-shaped shear blade with a cross-head speed of 400 mm/min.

### *Extraction of Muscle Proteins*

According to Jia's Method [19], frozen muscle tissue (30 mg) was placed in a mortar and ground in liquid nitrogen into a powder. Then, 0.5 g muscle tissue sample was added to 1 mL lysis buffer. Samples were lysed at room temperature for 30 min followed by homogenization with 22% vibrating amplitude via ultrasonic amplitude in ice water (pulsed ultrasound was programmed to turn on for 10 s and then off for 15 s, for a total of 250 s). Samples were placed at 4°C for 4 h and then centrifuged at 14.000 rpm at 4°C for 30 min. The supernatant was retained and protein solution was exchanged into 50 mmol/L triethy1-

ammonium bicarbonate, pH 9, using a PD10 column according to manufacturer instructions (GE Healthcare). Total protein content was measured using the Bradford method (IMPLEN, NanoPhotometerTM), and the lysates stored at -80°C.

### *Two-dimensional Electrophoresis (2-DE) and Mass Spectrometry Analysis*

For 2-DE, the muscle protein extract samples approximately 200 µg were performed to 17 cm nonlinear immobilized pH gradient (IPG) strips (pH 3-10, Bio-Rad). After hydration of the IPG strips at 50 V for 14 h, isoelectric focusing (IEF) was performed as follows: 1 h at 500 V, 1 h at 1000 V to remove salts, followed by 6 h at 1000-9000 V by a linear voltage for 80 000 Vh, and finally 500 V for the hold period. PROTEAN® IEF Cell PROTEAN i-12 (Bio-Rad) was selected for IEF, and the current limit was adjusted to 50 mA per strip; the run was carried out at 20°C. An 12.5% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used for secondary separation using a Protean II XL system (Bio-Rad). The SDS-PAGE steps were as follows: 50 V for 1 h and 150 V for 9 h. Gels were stained with Coomassie blue G-250 [20].

Five biological repeats were performed for each treatment. Every independent biological replicate 2DE gel image was captured using a GS-900® Calibrated Densitometer (Bio-Rad) at grayscale of 256 units and resolution of 600 dpi. PDQuest 8.0.1 software was used to perform the image filtration, background subtraction, spot detection, spot matching, and quantitative intensity analyses. Matched spots exhibiting a statistically significant ($P<0.05$) 2 fold or more intensity difference were considered as differentially abundant.

Matched spots were manually excised by micro pipette tips from the gels. The samples were analysis by mass-spectrophotometry by APT Co., LTD (Shanghai, China). Tryptic peptide analysis was performed using a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, USA). The mass spectrometry (MS) was performed as follows: enzymolysis, ziptip-desalination, peptide extraction in enzyme solution, MALDI-TOF/TOF, data analysis and protein identification [21]. The data of mass and mass/mass spectra were searched through Mascot (Version 2.1, Matrix Science, Boston,MA, USA) and the corresponding proteins were matched against bovine Information (NCBI) database.

### *Bioinformatics*

The differentially expressed proteins which were identified by PDQuest 8.0.1 software and MS were analyzed by Gene ontology (GO) by blast2GO 2.8 software. KEGG showed a pathway enrichment analysis of the differentially expressed proteins, the filter was e-value < $1e^{-10}$ and match score > 65. The PPi are the proteins, and the edges represent the predicted functional associations using Cyposcape 3.0 software.

### *Statistical Analysis*

Statistical significance was assessed with T-test using PROC TTEST or MIXED model (SAS®, Cary, NC, USA). Experiments adopted completely random design. The differences between means were detected using student's T test at 5% significance ($P<0.05$).

## RESULTS

### *Characteristics of Meat Quality in Datong Yak and Chaidamu Yellow Cattle*

The results for meat quality traits of Datong Yak (hereon referred to as Yak) and Chaidamu Yellow Cattle (hereafter referred to as cattle) LL muscles are displayed in *Table 1*.

There were significant differences in the pH of the intracellular water of yak and cattle ($P<0.05$) at 45 min (pH 7.06 Vs. pH 6.69), but no significant differences ($P>0.05$) at 24 h (pH 5.73 Vs. 5.61). This result could be related to living environment of yak and cattle and those life habits leading to a lower fibre conduit, and less binding water capacity in yak [22]. There were significant differences ($P<0.05$) between 0 h and 24 h (pH 7.06 Vs. pH 5.73 and pH 6.69 Vs. pH 5.61), respectively. Lactic acid would gradually product and accumulate inside the muscles during the storage, which had caused pH to decrease [23].

### *Identification of Differentially Expressed Proteins*

In present study, 61 spots displayed significant differences in the level of protein expression ($P<0.05$) at 0 h and 24 h or between yak and cattle LL muscles *(Fig. 1)*. Among them, 57 proteins were successfully identified by MALDI-TOF/TOF-MS. 4 spots were not identified because of the poor database search scores (score<65), which were lower than the 95% threshold required to yield unambiguous results. Because some spots identified repeat proteins, only 28 different proteins were successfully named *(Table 2)*. Some spots were identified as identical proteins, which indicates that they may represent multiple isoforms, fragments and cross linked proteins in the gel based analysis. The differently expressed proteins at 0 h and 24 h between yak and cattle were grouped into six functional classes

***Table 1.*** *Changes of quality characteristics in yak and cattle* LL muscle

| Meat Traits | Yak | Cattle |
|---|---|---|
| $pH_{0h}$ | 7.06±0.16** | 6.69±0.18 |
| $pH_{24h}$ | 5.73±0.12 | 5.61±0.11 |
| *a** value | 23.8±1.65** | 17.14±0.61 |
| *b** value | 9.03±1.21 | 9.71±1.89 |
| *L** value | 39.26±5.54 | 43.23±3.98** |
| WHC (%) | 56.22±1.48* | 51.87±1.28 |
| WBSF (kg/cm$^2$) | 4.91±0.13** | 4.22±0.14 |
| Intramuscular fat (g/100 g) | 2.05±0.15 | 3.07±0.18** |

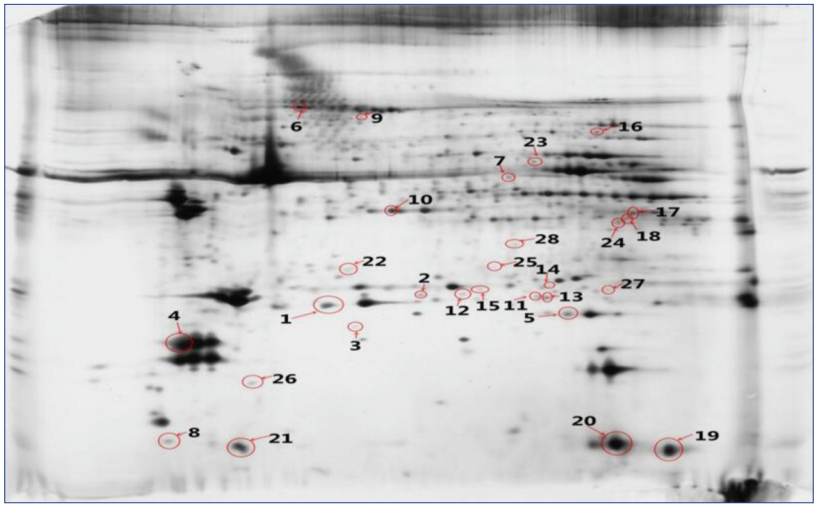



**Fig 1.** 2DE gel images of proteins extracted from yak and Cattle LL muscle at days 0 and 1 during post-mortem aging. The protein loading was 200 µg and the gel was stained with Coomassie blue 600G-250. 28 spots identified were found to be significantly different in abundance and listed in *Table 2*

***Table 2.*** *Identification of 28 differentially expressed proteins by 2DE and MALDI-TOF/TOF analysis in yak and cattle*

| Sample | | Protein Name | Accession No. | Species | MW | PI | Score/ Peptides |
|---|---|---|---|---|---|---|---|
| Structural Proteins | 1 | Myosin, light chain 6B | tr\|Q148H2 | *Bos taurus* | 23502.3 | 5.4 | 44/5 |
| | 2 | Keratin 10 | tr\|A6QNZ7 | *Bos taurus* | 54987.2 | 5.01 | 144/15 |
| | 3 | Actin, alpha | tr\|A4IFM8 | *Bos taurus* | 42338 | 5.23 | 412/7 |
| | 4 | Myosin regulatory light chain 2 | sp\|Q0P571 | *Bos taurus* | 19114.4 | 4.91 | 199/10 |
| | 5 | Alpha-crystallin B chain | sp\|P02510 | *Bos taurus* | 20024.4 | 6.76 | 308/13 |
| | 6 | Myosin-7 | sp\|Q9BE39 | *Bos taurus* | 223889.2 | 5.58 | 414/51 |
| | 7 | MYH1 protein | tr\|Q05B72 | *Bos taurus* | 34018 | 5.76 | 526/24 |
| | 8 | Keratin 4 | tr\|A4IFP2 | *Bos taurus* | 58466.6 | 7.46 | 119/16 |
| | 9 | Myosin-2 | tr\|F1MRC2 | *Bos taurus* | 224106.4 | 5.63 | 707/44 |
| | 10 | Troponin T | sp\|Q8MKH6 | *Bos taurus* | 31265.1 | 5.71 | 456/11 |
| Glycolytic Enzyme | 11 | Triosephosphate isomerase | sp\|Q5E956 | *Bos taurus* | 26900.9 | 6.45 | 634/14 |
| | 12 | Thioredoxin-dependent peroxide reductase | sp\|P35705 | *Bos taurus* | 28405.5 | 7.15 | 211/9 |
| | 13 | Adenylate kinase isoenzyme | sp\|P00570 | *Bos taurus* | 21764.3 | 8.4 | 467/100 |
| | 14 | Flavin reductase (NADPH) | sp\|P52556 | *Bos taurus* | 22232.4 | 6.58 | 159/4 |
| | 15 | Triosephosphate isomerase | sp\|Q5E956 | *Bos taurus* | 26900.9 | 6.45 | 695/17 |
| | 16 | ATP synthase subunit alpha | tr\|F1MLB8 | *Bos taurus* | 59766.7 | 9.21 | 358/21 |
| | 17 | Glyceraldehyde-3-phosphate dehydrogenase | tr\|Q712W6 | *Bos taurus* | 28908 | 9.21 | 147/7 |
| Stress Related Proteins | 18 | Heat shock 27kDa protein | sp\|Q5E956 | *Bos taurus* | 22436.3 | 5.98 | 720/14 |
| Transporter Proteins | 19 | Hemoglobin subunit alpha | sp\|P01966 | *Bos taurus* | 15174.9 | 8.07 | 571/12 |
| | 20 | Hemoglobin beta | tr\|D4QBB4 | *Bos taurus* | 16001.3 | 7.01 | 741/12 |
| Binding Proteins | 21 | Galectin-1 | sp\|P11116 | *Bos taurus* | 15076.4 | 5.37 | 289/8 |
| | 22 | Prohibitin | sp\|Q3T165 | *Bos taurus* | 29842.9 | 5.57 | 594/9 |
| | 23 | Elongation factor Tu | sp\|P49410 | *Bos taurus* | 49709.1 | 6.72 | 297/16 |
| | 24 | Myozenin-1 | sp\|Q8SQ24 | *Bos taurus* | 31653.8 | 9.17 | 256/10 |
| | 25 | Beta-defensin | tr\|G8CY11 | *Bos taurus* | 15026.3 | 8.17 | 42/4 |
| Other Proteins | 26 | Eukaryotic translation initiation factor | sp\|Q6EWQ7 | *Bos taurus* | 17049.5 | 5.08 | 269/5 |
| | 27 | Es1 protein | r\|Q3T0U3 | *Bos taurus* | 29023.1 | 8.76 | 58/8 |
| | 28 | Serine/arginine-rich-splicing factor 6 | tr\|F1MXY9 | *Bos taurus* | 39809.5 | 11.47 | 42/10 |

that included structural proteins, metabolic enzymes, stress related proteins, transporter proteins, binding proteins and other proteins.

There were 11 proteins for which expression was down-regulated at 24 h compared to 0 h in cattle ($P<0.05$, *Table 3*). Beta-defensin, Flavin reductase (NADPH), Hemoglobin

**Table 3.** *Fold change of cattle 0 h Vs 24 h in differentially expressed proteins*

| Spot No. | 0 h | 24 h | Protein Name |
|---|---|---|---|
| 1722 | -3.53173 | 3.53173 | Beta-defensin |
| 1853 | -1.89029 | 1.89029 | Flavin reductase (NADPH) |
| 2083 | 1.77176 | -1.77176 | Hemoglobin subunit alpha |
| 1369 | -1.67566 | 1.67566 | MYH1 protein |
| 1879 | -1.61470 | 1.61470 | Keratin 10 |
| 1601 | -1.59417 | 1.59417 | Heat shock 27kDa protein |
| 1718 | -1.59081 | 1.59081 | Serine/arginine-rich-splicing factor 6 |
| 1974 | 1.58358 | -1.58358 | Alpha-crystallin B chain |
| 1878 | -1.54175 | 1.54175 | Es1 protein |
| 1891 | -1.25692 | 1.25692 | Myosin, light chain 6B |
| 1883 | -1.23285 | 1.23285 | Thioredoxin-dependent peroxide reductase |

**Table 4.** *Fold change of yak 0 h Vs 24 h in differentially expressed proteins*

| Spot No. | 0 h | 24 h | Protein Name |
|---|---|---|---|
| 1588 | 3.78994 | -3.78994 | Triosephosphate isomerase |
| 1667 | 2.90849 | -2.90849 | Myosin, light chain 6B |
| 1600 | -2.77437 | 2.77437 | Heat shock 27kDa protein |
| 1735 | 2.56359 | -2.56359 | Keratin 10 |
| 1661 | -2.35641 | 2.35641 | Thioredoxin-dependent peroxide reductase |
| 1444 | 2.14926 | -2.14926 | Prohibitin |
| 1684 | 2.12196 | -2.12196 | Actin, alpha 1 |
| 1736 | -1.85353 | 1.85353 | Myosin regulatory light chain 2 |
| 1394 | 1.84813 | -1.84813 | MYH1 protein |
| 1815 | 1.82861 | -1.82861 | Eukaryotic translation initiation factor |
| 1905 | 1.69458 | -1.69458 | Galectin-1 |
| 1689 | 1.65970 | -1.65970 | Alpha-crystallin B chain |
| 1650 | 1.57537 | -1.57537 | Adenylate kinase isoenzyme |
| 1278 | -1.55399 | 1.55399 | Glyceraldehyde-3-phosphate dehydrogenase |

subunit alpha, MYH1 protein, Keratin 10, Heat shock 27 kDa protein, Serine/arginine-rich-splicing factor 6, Alpha-crystallin B chain, Es1 protein, Myosin light chain 6B, Thioredoxin-dependent peroxide reductase were upregulated at 24 h and the proteins Hemoglobin subunit alpha and Alpha-crystallin B chain were down-regulated.

There were 14 differently expressed proteins between 0 h and 24 h in yak were down- regulated at 24 h ($P<0.05$, *Table 4)*, including triosephosphate isomerase, myosin light chain 6B, heat shock 27 kDa protein, keratin 10, thioredoxin-dependent peroxide reductase, prohibitin, actin alpha, myosin regulatory light chain 2, MYH1 protein, eukaryotic translation initiation factor, myosin-2, galectin-1, alpha-crystallin B chain, adenylate kinase isoenzyme, glyceraldehyde-3-phosphate dehydrogenase. Whereas heat shock 27 kDa protein, thioredoxin-dependent peroxide reductase, myosin regulatory light chain 2 and glycer-aldehyde-3-phosphate dehydrogenase were up-regulated in expression at 24 h.

**Table 5.** *Fold change of yak Vs cattle at 0 h in differentially expressed proteins*

| Spot No. | Yak | Cattle | Protein Name |
|---|---|---|---|
| 1749 | 2.57159 | -2.57159 | Triosephosphate isomerase |
| 1851 | 2.18073 | -2.18073 | Alpha-crystallin B chain |
| 710 | -2.03445 | 2.03445 | Myosin-7 |
| 1970 | -1.83371 | 1.83371 | Hemoglobin beta |
| 1270 | 1.78043 | -1.78043 | MYH1 protein |
| 1812 | 1.76758 | -1.76758 | Adenylate kinase isoenzyme |
| 1950 | 1.70511 | -1.70511 | Keratin 10 |
| 1636 | 1.54484 | -1.54484 | Actin, alpha |
| 1450 | -1.51909 | 1.51909 | Troponin T |
| 1973 | 1.50355 | -1.50355 | KRT4 protein |

There were ten proteins for which expression were up-regulated in between yak compared to cattle at 0 h ($P<0.05$, *Table 5)*. Triosephosphate isomerase, Alpha-crystallin B chain, Myosin-7, Hemoglobin beta, MYH1 protein, Adenylate kinase isoenzyme, Keratin 10, Actin alpha, Troponin T, KRT4 protein). And Myosin-7, Hemoglobin beta and Troponin T were down-regulated expression in yak compared to cattle.

There were 13 proteins which were expressed more in cattle LL muscle than in yak at 24 h ($P<0.05$, *Table 6)*, namely keratin 10, myosin-2, troponin T, heat shock 27kDa protein, thioredoxin-dependent peroxide reductase, ATP synthase subunit alpha, KRT4 protein, myosin-7, myosin light chain 6B, elongation factor Tu, MYH1 protein, actin alpha and myozenin-1. Myosin-2, heat shock 27kDa protein, myosin-7 and MYH1 protein were up-regulated expression in yak LL muscle compared to cattle.

### *The Correlation of Differentially Expressed Proteins and Meat Quality*

The 7 target proteins (MLC, HSP 27, TIM, KRT 10, LGALS1, GAPDH and HBA) were screened in our study, they could play an important role in WHC of Yak and Cattle meat *(Table 7)*. The results showed that TIM had significant negative correlation with WBSF, HSP 27 had significant positive correlation with WBSF, and GAPDH had significant positive correlation with pH0h ($P<0.05$), those three proteins could be used the marker as tenderness. HBA was down-regulated during postmortem aging, and it had significant negative correlation with a* and L* ($P<0.05$). LGALS1 and KRT 10 were often considered as hypoxia-inducible factor. Baes on our analysis, we could presumptively use HBA, LGALS1 and KRT 10 as a meat color marker. MLC and HSP 27 had significant

**Table 6.** *Fold change of yak Vs cattle at 24 h in differentially expressed proteins*

| Spot No. | Yak | Cattle | Protein Name |
|---|---|---|---|
| 1958 | -3.11075 | 3.11075 | Keratin 10 |
| 899 | 2.92031 | -2.92031 | Myosin-2 |
| 1597 | -2.27384 | 2.27384 | Troponin T |
| 1975 | 2.21049 | -2.21049 | Heat shock 27kDa protein |
| 1883 | -1.96894 | 1.96894 | Thioredoxin-dependent peroxide reductase |
| 1015 | -1.88184 | 1.88184 | ATP synthase subunit alpha |
| 2028 | -1.85099 | 1.85099 | KRT4 protein |
| 1486 | 1.83274 | -1.83274 | Myosin-7 |
| 1891 | -1.67549 | 1.67549 | Myosin, light chain 6B |
| 1242 | -1.60325 | 1.60325 | Elongation factor Tu |
| 1432 | 1.56064 | -1.56064 | MYH1 protein |
| 1369 | -1.53975 | 1.53975 | Actin, alpha |
| 1601 | -1.38845 | 1.38845 | Myozenin-1 |

**Table 7.** *the correlation of differentially expressed proteins and meat quality*

| Protein Name | $pH_{0h}$ | $pH_{24h}$ | a* | b* | L* | WBSF | WHC |
|---|---|---|---|---|---|---|---|
| TIM | 0.59 | 0.56 | 0.22 | 0.21 | -0.23 | -0.68* | 0.13 |
| HSP 27 | 0.41 | 0.33 | 0.03 | 0.15 | -0.04 | 0.84* | 0.80* |
| KRT 10 | 0.04 | 0.39 | 0.85* | 0.11 | 0.08 | 0.37 | -0.76* |
| MLC | 0.34 | 0.32 | 0.41 | 0.18 | 0.29 | 0.36 | 0.91* |
| LGALS1 | 0.06 | 0.36 | 0.82* | 0.12 | 0.11 | 0.29 | 0.27 |
| GAPDH | -0.79* | 0.29 | 0.35 | 0.19 | 0.26 | 0.42 | 0.52 |
| HBA | 0.04 | 0.39 | 0.87* | 0.07 | 0.06 | 0.49 | 0.36 |

### *Bioinformatics Analysis*

Three structured terms (cellular component, molecular function and biological process) are widely used in proteome, metabolome, transcriptome and genome research and according to similarity properties, we grouped our proteins under these categories *(Fig. 2)*.

Functional enrichment analysis was conducted on all differentially expressed proteins by blast2GO 2.8 software [24]. As for GO term enrichment of cellular components, proteins related to Cell, Organelle, Extracellular region, Macromolecular complex and Membrane were the top 5 enriched. Similarly, as for GO term enrichment of molecular function, proteins related to Binding, Catalytic, Structural molecule, Transporter and Electron carrier were the top five enriched. Proteins involved in biological processes, such as Single-organism process, Cellular process, Metabolic process, Multicellular organismal process and Biological regulation, were found to be the top 5 enriched.

We used the KEGG database to understand the key role of each protein identified in our database [25] *(Fig. 3)*. It showed that proteins differentially expressed were significantly enriched in multiple biological processes, including carbon metabolism, gluconeogenesis, biosynthesis of aminoacids, pyruvate metabolism, TCA cycle, oxidative phosphorylation and MAPK signaling pathways *(Fig. 4)*.

## DISCUSSION

### *Characteristics of Meat Quality*

Myoglobin is the principal pigment responsible for the redcolor of meat, and may increase reflectance providing a

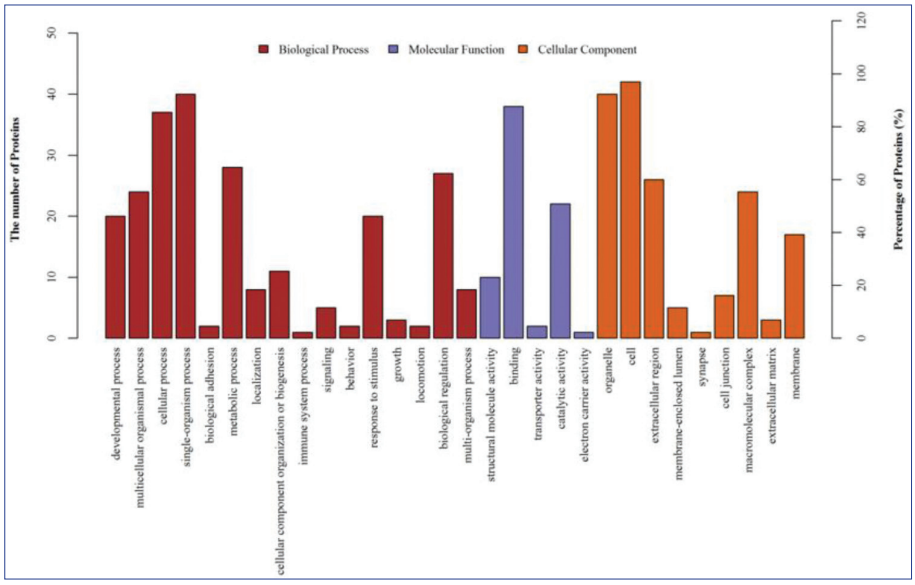



**Fig 2.** Classification of differential expression proteins identified by GO functional classification

negative correlation with WHC, and KRT 10 had significant positive correlation with WHC (P<0.05), all these findings highlight the importance of MLC, HSP 27 and KRT 10 in WHC.

lighter appearance and increased yellow coloration related to the increased free water content of tissue surface [26]. Yak was significantly higher than cattle in meat redness (a*)

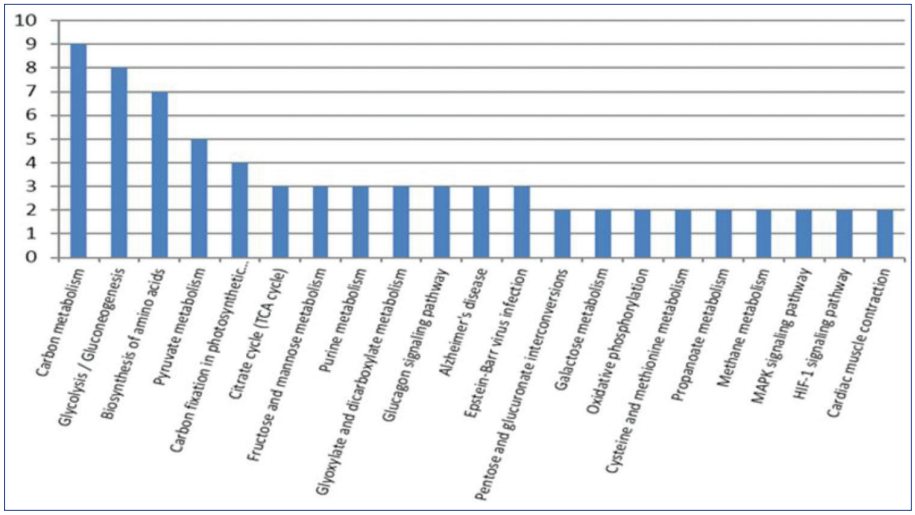
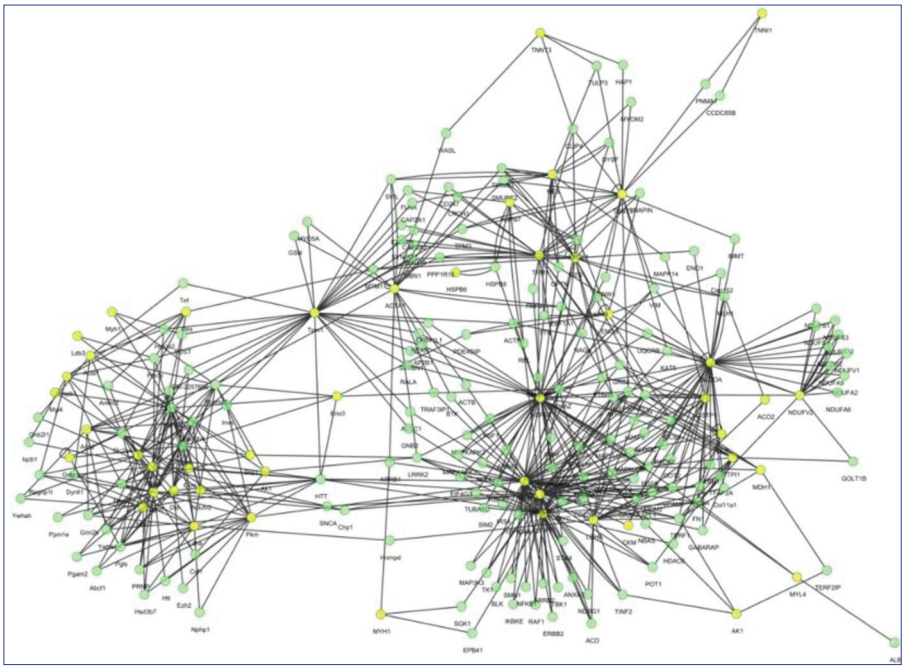



**Fig 3.** Classification of differential expression proteins identified by KEGG pathway enrichment analysis

**Fig 4.** Protein-protein interaction networks of the differential abundance proteins of Yak and Cattle groups in LL muscle based on Cytoscape 3.0 software. The nodes were proteins from Bos Taurus database and the lines were the predicted functional annotations (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)



value, and was significantly lower than cattle in lightness (L; $P<0.05$). There were no significant difference in yellowness (b*) between yak and cattle ($P>0.05$). The result showed that the $pH_{0h}$ was also strongly consistent in yak meat with surface color at 0 h after slaughter, which confirms that early postmortem pH might represent a significant quality predictor for meat color in both groups, as previously found [27].

Yak WBSF values and WHC value was significantly higher than cattle ($P<0.05$). Meanwhile, yak intramuscular fat content was significantly lower than cattle ($P<0.05$). Yaks have numerous physiological traits that equip them for life at high altitudes, including more myofibrillar and cytoskeletal proteins. It is well known that after examining some myofibrillar proteins degraded and declined in intensity, high-altitude hypoxia results in less tender in yak meat than cattle [28].

### *Categories of Proteins Identified in Yak and Cattle Tissue*

It was well known that the proteolytic degradation of structural proteins play a major role in increasing muscle moisture [29]. Structural proteins mainly included collagen, elastic protein and proteoglycan, which allow the binding of muscle tissue to other cellular material [30]. Once myofibrillar and cytoskeletal proteins are degraded during postmortem aging, muscle structures will become looser and muscle moisture content will descend [31]. Some myofibrillar and cytoskeletal proteins varied significantly between yak and cattle, namely Myosin and Keratin family proteins, Alpha-crystallin B chain, Troponin T and Actin [18].

Structural proteins are an important material component for cells and organisms. The myosin family of proteins are major structural protein associatedwith tubulin (such as actin); their soluble monomer can be made of long, rigid

fiber, and form the cytoskeleton by polymerization [32]. The solubility of some myosin proteins decreased and some myofibrillar proteins were fragmented and released during muscle aging [33]. Many scholars [34] showed that denaturation of the myosin could contribute to myofibrillar lateral shrinkage and reduced muscle WHC. Myosin light chain (MLC) proteins play an important role in forming muscle protease catalysis supramolecular complexes, and MLC could catalyze phosphorylation by MLCK which is a type of calmodulin dependent enzyme. Actomyosin could activate myosin ATPase to cause smooth muscle contraction activity [35]. MLC provided an important contribution to the structure and tensile strength of meat, and expression was up-regulated after 24 h of aging in yak and cattle. The result was that muscles need to improve the anaerobic energy metabolism for the supply of ATP during postmortem aging. This conclusion was similar to Jia [36] who found myosin family proteins could accelerate the systolic shrink of muscle fibers in overgrowth phenomenon of cattle. Therefore, MLC may act as a potential biomarker of WHC related to meat aging.

Keratin family, Alpha-crystallin B chain and Troponin T proteins could inhibit the actomyosin ATPase activity, and protect the overall integrity of muscle cells, and once those proteins are degraded, the integrity of meat myofibril could be disrupted [28]. In recent years, more and more study showed keratin 4 has more complex functions in cell growth, translation process, cell proliferation, organelle transport and stress response and protection. This study results showed that KRT4 has a significant positive correlation with a* value, we speculated that it played an important role in protecting cells against external environmental stress and injury.

When the animals were killed, it would stop their circulation of blood and cut off the oxygen supply [33]. The muscles can no longer remove metabolic products, leading to lactic acid accumulation by glycolysis of muscle glycogen. Muscle pH then lowers compared to within the than living body, meanwhile, myosin and actin were linked, the WHC of muscle was reduced and calcium was released [37]. In our study, eight kinds of proteins in metabolic enzymes *(Table 2)* were found to be present in different abundance comparing yak and cattle tissue. Thioredoxin-dependent peroxide reductase, Triosephosphate isomerase, ATP synthase subunit alpha, Glyceraldehyde-3-phosphate dehydrogenase, Flavin reductase (NADPH) were the key proteins for glycolysis and tricarboxylic acid cycle, in which it catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate, and it is essential for efficient energy production.

Through the research in recent years, it was shown that differences of protein expression in tender meat mainly included structural proteins, metabolic enzymes and stress related proteins [24,38]. The mechanism was that high glycolysis rate contributes to lactate accumulation, and the decrease of pH reduced the ions repulsion between the myofibrillar proteins and it could decrease the repulsion between filaments of myofibrillar, which contributed to lateral shrinkage of the muscle fibres, then sarcomere extension, and lead to higher WHC [39].

The study found that high degree of tender meat had a relatively high of Thioredoxin-dependent peroxide reductase, it could be used as a prediction of protein hydrolysis and tender meat of marker protein in beef. At the same time, the expression quantity of Triosephosphate isomerase and ATP synthase subunit alpha decreased during refrigeration. The active site of TIM was glutamate residues, when it bind to substrate, the catalytic reaction is quickly activated, and this reaction could accelerate meat tenderness.

Previous research found that the expression of stress related proteins could increase in hyperthermia, hypoxia, nutritional deficiency, oxidative stress, ultraviolet radiation and reperfusion following ischemic injury [40]. The expression and phosphorylation of HSP 27 could be up-regulation and prevent structural damage and degradation of proteins under the pressure. HSP 27 and Daxx could inhibit the apoptosis which was mediated by Fas. Apoptosis was closely related to shrinkage and water loss of cells. HSP 27 would inhibit the release of C cytochrome to prevent the expression of pro-apoptotic protein, C cytochrome regulates TNF-α and Fas ligand to induce apoptosis, so HSP27 would have effect on WHC by regulating cell apoptosis and have potential as biomarkers for moderate to good WHC [41].

Early thermo resistance was mainly influencing cytoskeleton, it could break actin filaments and resolve microtubules [26]. HSP 27 participated in thermo resistance process by regulating the polymerization of actin, and it was necessary to structural stability of actin microtubules in HSP 27 phosphorylation. The abundance of both the stress-induced thioredoxin-dependent peroxide reductase and glyceraldehyde-3-phosphate dehydrogenase was higher in 24 h than 0 h of yak and cattle meat. Thioredoxin-dependent peroxide reductase and Glyceraldehyde-3-phosphate dehydrogenase played a role in mechanisms of cellular detoxification and cellular resistance to oxidation, and under the condition of oxidation, the expression quantity of HSP 27 phosphorylation was up-regulation, and the affinity of actin poly was weaken, then HSP 27 was dissociation from actin poly, and the actin monomers enhanced the binding force with microtubules, and improved the resilience on oxidation of actin microtubules. All these findings highlight the importance of stress and related proteins in the proteomic response associated with WHC [42].

Our study, compared to cattle at 0 h, hemoglobin alpha was identified showing a lower abundance in 24 h compared to 0 h of both cattle and yak. Studies had shown that

Hemoglobin alpha had strong ability of the combination to nucleotide, so we think that Hemoglobin alpha could play a role in nucleotide carrier for muscles, meanwhile, the expression quantity of Hemoglobin alpha would be greatly up-regulated under oxidation, and it also could play a role in cell growth and development by the pathway of cell mitochondrial synthesis and transport [43]. Results of the aforementioned studies indicated the need for further research on the role of this protein in yak and cattle meat.

The meat color was determined by concentration and chemical state of Hemoglobin alpha [44]. Yak increase the Hemoglobin alpha content of meat to meet the demand for oxygen, so the yak meat had a darker red color. In our study, we showed that the content of Hemoglobin alpha had positive correlation with a*.

Binding proteins are a type of glycoprotein macromolecule, commonly found in Animal tissues, which play an important role in many physiological and pathological process, such as cell adhesion, apoptosis, inflammatory response and neoplasm metastasis [45].

In Doutaud's study, the expression of Galectin-1, which is a type of hypoxia regulatory proteins, was up-regulated in a hypoxic environment, possibly to promote the growth of vascular tissue. On the contrary, the expression of Galectin-1 was invariant in normal tissue. We could use Galectin-1 as the marker of hypoxia. In our study, there were Galectin-1 in yak and cattle meat and we speculate that the phenomenon could be associate with the low oxygen environment in which both animals live.

Proteins serve as fundamental parts of protein complexes in living cells, and adjust and mediate by other proteins to carry out shared functions. It was important for revealing the function of the protein to explore the protein-protein interaction networks [36]. Some proteins acted as core proteins as shown by gene name in biological interaction networks (BIN), such as MLC, HSP 27, TIM, KRT 10, LGALS1, GAPDH and HBA *(Fig. 4)*. In BIN, gene names of differentially expressed proteins could be summarized into two major categories. One showed the enzymes of glycolytic and energy metabolism, and the other was cell structure.

This study provides a better understanding of proteome changes in yak and cattle muscle.

HSP 27, MLC and KRT 10 indicate differential expression patterns between yak and cattle groups after 0 h and 24 h of postmortem aging. The bioinformatics results showed that differential expressed proteins involved in glycolytic and energy metabolism enzymes and structural proteins. Further studies about post-translational modifications and the changes in metaboliteof the related proteins remain to be explored. The functions of the identified proteins contribute to a more detailed molecular view of the processes behind WHC and are a valuable resource for future investigations.

## Conflicts of Interest

The authors declare no conflict of interest.

## Acknowledgments

This work was supported by National Natural Science Foundation of China (No. 31660662; No. 31560701), the program for International Cooperation and Exchange of the Ministry of Education (No. 2015-12), Natural Science Foundation of Qinghai (2016-ZJ-935Q) and Special Science and Technology Cooperation Foundation of Qinghai (No.2016-HZ-823).

## REFERENCE

**1. Zhang L, Sun B, Xie P, Li H, Su H, Sha K, Huang C, Lei Y, Liu X, Wang H:** Using near infrared spectroscopy to predict the physical traits of *Bos grunniens* meat. *LWT Food Sci Technol,* 64, 602-608, 2015. DOI: 10.1016/j.lwt.2015.06.022

**2. Kuchtík J, Zapletal D, Šustovác K:** Chemical and physical characteristics of lamb meat related to crossbreeding of Romanov ewes with Suffolk and Charollais sires. *Meat Sci,* 90 (2): 426-430, 2012. DOI: 10.1016/j.meatsci.2011.08.012

**3. Bowker B, Zhuang H:** Relationship between water-holding capacity and protein denaturation in broiler breast meat. *Poult Sci,* 94 (7): 1657-1664, 2015. DOI: 10.3382/ps/pev120

**4. D'Alessandro A, Marrocco C, Zolla V, D'Andrea M, Zolla L:** Meat quality of the *longissimus lumborum* muscle of Casertana and Large White pigs: Metabolomics and proteomics intertwined. *J Proteomics,* 75 (2): 610-627, 2011. DOI: 10.1016/j.jprot.2011.08.024

**5. Di Luca A, Mullen AM, Elia G, Davey G, Hamill RM:** Centrifugal drip is an accessible source for protein indicators of pork ageing and water-holding capacity. *Meat Sci,* 88, 261-270, 2011. DOI: 10.1016/j.meatsci.2010.12.033

**6. Kristensen L, Purslow PP:** The effect of ageing on the water-holding capacity of pork: Role of cytoskeletal proteins. *Meat Sci,* 58, 17-23, 2001. DOI: 10.1016/S0309-1740(00)00125-X

**7. Pearce KL, Rosenvold K, Andersen HJ, Hopkins DL:** Water distribution and mobility in meat during the conversion of muscle to meat and ageing and the impacts on fresh meat quality attributes. *Meat Sci,* 89, 111-124, 2011. DOI: 10.1016/j.meatsci.2011.04.007

**8. Farouk MM, Mustafa NM, Wu G, Krsinic G:** The "sponge effect" hypothesis: An alternative explanation of the improvement in the waterholding capacity of meat with ageing. *Meat Sci,* 90, 670-677, 2012. DOI: 10.1016/j.meatsci.2011.10.012

**9. Gui LS, Wang ZY, Jia JL, Zhang CT, Chen YZ, Hou SZ:** IGF-1 gene polymorphisms influence bovine growth traits in Chinese Qinchuan Cattle. *Kafkas Univ Vet Fak Derg,* 24 (3): 387-392, 2018. DOI: 10.9775/kvfd.2017.19036

**10. Zheng AJ, Liu GH, Zhang YS, Hou S, Chang W, Zhang S, Cai H, Chen G:** Proteomic analysis of liver development of lean Pekin duck *(Anas platyrhynchos domestica). J Proteomics,* 75, 5396-5413, 2012. DOI: 10.1016/j.jprot.2012.06.019

**11. Laville E, Sayd T, Morzel M, Blinet S, Chambon C, Lepetit J, Renand G, Hocquette JF:** Proteome changes during meat aging in tough and tender beef suggest the importance of apoptosis and protein solubility for beef aging and tenderization. *J Agric Food Chem,* 57, 10755-10764, 2009. DOI: 10.1021/jf901949r

**12. Suman SP, Rentfrow G, Nair MN, Joseph P:** Proteomics of muscle- and species-specificity in meat color stability. *J Anim Sci,* 92, 875-882,

2014. DOI: 10.2527/jas.2013-7277

**13. Franco D, Mato A, Salgado FJ, López-Pedrouso M, Carrera M, Bravo S, Parrado M, Gallardo JM, Zapata C:** Tackling proteome changes in the *longissimus thoracis* bovine muscle in response to pre-slaughter stress. *J Proteomics,* 122, 73-85, 2015. DOI: 10.1016/j.jprot.2015.03.029

**14. Zuo H, Han L, Yu Q, Niu K, Zhao S, Shi H:** Proteome changes on water-holding capacity of yak *longissimus lumborum* during postmortem aging. *Meat Sci*, 121, 409-419, 2016. DOI: 10.1016/j.meatsci.2016.07.010

**15. Honikel KO:** Reference methods for the assessment of physical characteristics of meat. *Meat Sci,* 49, 447-457, 1998. DOI: 10.1016/S0309-1740(98)00034-5

**16. Laghi L, Venturi L, Dellarosa N, Petracci M:** Water diffusion to assess meat microstructure. *Food Chem,* 236 (1): 15-20, 2017. DOI: 10.1016/j.foodchem.2016.12.043

**17. Dai Y, Lu Y, Wu W, Lu XM, Han ZP, Liu Y, Li XM, Dai RT:** Changes in oxidation, color and texture deteriorations during refrigerated storage of ohmically and water bathcooked pork meat. *Innov Food Sci Emerg Technol,* 26, 341-346, 2014. DOI: 10.1016/j.ifset.2014.06.009

**18. Wheeler TL, Shackelford SD, Koohmaraie M:** Sampling, cooking, and coring effects on Warner-Bratzler shear force values in beef. *J Anim Sci,* 74, 1553-1562, 1996.

**19. Jia JL, Zhang LP, Wu JP, Wang J, Ding Q:** Establishment of the optimum two-dimensional electrophoresis system of ovine ovarian tissue. *Genet Mol Res*, 13 (3): 6528-6538, 2014. DOI: 10.4238/2014.August.26.3

**20. Wu W, Gao XG, Dai Y, Fu Y, Li XM, Dai RT:** Post-mortem changes in sarcoplasmic proteome and its relationship to meat color traits in *M. semitendinosus* of Chinese Luxi yellow cattle. *Food Res Int,* 72, 98-105, 2015. DOI: 10.1016/j.foodres.2015.03.030

**21. Zuo H, Han L, Yu Q, Niu K, Zhao S, Shi H:** Proteome changes on water-holding capacity of yak *longissimus lumborum* during postmortem aging. *Meat Sci,* 121, 409-419, 2016. DOI: 10.1016/j.meatsci.2016.07.010

**22. Polati R, Menini M, Robotti E, Millioni R, Marengo E, Novelli E, Balzan S, Cecconi D:** Proteomic changes involved in tenderization of bovine *Longissimus dorsi* muscle during prolonged ageing. *Food Chem,* 135, 2052-2069, 2012. DOI: 10.1016/j.foodchem.2012.06.093

**23. Lomiwes D, Farouk MM, Wiklund E, Young OA:** Small heat shock proteins and their role in meat tenderness: A review. *Meat Sci,* 96, 26-40, 2014. DOI: 10.1016/j.meatsci.2013.06.008

**24. Yates JR, Ruse CI, Nakorchevsky A:** Proteomics by mass spectrometry: Approaches, advances, and applications. *Annu Rev Biomed Eng,* 11, 49-79, 2009. 10.1146/annurev-bioeng-061008-124934

**25. Qiang P, Jia L, Linyuan S, Xuewei L, Shunhua Z, Li Z:** Application of modification-specific proteomics in the meat-quality study. *Hereditas,* 37 (4): 327-335, 2015. DOI: 10.16288/j.yczz.14-417

**26. Kim YHB, Lonergan SM, Grubbs JK, Cruzen SM, Fritchen AN, della Malva A, Marino R, Huff-Lonergan E:** Effect of low voltage electrical stimulation on protein and quality changes in bovine muscles during postmortem aging. *Meat Sci,* 94, 289-296, 2013. DOI: 10.1016/j.meatsci.2013.02.013

**27. Hwang IH, Park BY, Kim JH, Cho SH, Lee JM:** Assessment of postmortem proteolysis by gel-based proteome analysis and its relationship to meat quality traits in pig longissimus. *Meat Sci,* 69, 79-91, 2005. DOI: 10.1016/j.meatsci.2004.06.019

**28. Huff-Lonergan E, Zhang WG, Lonergan SM:** Biochemistry of postmortem muscle-Lessons on mechanisms of meat tenderization. *Meat Sci,* 86, 184-195, 2010. DOI: 10.1016/j.meatsci.2010.05.004

**29. Zhang WG, Lonergan SM, Gardner MA, Huff-Lonergan E:** Contribution of postmortem changes of integrin, desmin and μ-calpain to variation in water holding capacity of pork. *Meat Sci,* 74, 578-585, 2006. DOI: 10.1016/j.meatsci.2006.05.008

**30. Kristensen L, Purslow PP:** The effect of ageing on the water-holding capacity of pork: Role of cytoskeletal proteins. *Meat Sci,* 58, 17-23, 2001. DOI: 10.1016/S0309-1740(00)00125-X

**31. Schäfer A, Rosenvold K, Purslow PP, Andersen HJ, Henckel P:** Physiological and structural events post mortem of importance for drip loss in pork. *Meat Sci,* 61, 355-366, 2002. DOI: 10.1016/S0309-1740(01)00205-4

**32. Pearce KL, Rosenvold K, Andersen HJ, Hopkins DL:** Water distribution and mobility in meat during the conversion of muscle to meat and ageing and the impacts on fresh meat quality attributes. *Meat Sci,* 89, 111-124, 2011. DOI: 10.1016/j.meatsci.2011.04.007

**33. Lund MN, Heinonen M, Baron CP, Estévez M:** Protein oxidation in muscle foods: A review. *Mol Nutr Food Res,* 55 (1): 83-95, 2011. DOI: 10.1002/mnfr.201000453

**34. Marino R, Albenzio M, della Malva A, Caroprese M, Santillo A, Sevi A:** Changes in meat quality traits and sarcoplasmic proteins during aging in three different cattle breeds. *Meat Sci,* 98 (2): 178-186, 2014. DOI: 10.1016/j.meatsci.2014.05.024

**35. Au Y:** The muscle ultrastructure: A structural perspective of the sarcomere. *Cell Mol Life Sci,* 61, 3016-3033, 2004. DOI: 10.1007/s00018-004-4282-x

**36. Jia X, Ekman M, Grove H, Faergestad EM, Aass L, Hildrum KI, Hollung K:** Proteome changes in bovine *longissimus thoracis* muscle during the early postmortem storage period. *J Proteome Res,* 6, 2720-2731, 2007. DOI: 10.1021/pr070173o

**37. Bouley J, Chambon C, Picard B:** Mapping of bovine skeletal muscle proteins using two-dimensional gel electrophoresis and mass spectrometry. *Proteomics,* 4, 1811-1824, 2004. DOI: 10.1002/pmic.200300688

**38. Canto AC, Suman SP, Nair MN, Li S, Rentfrow G, Beach CM, Silva TJ, Wheeler TL, Shackelford SD, Grayson A, McKeith RO, King DA:** Differential abundance of sarcoplasmic proteome explains animal effect on beef *Longissimus lumborum* color stability. *Meat Sci,* 102, 90-98, 2015. DOI: 10.1016/j.meatsci.2014.11.011

**39. Van de Wiel DFM, Zhang WL:** Identification of pork quality parameters byproteomics. *Meat Sci,* 77, 46-54, 2007. DOI: 10.1016/j.meatsci.2007.04.017

**40. Zhang WG, Lonergan SM, Gardner MA, Huff-Lonergan E:** Contribution of postmortem changes of integrin, desmin and μ-calpain to variation in water holding capacity of pork. *Meat Sci,* 74, 578-585, 2006. DOI: 10.1016/j.meatsci.2006.05.008

**41. Lo Bello M, Nuccetelli M, Caccuri AM, Stella L, Parker MW, Rossjohn J, McKinstry WJ, Mozzi AF, Federici G, Polizio F, Pedersen JZ, Ricci G:** Human glutathione transferase P1-1 and nitric oxide carriers: A new role for an old enzyme. *J Biol Chem,* 276, 42138-42145, 2001. DOI: 10.1074/jbc.M102344200

**42. Lametsch R, Karlsson A, Rosenvold K, Andersen HJ, Roepstorff P, Bendixen E:** Postmortem proteome changes of porcine muscle related to tenderness. *J Agric Food Chem,* 51 (24): 6992-6997, 2003. DOI: 10.1021/jf034083p

**43. Paredi G, Raboni S, Bendixen E, de Almeida AM, Mozzarelli A:** "Muscle to meat" molecular events and technological transformations: The proteomics insight. *J Proteomics,* 75 (14): 4275-4289, 2012. DOI: 10.1016/j.jprot.2012.04.011

**44. Bendixen E:** The use of proteomics in meat science. *Meat Sci,* 71 (1): 138-149, 2005. DOI: 10.1016/j.meatsci.2005.03.013

**45. Zheng A, Liu G, Zhang Y, Hou S, Chang W, Zhang S, Cai H, Chen G:** Proteomic analysis of liver development of lean Pekin duck *(Anas platyrhynchosdomestica). J Proteomics,* 75, 5396-5413, 2012. DOI: 10.1016/j.jprot.2012.06.019

Kafkas Univ Vet Fak Derg
24 (5): 701-707, 2018
DOI: 10.9775/kvfd.2018.19741

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Prevalence and Antimicrobial Resistance of Thermophilic *Campylobacter* Isolates from Raw Chicken Meats [1] [2]

Ghassan ISSA [1] Beren BASARAN KAHRAMAN [2] Mehmet Cemal ADIGUZEL [3]
Funda YILMAZ EKER [4] Esra AKKAYA [4] Gülay Merve BAYRAKAL [4]
Ahmet KOLUMAN [5] Tolga KAHRAMAN [4]

[1] *Supported by the Scientific Research Projects Coordination Unit of Istanbul University (Project No. 44016)*
[2] *This study was presented in 3rd International VETistanbul Group Congress, 17-20 May 2016, Bosnia and Herzegovina*

[1] Avrupa Vocational School, TR-34020 Kazlıçeşme, Istanbul - TURKEY
[2] Department of Microbiology, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcılar, Istanbul - TURKEY
[3] Department of Microbiology, Faculty of Veterinary Medicine, Atatürk University, TR-25240 Yakutiye, Erzurum - TURKEY
[4] Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcılar, Istanbul - TURKEY
[5] Faculty of Biomedical Engineering, Pamukkale University, TR-20070 Denizli - TURKEY

**Article Code: KVFD-2018-19741 Received: 12.03.2018 Accepted: 11.06.2018 Published Online: 11.06.2018**

**How to Cite This Article**

**Issa G, Basaran Kahraman B, Adiguzel MC, Yilmaz Eker F, Akkaya E, Bayrakal GM, Koluman A, Kahraman T:** Prevalence and antimicrobial resistance of thermophilic *Campylobacter* isolates from raw chicken Meats. *Kafkas Univ Vet Fak Derg,* 24 (5): 701-707, 2018. DOI: 10.9775/kvfd.2018.19741

## Abstract

Globally, the spread of antibiotic resistance via chicken meat consumption cause serious public health concerns. With this respect, the current study aimed to investigate the prevalence of thermophilic *Campylobacter* species isolated from raw meat chicken samples and their genetic determinants of resistance to various classes of antibiotics. A total of 540 chicken raw meat samples collected from various supermarkets and slaughterhouses in Istanbul, Turkey were analyzed according to EN ISO 10272-1:2006 standard procedure. For identification of the genus and species of the isolates, multiplex PCR assay was held. Minimum inhibitory concentrations of the antimicrobial agents (nalidixic acid, ciprofloxacin, tetracycline, gentamicin, kanamycin, and erythromycin) were initially determined using the broth microdilution method. In addition, the genetic determinants of antimicrobial resistance were investigated by PCR assays. In total, 357 (66.1%) *Campylobacter* isolates were obtained including 268 *Campylobacter jejuni* and 89 *Campylobacter coli*. Resistance to quinolones (nalidixic acid and ciprofloxacin) was the most common in all strains (80.1%), followed by resistance to tetracycline's (70.3%). The lowest resistance was determined as resistance to kanamycin (4.2%). Gentamicin and erythromycin resistance was not observed in this study. Only five *C. coli* isolate (1.4%) was classified as multidrug resistant. On the basis of these data, execute widely presence of antimicrobial resistance to quinolones and tetracycline's in *C. jejuni* and *C. coli* isolates from chicken raw meat samples and emphasizes that further multidisciplinary studies and novel strategies in the concept of 'One Health' are needed.

***Keywords:*** *Campylobacter, Raw chicken meat, Prevalence, Antimicrobial resistance, PCR*

# Çiğ Tavuk Etlerinden İzole Edilen Termofilik *Campylobacter* İzolatlarının Prevalansı ve Antimikrobiyal Direnci

## Öz

Dünyada, tavuk eti tüketimi yoluyla antibiyotik direncinin yayılması ciddi halk sağlığı sorunlarına neden olmaktadır. Bu bağlamda, bu çalışmada, çiğ tavuk eti örneklerinden izole edilen termofilik *Campylobacter* türlerinin prevalansını ve çeşitli antibiyotik sınıflarına direnci gösteren genetik belirleyicileri araştırmayı amaçlandı. İstanbul'daki çeşitli süpermarketlerden ve kesimhanelerden toplanan toplam 540 çiğ tavuk eti numunesi, EN ISO 10272-1:2006 standart prosedürüne göre analiz edildi. İzolatların cins ve türlerinin belirlenmesi için multipleks PCR testi yapıldı. Antimikrobiyal ajanların (nalidiksik asit, siprofloksasin, tetrasiklin, gentamisin, kanamisin ve eritromisin) minimum inhibisyon konsantrasyonları sıvı mikrodilüsyon yöntemi kullanılarak tespit edildi. Bununla beraber, antimikrobiyal direncin genetik belirleyicileri de PCR ile araştırıldı. Toplamda 357 (%66.1) *Campylobacter* izolatı, 268 *Campylobacter jejuni* ve 89 *Campylobacter coli* saptandı. Kinolonlara (nalidiksik asit ve siprofloksasin) karşı direnç, tüm suşlarda en sık görülen direnç (%80.1) olarak saptandı, bunu tetrasiklinlere (%70.3) direnç izledi. En düşük direnç, kanamisin direnci (%4.2) olarak belirlendi. Bu çalışmada gentamisin ve eritromisin direnci gözlenmedi. Sadece beş *C. coli* izolatı (%1.4) çok ilaca dirençli olarak sınıflandırıldı. Bu verilere dayanarak, çiğ tavuk eti örneklerinden elde edilen *C. jejuni* ve *C. coli* izolatlarında kinolonlara ve tetrasiklinlere karşı yaygın antimikrobiyal direnç varlığı saptandı ve "Tek Sağlık" konsepti içinde daha fazla disiplinler arası çalışmalara ve yeni stratejilere ihtiyaç duyulduğu vurgulandı.

***Anahtar sözcükler:*** *Campylobacter, Çiğ tavuk eti, Prevalans, Antimikrobiyal direnç, PCR*

**İletişim (Correspondence)**
+90 212 4737070/17360
beren@istanbul.edu.tr

# INTRODUCTION

Poultry is an extremely high nutritive versatile meat, which is of a great importance for human nutrition, so the safety protection measures of poultry meat are very important subject [1]. Thermophilic *Campylobacter*, including *Campylobacter jejuni* and *Campylobacter coli*, is a main bacterial cause of acute gastroenteritis in humans. Raw poultry products are the main reservoir of thermophilic *Campylobacter* infection in particular via consumption of undercooked products or cross-contamination of ready-to-eat products [2,3]. Also, *Campylobacter* infection is associated with the development of Guillain-Barre´ syndrome, a neurological disorder affecting the peripheral nervous system. Particularly, the chicken is a natural host of *C. jejuni* and serves as a major reservoir for this pathogenic organism. Contamination of chicken carcasses often occurs during the slaughtering process and consumption of chicken meat is a significant source of human *Campylobacter* infections [2-4].

The emergence of antimicrobial resistance is not a new phenomenon, nor an unexpected one. Several reports have been published about antibiotic resistance problem and the reasons behind the increasing rates. These reports have highlighted that poultry meat may play a major role in transmission [3-9]. The uncontrolled and excessive use of antibiotics in the treatment of infections in humans and veterinary medicine may be the reason for high rates of resistance, in poultry [1,10]. In Turkey, antibiotics feed additives were widely used for control of the growth in poultry, but in 2006 the usage of antibiotics in broiler flocks were forbidden by the European Union (EU) Council Directive 90/167/EEC [11,12]. All of the countries in EU have been started to investigate the prevalence of *Campylobacter* spp. in broiler carcasses and the antimicrobial resistance in broiler flocks [10]. However, the number of the studies on antibiotic-resistant *Campylobacter*s isolated from poultry meat in Turkey, is rather limited.

This study was aimed to carry out to determine the prevalence and antimicrobial resistance of thermophilic *Campylobacter* species isolated from chicken raw meat samples available in retail trade in İstanbul, Turkey.

# MATERIAL and METHODS

### *Sample Collection*

A total of 540 chicken raw meat samples including chicken thigh, breast and wings were collected from various supermarkets and slaughterhouses in Istanbul, Turkey, between January 2015 and March 2016. With this aim each month, 6 thigh, 6 breast, and 6 wings were obtained from slaughterhouse and same amounts were collected from different markets. A sum of 540 samples were analysed for *Campylobacter* contamination.

### *Isolation and Species Identification*

*Campylobacter* species detection and isolation were performed according to EN ISO 10272-1:2006 standard procedures [13]. A 25 g portion of each sample was homogenized in a stomacher and were enriched in Bolton broth (Oxoid, USA) for 4 h at 37°C and then incubated for up to 44 h at 42°C under microaerophilic conditions created by using a CampyGen gas pack (Oxoid, USA). The enriched samples were subsequently subcultured by spreading 10 µL aliquots on modified Charcoal Cefoperazone Deoxycholate agar (CCDA, Oxoid, USA) and incubated for up to 48 h at 42°C under microaerophilic conditions. Suspected colonies were cultured onto plates of Columbia Blood agar (Oxoid, USA) containing 5% horse blood, and were confirmed by microscopic analysis, oxidase testing (Oxoid, USA), microaerophilic growth at 25°C and aerobic growth at 42°C. The remainder of each plate was harvested and stored in 1 mL of nutrient broth plus 10% glycerol at 80°C. Conventional culture method was verified using ISO 16140 method. According to this method 30 positive and 30 negative samples were analysed using the method. The results obtained showed a specifity and sensitivity of 95%.

For identification of the genus and species of the isolates, multiplex PCR was carried out following the PCR assay method described by Linton et al.[14] and Denis et al.[15]. Simultaneous amplification of 16SrRNA gene fragment (genus-specific), *map*A gene (for *C. jejuni)* and *ceu*E gene (for *C. coli)* was carried using primers and protocol. The details of primers and cycling conditions are given in *Table 1*. Amplified PCR products were visualized by electrophoresis in 1.5% agarose gel stained with ethidium bromide. For quality control, *C. jejuni* ATCC 33291, *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 strains were used.

### *Antimicrobial Susceptibility Testing*

Minimum inhibitory concentrations (MIC) of antimicrobial agents (ciprofloxacin, erythromycin, gentamicin, kanamycin, nalidixic acid and tetracycline) was determined with a microbroth dilution method [16].

The clinical breakpoints were interpreted according to the EUCAST [16] guidelines for *Campylobacter* as regards erythromycin, nalidixic acid, gentamicin, ciprofloxacin and tetracycline, and to CLSI guidelines for Enterobacteriaceae [17] as regards kanamycin (MIC≤16 susceptibility, MIC=32 intermediate, MIC≥64 resistant), because there was no ECOFFS for *Campylobacter*.

*Campylobacter jejuni* ATCC 33560 was used as reference strains for quality control assurance in each batch of broth microdilution plates [16].

### *Detection of Antimicrobial Resistance Genes*

All of the phenotypically resistant isolates were analyzed for the presence of *ery*, *tet*(O), *aph*A-3, *gyr*A (Thr-86-Ile

**Table 1.** *Primer sequences, product sizes and cycling conditions*

| Primer Specific For | Target(s) | Primers (5′ to 3′, as synthesized) | Size (bp) | Cycling Conditions |
|---|---|---|---|---|
| *Campylobacter* | 16SrRNA | ATCTAATGGCTTAACCATTAAAC<br>GGACGGTAACTAGTTTAGTATT | 857 | 95°C 60 s; 95°C 15 s; 59°C 60 s; 72°C 90 s (35 cycles); 72°C 3 min |
| *C. jejuni* | *map*A | CTATTTTATTTTTGAGTGCTTGTG<br>GCTTTATTTGCCATTTGTTTTATTA | 589 | |
| *C. coli* | *ceu*E | AATTGAAAATTGCTCCAACTATG<br>TGATTTTATTATTTGTAGCAGCG | 462 | |

**Table 2.** *Primer sequences, product sizes and cycling conditions*

| Primer Specific For | Target(s) | Primers (5′ to 3′, as synthesized) | Size (bp) | Cycling Conditions |
|---|---|---|---|---|
| Erythromycin resistance | 23S rRNA-F<br>23S rRNA-R | TTAGCTAATGTTGCCCGTACCG<br>AGCCAACCTTTGTAAGCCTCCG | 697 | 94°C 5 min; 94°C′ 30 s; 59°C′ 30 s; 72°C 45 s; (30 cycles); 72°C 5 min |
| | ERY2075-R | TAGTAAAGGTCCACGGGGTCGC | 485 | |
| | ERY2074-R | AGTAAAGGTCCACGGGGTCTGG | 485 | |
| Quinolones resistance | GZgyrA5-F<br>GZgyrA6-R | ATTTTTAGCAAAGATTCTGAT<br>CCATAAATTATTCCACCTGT | 673 | 94°C 3 min; 94°C′ 30 s; 50°C′ 30 s 72°C′ 20 s; (30 cycles); 72°C 5 min |
| | CampyMAMAgryA-F<br>CampyMAMAgyrA-R | TTTTTAGCAAAGATTCTGAT<br>CAAAGCATCATAAACTGCAA | 265 | |
| | CampyMAMAgryA1-F<br>GZgyrA4 | TTTTTAGCAAAGATTCTGAT<br>CAGTATAACGCATCGCAGCG | 368 | |
| | GZgyrACcoli3F-F<br>CampyMAMAgyrA8-R | TATGAGCGTTATTATCGGTC<br>TAAGGCATCGTAAACAGCCA | 192 | |
| | GZgyrACcoli3F-F<br>GZgyrACcoli4R-R | TATGAGCGTTATTATCGGTC<br>GTCCATCTACAAGCTCGTTA | 505 | |
| Aminoglycoside resistance | *aph*A-3 F<br>*aph*A-3 R | GGGACCACCTATGATGTGGAACG<br>CAGGCTTGATCCCCAGTAAGTC | 600 | 95°C 30s; 55°C 1 min; 72°C 1 min (30 cycles); 72°C 5 min |
| Tetracycline resistance | *tet*O F<br>*tet*O R | GGCGTTTTGTTTATGTGCG<br>ATGGACAACCCGACAGAAGC | 559 | 95°C 1 min; 95°C 15 s; 58°C 15 s 72°C 30 s; (30 cycles); 72°C 5 min |
| cmeABC | *cme*A- F<br>*cme*A- R | TAGCGGCGTAATAGTAAATAAAC<br>ATAAAGAAATCTGCGTAAATAGGA | 435 | 94°C 7 min; 94°C 1 min; for cmeA 49.8°C, for cmeB 50.8°C, for cmeC 52.3°C 90 s; 72°C 2.5 min (31 cycles); 72°C 5 min |
| | *cme*B- F<br>*cme*B- R | AGGCGGTTTTGAAATGTATGTT<br>TGTGCCGCTGGGAAAAG | 444 | |
| | *cme*C- F<br>*cme*C- R | CAAGTTGGCGCTGTAGGTGAA<br>CCCCAATGAAAAATAGGCAGAGTA | 431 | |

mutation), *cme*A, *cme*B and *cme*C genes, representing resistance to erythromycin, tetracycline, aminoglycoside, and quinolones, and CmeABC efflux system components, respectively.

Mismatch Amplification Mutation Assay (MAMA-PCR) for the detection of point mutations at position 2075 and 2074, which present high-level erythromycin resistance, were performed [18]. Genes *tet*(O) and *aph*A-3 were detected by PCR assay as described [19]. Thr-86-Ile mutations in the quinolones resistance determining region (QRDR) of gene *gyr*A were detected by MAMA-PCR [20,21]. The presence of the *cme*A, *cme*B and *cme*C genes were determined by PCR assays [22]. The primers sequences, product sizes and cycling conditions are listed in *Table 2*.

Multi-drug resistance (MDR) was defined as resistance to three or more antimicrobial agents with different mechanisms of action, as previously described [23].

## RESULTS

The prevalence rate of *Campylobacter* spp. in chicken raw meat samples were found in 66.1%. Monthly distribution is summarized in *Fig. 1*. Totally 357 *Campylobacter* isolates, whereas *C. jejuni* was identified in the remaining 268 (75.07%) and *C. coli* 89 (24.93%).

Distribution of *C. jejuni* according to tight, breast and wing samples were 73 (27.23%), 106 (39.55%) and 89 (33.22%). Distribution of *C. jejuni* from different parts at slaughterhouse level was not significant with months and no seasonal change was observed. On the contrary, seasonal distribution of *C.jejuni* was observed in market samples. *C. jejuni* was mostly isolated during summer months with a rate of 88.88% (48 of 54 samples) and was lowest during January with a rate of 5.56% (1 of 18 samples).

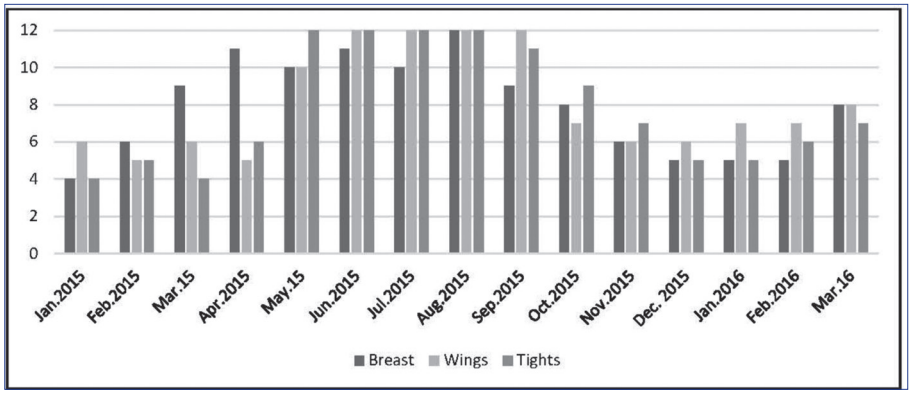



**Fig 1.** Monthly distribution of *Campylobacter* isolates

***Table 3.*** *Antibacterial resistance profiles and MIC distributions of the isolates*

| Antimicrobial | MIC Range (µg/mL) | | Number of Isolates According to MIC | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S ≤ | R > | Isolates | 0.094 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 |
| Erythromycin | 4 | 4 | Cj | | 3 | 182 | 53 | 30 | | | | | | | | | |
| | 2 | 2 | Cc | | 6 | 56 | 13 | 14 | | | | | | | | | |
| Gentamicin | 2 | 2 | Cj | | 8 | 214 | 34 | 12 | | | | | | | | | |
| | | | Cc | | | 66 | 15 | 8 | | | | | | | | | |
| Kanamycin | 4 | 4 | Cj | | | | 9 | 34 | 197 | 18 | 10 | | | | | | |
| | | | Cc | | | | | 4 | 80 | | | | | | 5 | | |
| Nalidixic acid | 16 | 16 | Cj | | | | | | | 16 | 31 | 4 | 100 | 117 | | | |
| | | | Cc | | | | | | 2 | 6 | 12 | | 10 | 59 | | | |
| Ciprofloxacin | 0.5 | 0.5 | Cj | | | | | | | 8 | 52 | | 52 | 156 | | | |
| | | | Cc | | | | | | | 10 | 1 | | 22 | 56 | | | |
| Tetracycline | 1 | 1 | Cj | | 80 | 19 | | | | | | 5 | 109 | 16 | 35 | 4 | |
| | 2 | 2 | Cc | | 3 | 4 | | | | | | 4 | 56 | 8 | 12 | 2 | |

***MIC:*** *Minimum Inhibitory Concentration,* ***S:*** *Susceptible,* ***R:*** *Resistant,* ***Cj:*** *C. jejuni,* ***Cc:*** *C. coli*

***Table 4.*** *Comparison of phenotypic and genotypic resistance to antimicrobial agents*

| Isolates | Number of Strains Resistant to Antimicrobial Agents | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Quinolones | | | | Tetracycline | | Kanamycin | |
| | Nalidixic Acid | | Ciprofloxacin | | | | | |
| | Broth Microdilution | Mutation Thr86Ile | Broth Microdilution | Mutation Thr86Ile | Broth Microdilution | tet(O) gene | Broth Microdilution | aphA-3 gene |
| *C. jejuni* (n=268) | 217 | 217 | 208 | 208 | 169 | 169 | 10 | 10 |
| *C. coli* (n=89) | 69 | 69 | 78 | 78 | 82 | 82 | 5 | 5 |
| Total (n=357) | 286 (80.1%) | | | | 251 (70.3%) | | 15 (4.2%) | |

Resistance to quinolones (nalidixic acid and ciprofloxacin) was the most common finding (80.1%), followed by resistance to tetracyclines (70.3%). Conversely, the lowest resistance was recorded against to kanamycin (4.2%). Furthermore, all isolates were detected susceptible to gentamicin and erythromycin. Only five *C. coli* isolates (1.4%) were evaluated as multidrug resistant.

The antibacterial susceptibility testing results of 357 *Campylobacter* isolates against six different antibacterial agents are exhibited in *Table 3*.

The phenotypic and genotypic results were fully concordant. Comparison of phenotypic and genotypic resistance to antimicrobial agents was shown in *Table 4*.

In this study, all isolates were resistant to at least one antibacterial agent, while most of the isolates were resistant to tetracycline, nalidixic acid, and ciprofloxacin. 20% of the isolates were resistant to two antibacterial agents and 1.4% of the isolates to more than two antibiotics.

## DISCUSSION

Poultry products are the most important single source of human Campylobacteriosis. The European Food Safety Authority (EFSA) reported 246.307 laboratory confirmed cases in the EU [24]. Turkey was one of the most often reported as the probable country of infection outside EU (5.5%). Han et al.[3] in Korea, Guyard-Nicodème et al.[7] in France, and Maesaar et al.[8] in Estonia were reported *Campylobacter* spp. prevalence from broiler chicken meat 68.3%, 76%, and 88.8%, respectively.

Regarding previous studies in Turkey, Hızlısoy et al.[25] found 100% of the chicken meat samples positive for *Campylobacter* species. Abay et al.[6] reported that among 100 carcass samples examined, 96°*C. jejuni* strains were isolated. In this study, it has been demonstrated that *Campylobacter* spp. are frequently present (66.1%). Withal, when comparing the reported prevalence of *Campylobacter* spp. among our country during recent years, the results of the present study are considerably lower. Seasonal distribution of samples were showing similarity with the results of Koluman [26] and Pamuk [27].

High fluoroquinolones resistance levels among *Campylobacter* poultry meat isolates have been widely stated, in Poland [5,28], Italy [29], Turkey [6], Korea [9] and many other European countries [30]. In the current study, resistance to quinolones (nalidixic acid and ciprofloxacin) was the most common and these results substantiate other authors' findings. The broad use of this class of antibiotics in poultry may be the reason for this crucial problem.

The tetracycline's, being the first major group of antimicrobial agents, are among the most frequently used therapeutics in veterinary medicine. In the current study, the resistance rate to tetracycline was determined as 70.3%. The prevalence was higher in comparison to those detected by Abay et al.[6], Guyard-Nicodème et al.[7], Maesaar et al.[8], Wei et al.[9], Wieczorak et al.[15].

The aminoglycosides are a group of antimicrobials used both in human and veterinary medicine. Gentamicin is the most widely used aminoglycosides in poultry. EFSA [31] reported that the gentamicin resistance was comparatively very low (0.3%) in *C. jejuni* isolates and resistance were not detected in *C. coli* isolates from broiler meat. Wei et al.[9] call attention to the high prevalence of gentamicin-resistant Campylobacter isolated in food-producing animals in China. Moreover, low to moderate resistance ranging from 0 to 27% was observed in various studies [32-34]. Kanamycin is an aminoglycoside antibiotic which is effective in the treatment of severe infections caused by Gram-negative bacteria [5]. In the current study, all isolates were susceptible to gentamicin. Also, kanamycin resistance was determined in 15 strains (4.2%).

Macrolides are still the most effective antibiotics against *Campylobacter* infections. Macrolide resistance in *Campylobacter* spp. has been the result of the point mutation(s) occurring in ribosomal RNA or proteins. The authors reported high resistance to erythromycin in Spain [35]. However, in European countries, low resistance levels were stated from 0 to 8% [32]. According to EU summary report, the variable occurrence of resistance to erythromycin among *Campylobacter* species were reported, depending on the country of isolation [24]. In this study, all of the isolates were susceptible to erythromycin which is the drug of choice for the treatment of human Campylobacteriosis. This result is in agreement with those reported for chicken meat isolates Wieczorak et al.[5] in Poland and Guyard-Nicodème et al.[7] in France. Because of the low level of resistance might be consequences of the ban of macrolides as a growth promoter in broilers.

Otherwise, except these individual resistance mechanisms, multidrug efflux system CmeABC contributes to *Campylobacter* resistance to multiple drugs, including fluoroquinolones, β-lactams, erythromycin, and tetracycline [19,36]. The authors indicated that the effect of CmeABC on aminoglycoside resistance (like gentamicin) was less apparent [36]. In this study, only five *C. coli* isolate (1.4%) was classified as multidrug resistant. Contrary, the authors reported much higher percentages ranged from 44.9 to 86% [3,4,37]. The use of antimicrobial drugs in food animals has been regulated in European countries, the conflicted results may base on the implementation of legislation. In some developing countries, even where legislation does exist and is enforced, their enforcement may be a problem and virtually non-existent [10,37]. The absence and/or weakness of regulations and implementation particularly about usage of antibiotics in the food animals, also inadequate hygiene and sanitation, may have accelerated the emergence and dissemination of antimicrobial resistance.

Over recent decades, antibiotic resistance undoubtedly represents a global public health problem. The global author's highlight that poultry meat is an important risk of human exposure to antimicrobial resistance due to residual resistance of high impact antibiotic application of 20$^{th}$ century or illegal applications as growth promoters [38,39]. In the current study provides baseline information on the highlights the widespread presence of this emerging foodborne pathogen and resistance profiles in poultry meat. These data emphasize that further multidisciplinary studies, surveillance programmes and reports in animals, and humans, as well as food, are important in terms of manifesting the current status of resistance against antimicrobial drugs and emerging

health problems. Therewithal, the data acquired here will be useful for risk assessment for public health hazard *C. jejuni*. It is significant that the population and demographic character of Istanbul is highly variable with widespread chicken consuming behaviour. This picture can be generalized with significant variations of other cities to determine a significant hazard map to prevent *C. jejuni* borne infections.

## REFERENCES

**1. Ljubojević D, Pelić M, Puvača N, Milanov D:** Resistance to tetracycline in *Escherichia coli* isolates from poultry meat: Epidemiology, policy and perspective. *Worlds Poult Sci J*, 73 (2): 409-417, 2017. DOI: 10.1017/S0043933917000216

**2. Fontanot M, Iacumin L, Cecchini F, Comi G, Manzano M:** Rapid detection and differentiation of important *Campylobacter* spp. in poultry samples by dot blot and PCR. *Food Microbiol*, 43, 28-34, 2014. DOI: 10.1016/j.fm.2014.05.001

**3. Han K, Jang SS, Choo E, Heu S, Ryu S:** Prevalence, genetic diversity, and antibiotic resistance patterns of *Campylobacter jejuni* from retail raw chickens in Korea. *Int J Food Microbiol*, 114 (1): 50-59, 2007. DOI: 10.1016/j.ijfoodmicro.2006.10.042

**4. Chen X, Naren GW, Wu CM, Wang Y, Dai L, Xia LN, Luo PJ, Zhang Q, Shen JZ:** Prevalence and antimicrobial resistance of *Campylobacter* isolates in broilers from China. *Vet Microbiol*, 144 (1-2): 133-139, 2010. DOI: 10.1016/j.vetmic.2009.12.035

**5. Wieczorek K, Szewczyk R, Osek J:** Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter jejuni* and *C. coli* isolated from retail raw meat in Poland. *Vet Med Czech*, 57 (6): 293-299, 2012.

**6. Abay S, Kayman T, Otlu B, Hizlisoy H, Aydin F, Ertas N:** Genetic diversity and antibiotic resistance profiles of *Campylobacter jejuni* isolates from poultry and humans in Turkey. *Int J Food Microbiol*, 178, 29-38, 2014. DOI: 10.1016/j.ijfoodmicro.2014.03.003

**7. Guyard-Nicodème M, Rivoal K, Houard E, Rose V, Quesne S, Mourand G, Rouxel S, Kempf I, Guillier L, Gauchard F, Chemaly M:** Prevalence and characterization of *Campylobacter jejuni* from chicken meat sold in French retail outlets. *Int J Food Microbiol*, 203, 8-14, 2015. DOI: 10.1016/j.ijfoodmicro.2015.02.013

**8. Mäesaar M, Kramarenko T, Meremäe K, Sõgel J, Lillenberg M, Häkkinen L, Ivanova M, Kovalenko K, Hörman A, Hänninen ML, Roasto M:** Antimicrobial resistance profiles of *Campylobacter* spp. isolated from broiler chicken meat of Estonian, Latvian and Lithuanian origin at estonian retail level and from patients with severe enteric infections in Estonia. *Zoonoses Public Health*, 63 (2): 89-96, 2016. DOI: 10.1111/zph.12208

**9. Wei B, Cha SY, Yoon RH, Kang M, Roh JH, Seo HS, Lee JA, Jang HK:** Prevalence and antimicrobial resistance of *Campylobacter* spp. isolated from retail chicken and duck meat in South Korea. *Food Control*, 62, 63-68, 2016. DOI: 10.1016/j.foodcont.2015.10.013

**10. Maćkiw E, Korsak D, Rzewuska K, Tomczuk K, Rożynek E:** Antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from food in Poland. *Food Control*, 23 (2): 297-301, 2012. DOI: 10.1016/j.foodcont.2011.08.022

**11. EC.** Council Directive 90/167/EEC of 26 March 1990 laying down the conditions governing the preparation, placing on the market and use of medicated feeding stuffs in the Community. *Offic J Eur Comm*, L92: 42-48, 1990.

**12. Anonymous:** Yem katkıları ve premikslerin üretimi, ithalatı, ihracatı, satışı ve kullanımı hakkında tebliğde değişiklik yapılmasına dair tebliğ. Tarım ve Köy İşleri Bakanlığından. *Resmi Gazete.* 21 Ocak 2006; Sayı: 26056 Tebliğ No: 2006/1.

**13. Anonymous.** Microbiology of food and animal feeding stuffs - horizontal method for detection and enumeration of *Campylobacter* spp. - Part 1: Detection method (EN ISO 10272-1:2006). International Organization for Standardisation, Geneva, Switzerland. 2006.

**14. Linton D, Owen RJ, Stanley J:** Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. *Res Microbiol*, 147, 707-718, 1996. DOI: 10.1016/S0923-2508(97)85118-2

**15. Denis M, Petton JR, Laisney MJ, Ermel G, Salvat G:** *Campylobacter* contamination in French chicken production from farm to consumers. Use of a PCR assay for detection and identification of *Campylobacter jejuni* and *Campylobacter coli*. *J Appl Microbiol*, 91, 255-267, 2001. DOI: 10.1046/j.1365-2672.2001.01380.x

**16. EUCAST (The European Committee on Antimicrobial Susceptibility Testing):** Breakpoint tables for interpretation of MICs and zone diameters. version 5.0, 2015.

**17. CLSI (Clinical and Laboratory Standards Institute):** Performance standards for antimicrobial susceptibility testing. Twenty-second informational supplement. Document M100-S22. Wayne, PA, USA, 2012.

**18. Alonso R, Mateo E, Churruca E, Martinez I, Girbau C, Fernández-Astorga A:** MAMA-PCR assay for the detection of point mutations associated with high-level erythromycin resistance in *Campylobacter jejuni* and *Campylobacter coli* strains. *J Microbiol Methods*, 63 (1): 99-103, 2005. DOI: 10.1016/j.mimet.2005.03.013

**19. Gibreel A, Sköld O, Taylor DE:** Characterization of plasmid-mediated *aphA-3* kanamycin resistance in *Campylobacter jejuni*. *Microb Drug Resist,* 10 (2), 98-105, 2004. DOI: 10.1089/1076629041310127

**20. Zirnstein, G, Helsel L, Li Y, Swaminathan B, Besser J:** Characterization of gyrA mutations associated with fluoroquinolone resistance in *Campylobacter coli* by DNA sequence analysis and MAMA PCR. *FEMS Microbiol Lett*, 190, 1-7, 2000. DOI: 10.1111/j.1574-6968.2000.tb09253.x

**21. Zirnstein G, Li Y, Swaminathan B, Angulo F:** Ciprofloxacin resistance in *Campylobacter jejuni* isolates: Detection of gyrA resistance mutations by mismatch amplification mutation assay PCR and DNA sequence analysis. *J Clin Microbiol*, 37, 3276-3280, 1999.

**22. Olah PA, Doetkott C, Fakhr MK, Logue CM:** Prevalence of the *Campylobacter* multi-drug efflux pump (CmeABC) in *Campylobacter* spp. isolated from freshly processed Turkeys. *Food Microbiol,* 23, 453-460, 2006. DOI: 10.1016/j.fm.2005.06.004

**23. Kashoma IP, Kassem II, John J, Kessy BM, Gebreyes W, Kazwala RR, Rajashekara G:** Prevalence and antimicrobial resistance of *Campylobacter* isolated from dressed beef carcasses and raw milk in Tanzania. *Microb Drug Resist*, 22 (1): 40-52, 2016. DOI: 10.1089/mdr.2015.0079

**24. EFSA (European Food Safety Authority):** The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA J*, 15 (12): 5077, 2017. DOI: 10.2903/j.efsa.2017.5077

**25. Hızlısoy H, Kılıç H:** The resistance state of *Campylobacter jejuni* isolated from broiler carcasses against to macrolide, quinolone and tetracycline groups of antibiotics. *Erciyes Univ Vet Fak Derg*, 12 (2): 81-92, 2015.

**26. Koluman A:** Detection of *Campylobacter jejuni* contamination in poultry houses and slaughterhouses. *Turk Hij Den Biyol Derg*, 67 (2): 57-64, 2010.

**27. Pamuk S:** Afyon'da paketlenmeden satılan piliç karkaslarında termofilik *Campylobacter* türlerinin saptanması ve *C. jejuni* izolatlarının PCR ile doğrulanması. *Doktora Tezi,* Ankara Üniversitesi Sağlık Bilimleri Enstitüsü, 2010.

**28. Rozynek E, Dzierzanowska-Fangrat K, Korsak D, Konieczny P, Wardak S, Szych J, Jarosz M, Dzierzanowska D:** Comparison of antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolated from humans and chicken carcasses in Poland. *J Food Prot*, 71 (3): 602-607, 2008.

**29. Di Giannatale E, Di Serafino G, Zilli K, Alessiani A, Sacchini L, Garofolo G, Aprea G, Marotta F:** Characterization of antimicrobial resistance patterns and detection of virulence genes in *Campylobacter* isolates in Italy. *Sensors*, 14 (2): 3308-3322, 2014. DOI: 10.3390/s140203308

**30. EFSA (European Food Safety Authority):** The European Union Summary Report on antimicrobial resistance in zoonotic and indicator

bacteria from humans, animals and food in 2011. *EFSA J*, 11 (5): 3196, 2013. DOI: 10.2903/j.efsa.2013.3196

**31. EFSA (European Food Safety Authority):** The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014. *EFSA J*, 14 (2): 4380, 2016. DOI: 10.2903/j.efsa.2016.4380

**32. EFSA (European Food Safety Authority):** Analysis of the baseline survey on the prevalence of *Campylobacter* on broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008. Part A: *Campylobacter* and Salmonella prevalence estimates. *EFSA J*, 8, 1503, 2010. DOI: 10.2903/j.efsa.2010.1503

**33. EFSA (European Food Safety Authority):** The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. *EFSA J*, 10 (3): 2597, 2012. DOI: 10.2903/j.efsa.2012.2597

**34. Economou V, Zisides N, Gousia P, Petsios S, Sakkas H, Soultos N, Papadopoulou C:** Prevalence and antimicrobial profile of *Campylobacter* isolates from free-range and conventional farming chicken meat during a 6-year survey. *Food Control*, 56, 161-168, 2015. DOI: 10.1016/j.foodcont.2015.03.022

**35. Sáenz Y, Zarazaga M, Lantero M, Gastañares MJ, Baquero F, Torres C:** Antibiotic resistance in *Campylobacter* strains isolated from animals, foods, and humans in Spain in 1997-1998. *Antimicrob Agents Chemother*, 44 (2): 267-271, 2000. DOI: 10.1128/AAC.44.2.267-271.2000

**36. Lin J, Michel LO, Zhang Q:** CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. *Antimicrob Agents Chemother,* 46, 2124-2131, 2002. DOI: 10.1128/AAC.46.7.2124-2131.2002

**37. Rodrigo S, Adesiyun A, Asgarali Z, Swanston W:** Prevalence of *Campylobacter* spp. on chickens from selected retail processors in Trinidad. *Food Microbiol*, 22, 125-131, 2005. DOI: 10.1016/j.fm.2004.03.008

**38. Bilge N, Vatansever L, Sezer Ç:** Antibiotic resistance of *Salmonella* spp. isolated from raw chicken wings. *Kafkas Univ Vet Fak Derg*, 24 (3): 431-435, 2018. DOI: 10.9775/kvfd.2017.19134

**39. Büyükünal SK:** Prevalence and antibiotic resistance of thermotolerant *Campylobacter* spp. in chicken meat sold in İstanbul. *J Fac Vet Med Istanbul Univ*, 43 (2): 98-109, 2017. DOI: 10.16988/iuvfd.282027

Kafkas Univ Vet Fak Derg
24 (5): 709-716, 2018
DOI: 10.9775/kvfd.2018.19772

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# The Clinical and Radiological Evaluation of Medial Coronoid Diasease in Dogs: 20 Cases

Didar AYDIN KAYA [1] Kemal ALTUNATMAZ [1]

[1] Istanbul University, Veterinary Faculty, Surgery Department, TR-34320 Avcilar, Istanbul - TURKEY

**Article Code: KVFD-2018-19772 Received: 17.03.2018 Accepted: 27.06.2018 Published Online: 27.06.2018**

**How to Cite This Article**

**Aydın Kaya D, Altunatmaz K:** The clinical and radiological evaluation of medial coronoid diasease in dogs: 20 cases. *Kafkas Univ Vet Fak Derg,* 24 (5): 709-716, 2018. DOI: 10.9775/kvfd.2018.19772

## Abstract

In this study, clinical status and treatment outcomes of 20 dogs, which were referred to Istanbul University, Faculty of Veterinary Medicine, and Department of Surgery with the complaint of forelimb lameness and diagnosed with medial coronoid disease (MCD), were assessed. The diagnosis was made based on radiographic findings in 16 patients, on arthroscopic findings in 2 and on computed tomographic findings in other 2 patients. Arthrotomy aimed at removal of fragmented medial coronoid process and debridement of the surrounding tissues was carried out on a total of 20 elbow joints. Average functional recovery period after arthrotomy was 31 days and postoperative lameness scores on day 30 were "1" and "0" in 8 and 12 cases, respectively. Recovery rates on postoperative day 60 were detected to be moderate, good and very good in 9, 5 and 6 dogs, respectively. In the current study, treatment options were evaluated in the dogs with medial coronoid disease and the outcomes were aimed to be a contribution to veterinary practice.

***Keywords:*** *Medial coronoid process, Fragmentation, Dog*

# Köpeklerde Medial Koronoid Hastalığının Klinik ve Radyografik Olarak Değerlendirilmesi: 20 Olgu

## Öz

Bu çalışmada İstanbul Üniversitesi Veteriner Fakültesi Cerrahi Anabilim Dalı'na ön bacakta topallık şikayetiyle getirilip, medial koronoid hastalığı (MKH) tanısı konulan 20 adet köpeğin klinik durumları ve sağaltım sonuçları değerlendirilmiştir. MKH'nın tanısı 16 hastada direkt radyografi, 2 hastada artroskopi ve 2 hastada bilgisayarlı tomografi ile konulmuştur. MKH'na bağlı medial koronoid prosesin kırık parçasının uzaklaştırılması ve bölgenin debridmanına yönelik artrotomi işlemi toplam 20 dirsek eklemine uygulanmıştır. Artrotomi sonrasında fonksiyonel iyileşme ortalama 31 gün, postoperatif 30. gün topallık dereceleri 8 olguda "1", 12 olguda "0" olarak belirlenmiş olup 60. gün sonunda iyileşmeleri 9 olguda vasat, 5 olguda "iyi", 6 olguda ise "çok iyi" olarak değerlendirildi. Sunulan bu çalışmada medial koronoid hastalığı olan köpeklere uygulanan sağaltım yöntemleri değerlendirilmiş olup sonuçlarının meslek pratiğine aktarılması amaçlanmıştır.

***Anahtar sözcükler:*** *Medial koronoid proses, Kırık, Köpek*

## INTRODUCTION

Medial coronoid disease (MCD) which is one of the most frequently encountered pathology of the forelimb lameness is most particularly seen in the young individuals of medium or large dog breeds [1-5]. Medial coronoid disease is a component of a phenomenon called as canine elbow dysplasia complex which includes ununited anconeal process (UAP), osteochondrosis (OC) or osteochondritis dissecans of humerus condyle (OCD) and radioulnar joint incongruence [6-8]. Fragmentation or fissuring of the medial coronoid process (FMCP) and occurrence of pathological changes in the subchondral bone and at the periphery of the articular cartilage play an important role in the diagnostic approach of the disease [2,9,10]. Clinical manifestations in dog may be seen at 4-8 months of age and were reported to have occurred earlier (3 months of age) or later (>6 years) ages, as well [2,11-14]. Clinical signs comprise lameness, timid and stiff gait and tendency to keep the affected leg in the abducted position. Articular effusion, crepitus and pain reaction may be observed at the physical examination [8,13]. Disorders in endochondral ossification, abnormalities in subchondral bone and abnormal load distribution due to radioulnar joint non-

**İletişim (Correspondence)**
+90 532 3921994
didaraydin@hotmail.com

conformity rank among the etiological factors apart from the mostly pronounced genetic predilection [3,8,9]. Miscellaneous breeds such as Labrador and Golden Retrievers, German Shepherds, Bernese Mountain Dogs and Rottweilers are predisposed to the disease. Male dogs are affected two times more often than the females [9,14,15]. Radiography, computed tomography (CT), arthrotomy/arthroscopy, scintigraphy and ultrasonography, magnetic resonance imaging (MRI) are utilized in the diagnosis of MCD [16,17]. The disease is identified best on mediolateral projection of the flexed elbow joint and radiographic view of the elbow joint extended in mediolateral position with the antebrachium pronated at 15 degrees. Nonetheless, computed tomography which is known to be more sensitive than radiography is of great diagnostic significance at earlier ages (14 months of age) [18,19].

Treatment for MCD may consist of different surgical techniques. The set of alternatives can be listed as follows: Surgical removal of the fragment by arthrotomy or arthroscopy, subtotal coronoidectomy, articular cartilage debridement, distal ulnar ostectomy, proximal ulnar osteotomy, bi-oblique dynamic proximal ulnar osteotomy and the releasing procedures of the biceps/brachialis muscle [12,20-23].

This retrospective study has been designed to evaluate and compare the therapeutic approaches in the dogs diagnosed with MCD between the years 2007 and 2017 in an attempt to establish an applied guidance for the veterinary surgeons.

## MATERIAL and METHODS

The study material consisted of 20 dogs of different breeds, ages and genders which were referred to the clinic of the Department of Surgery, Faculty of Veterinary Medicine, Istanbul University between 2007-2017 with the complaint of forelimb lameness. After having received a complete medical history, the dogs were subjected to a throughout orthopedic examination followed by radiographic imaging of both elbow joints positioned mediolaterally in complete flexion and extended craniocaudally with pronation of the antebrachium at 15 degrees. After radiographic examination was performed in all patients, of 4 suspected cases *(Fig. 1)* with MCD diagnosis of 2 (case numbers 19 and 20) were based computed tomographic findings *(Fig. 2)* and other 2 (case numbers 4 and 5) were diagnosed according to arthroscopic findings *(Fig. 3)*.

For arthroscopic examination equipment consisted of a 2.7 mm diameter, 175 mm lenghth 0° angle Rema arthroscope and accessories, 250 Watt Rema HLS-M-250 halogen cold light source, 170 cm fiberoptic cable, Rema CCD 950 camera system, 15" 105S Phillips digital colored monitor and Sony UP-230P/2 video printer were used. Dogs were positioned in lateral recumbency. The irrigation of the joint was maintained using saline solution (0.9% NaCl). At the beginning, in order to distend the joint, saline was injected inside the joint by using a 18 G cannula in a craniodistal and slight lateral direction, beginning just proximal or adjacent to the anconeus. The joint was filled until moderate pressure was felt by digital touching of the distended joint capsule. The arthroscope cannula was established caudally and distally to the medial epicondyle of the humerus. The thumb was placed on the medial epicondyle and drawn down distally and caudally until the approximate level of the joint was reached. A short incision through the skin and superficial soft tissues was made with a small blade (no. 11). The articular capsule was penetrated with the arthroscope cannula fitted with a

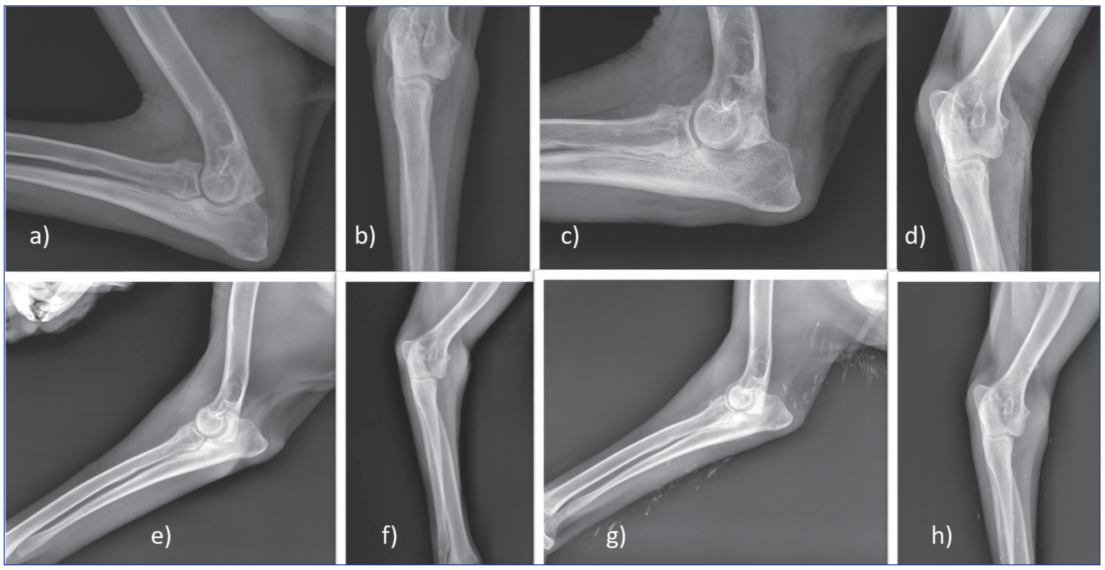



**Fig 1.** One of suspected cases **(**Case number 19): pre-operative (a, b) and immediately post-operative (c, d), postoperatively 30th day (e, f) and 60th day (g, h) later radiographic view of right humeroradial joint. **a, c, e, g-** mediolateral and **b, d, f, h-** craniocaudal obliqe positions

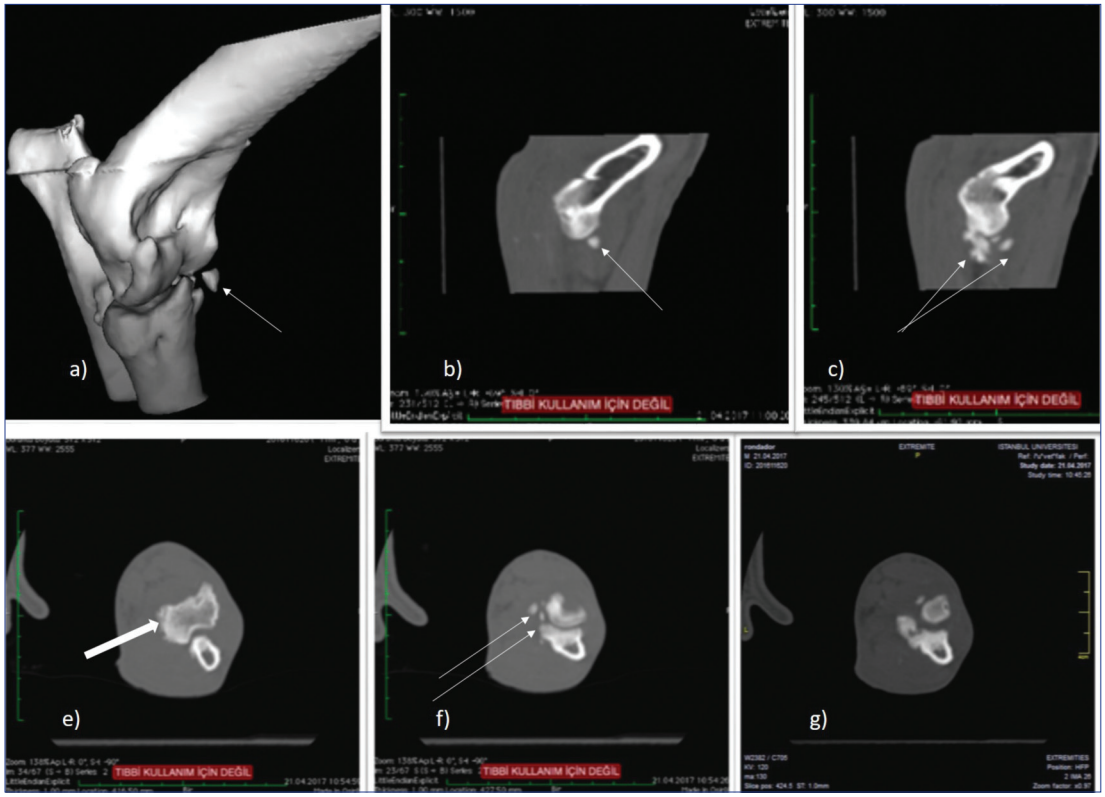



**Fig 2.** Transverse computed tomography (CT) images 1.0 mm thick were obtained and reconstructed into 3D image and sagittal view of right humeroradial joint of case number 19. **a, b, c, f, g-** *thin arrows:* abnormal shape of the medial coronoid process (MCP) with a distinct fragments, **d-** *thick arrow:* degenerative changes in distal humeral condyle

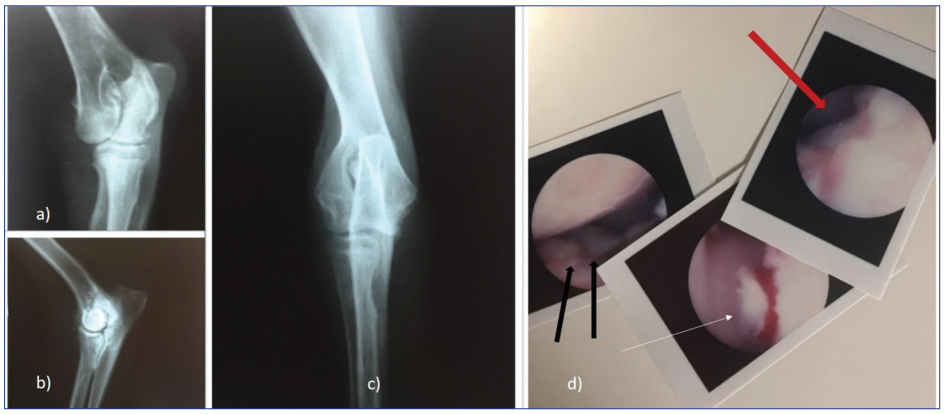



**Fig 3.** One of suspected cases (case number 5) and preoperative a- oblique, b- mediolateral, c- craniocaudal radiographic, arthroscopic view of the right humeroradial joint. *black arrows:* fragmented bones in the joint cavity, *white arrow:* distinct nondisplaced fragment of the coronoid process, *red arrow:* irregularity of medial coronoid process

sharp obturator. Sequentially, the cannula was substituted by the arthroscope. After the visualization of the joint that seen nondisplaced fragments, observative arthrotomy were ended.

In CT study, general anaesthesia was achieved with slow IV injection of propofol (Propofol 1% Fresenius® 200 mg/20 mL-Sweden-10 mg/kg) and dogs were positioned in dorsal recumbency on the CT scanning table with the elbow joint extended approximately 135°. CT images of elbow joint was obtained with a single slice scanner Shimadzu, SCT7800 TC, Japan using 120 KV, 130 mA parameters. Transverse images 1.0 mm thick were obtained and reconstructed into sagittal and 3D images.

All preoperative laboratory data (total blood counts and biochemical parameters) were evaluated prior to the surgical procedure. Anesthesia was induced by Ketamine HCl (5 mg/kg, IV, Ketamine, Eczacıbaşı®, Turkey) followed by Xylazine HCl (2 mg/kg, IM, Rompun, Bayer®, Germany) premedication and then maintained by Isoflurane (Forane, 100ml, Abbott, Switzerland) at an initial concentration of 4% followed by 2% via endotracheal intubation. The subjects were tilted on the operation desk enabling the affected leg to be seated underneath. Strictly following the principles for asepsis and antisepsis, a skin incision was made with a slight curve, starting from the medial condylus of the humerus and proceeding on the middle

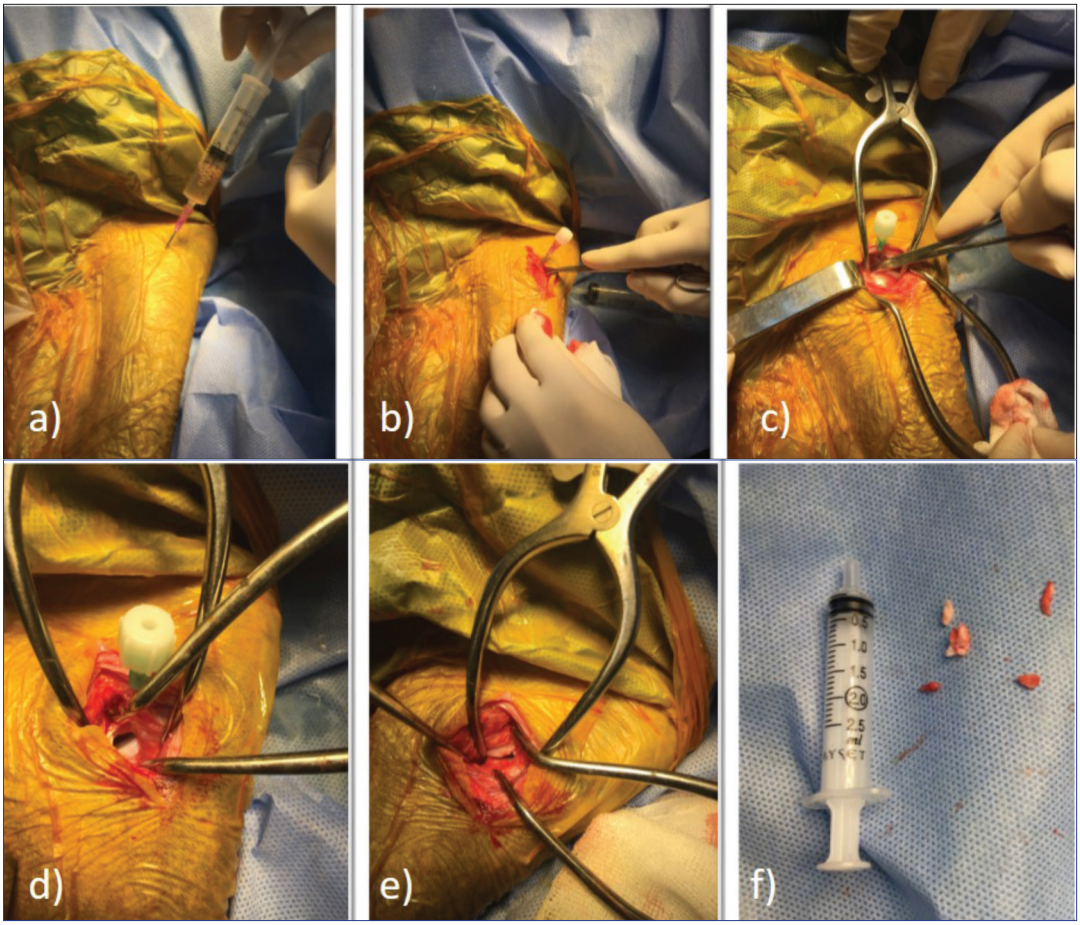



**Fig 4.** Intraoperative views of case number 19

plane of the joint. Antebrachial fascia was dissected and pronator teres and flexor carpi radialis muscles were separated by blunt dissection. Gelpi retractors were used not to damage the arteries and the nerves located on the medial region. Then the articular space was distinguished by the aid of a 20 G cannula and 5-10 mL of physiological saline was injected enabling the distention of the articular capsule. A horizontal incision was made on the articular capsule and Gelpi retractors were placed beneath. Then the fragment was released and removed from the joint. Articular space was rinsed with abundant physiological saline before the articular capsule and the skin incisions were closed in accordance with the routine surgical principles *(Fig. 4)*. Postoperatively, the patients received 20-40 mg/kg of Ceftriaxone (Forsef, IM, 1000 mg/mL Bilim İlaç, Turkey) daily for a week and were strictly prescribed exercise restriction for the following 4 weeks.

As for the patients with bilateral FMCP, the second limb was operated after the achievement of complete functional recovery of the first limb. The ununited process was removed at the same session in the patients with FMCP accompanied by UAP.

Pre- and postoperative scores of all patients were comparatively evaluated *(Fig. 1)*. In the patients with bilateral lesions, the following 30$^{th}$ day of the first arthrotomy was determined as the baseline. Functional recovery periods were recorded according to face to face meetings or on-phone dialoging with the owners. Lameness scores were based on a 5-point scale: 0 = no detectable lameness; 1 = mild weightbearing lameness; 2 = moderate weight-bearing lameness; 3 = marked weight-bearing lameness; and 4 = nonweight-bearing lameness [24]. Clinical and radiographic *(Fig 1. e, f, g, h)* evaluations were carried out on days, 30 and 60 and all data collected were assessed.

## RESULTS

The demographic features like breed, body weight and gender of the dogs, diagnostic methods, the localization of the lesions, and presence of an accompanying lesion and time span of lameness were recapitulated in *Table 1*.

Reluctance to move and lameness were noted in the anamnesis received from the patients' owners. In cases of unilateral involvement, owners reported intermittent lameness, and in cases of bilateral MCD patients manifested a progressive lameness with a sudden onset. Severe pain reaction was detected when the elbow joint was positioned in flexion or extension during the clinical

**Table 1.** *Summary of the case details included in the study*

| Dog | Breed | Sex | Age (month) | Weight (kg) | Duration of Lameness | LS | Co-existance of UAP | Pre-op LS | Post-op LS | FHT | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | GSD | ♂ | 9 | 28 | 45 | L | - | 2 | 0 | 20 | G |
| 2 | Ptb | ♀ | 8 | 35 | 30 | R | - | 3 | 0 | 14 | VG |
| 3 | BM | ♀ | 8 | 43 | 15 | B | + | 2 | 1 | 60 | MOD |
| 4 | GSD | ♂ | 8 | 29 | 20 | L | - | 3 | 1 | 45 | MOD |
| 5 | ASD | ♂ | 8 | 40 | 30 | R | - | 2 | 0 | 18 | VG |
| 6 | GSD | ♂ | 8 | 27 | 20 | B | - | 2 | 0 | 16 | VG |
| 7 | Lab | ♂ | 9 | 28 | 30 | L | + | 3 | 1 | 35 | MOD |
| 8 | Lab | ♂ | 9 | 29 | 30 | R | - | 3 | 0 | 14 | VG |
| 9 | BM | ♂ | 8 | 55 | 20 | R | - | 2 | 0 | 18 | G |
| 10 | GR | ♀ | 9 | 28 | 40 | L | + | 2 | 0 | 35 | MOD |
| 11 | GR | ♀ | 36 | 39 | 30 | B | - | 2 | 0 | 21 | G |
| 12 | GR | ♂ | 14 | 29 | 15 | L | - | 3 | 0 | 14 | VG |
| 13 | Lab | ♀ | 10 | 32 | 30 | L | - | 3 | 0 | 30 | MOD |
| 14 | GSD | ♂ | 10 | 34 | 60 | B | - | 3 | 1 | 45 | MOD |
| 15 | ASD | ♂ | 12 | 43 | 90 | R | - | 2 | 1 | 40 | MOD |
| 16 | Aust SD | ♂ | 7 | 22 | 45 | R | - | 3 | 0 | 40 | G |
| 17 | BM | ♂ | 7 | 60 | 60 | B | + | 4 | 1 | 45 | MOD |
| 18 | DA | ♂ | 12 | 45 | 45 | R | - | 4 | 1 | 42 | MOD |
| 19 | GSD | ♀ | 8 | 30 | 20 | R | - | 3 | 0 | 25 | VG |
| 20 | Ptb | ♂ | 9 | 38 | 30 | B | - | 4 | 1 | 35 | G |

*GSD: German Shepherd Dog, Ptb: Pitbull, BM: Bernese Mountain, ASD: Anatolian Sheep Dog, Lab: Labrador, GR: Golden Retriever, Aust SD: Austrian Sheep Dog;*
*Pre-op LS: Preoperatively lameness score, Post-op LS: Postoperatively lameness score, FHT: Functional healing time;*
*L: left, R: right, B: bilateral;*
*VG: Very good, G: Good, MOD: Moderate*

examination. On physical examination, mild/moderate muscular atrophy was noted particularly in dogs with unilateral lesion. Lameness was recorded to have lasted for an average of 35 days before the referral of the dogs to the clinic. All patients were admitted to the Surgery Clinic with unilateral forelimb lameness however; clinical and radiographic examinations revealed bilateral involvement in 6 cases (case numbers: 3, 6, 11, 14, 17 and 20).

The diagnosis was made based on radiographic findings in 16 patients, on arthroscopic findings in 2 (case numbers 4 and 5) and on computed tomographic findings in other 2 (case numbers 19 and 20) patients.

*Radiographic Findings:* Radiography revealed *(Fig. 5, Fig. 6)* subtrochlear notch sclerosis *(Fig. 6b)*; osteophyte formations on the medial surfaces of epicondyles *(Fig. 5a, thin white arrow)* radial head and anconeus *(Fig. 4b,c)* and structural deformities on the medial coronoid process in most of the cases. Coronoid fracture was apparent in 16 (6 were bilaterally involved) out of 20 cases. Furthermore, UAP *(Fig. 6)* was diagnosed concurrently with FMCP in 4 cases (case numbers: 3, 7, 10, 17). Humeroulnar joint incongruence was detected in none of the cases.

*Arthroscopic Findings:* Arthroscopy revealed non displaced fragments and irregular fibrocartilagenous tissue in case number 4 and 5 *(Fig. 3d)*.

*Computed Tomographic Findings:* Computed tomography showed heterogenous aspect of medial coronoid process with distinct rounded fragments in case number 19 *(Fig. 2)* and 20.

Average duration of preoperative lameness was 35 days whereas functional recovery period was recorded to be 31 days. Postoperative lameness scores on day 30 revealed 0 = no detectable lameness and 1 = mild weightbearing lameness in 12 and 8 cases, respectively. When the preoperative lameness and weight-bearing of extremities were compared with those of postoperative day 60; 6, 5 and 9 cases revealed excellent, good and moderate recovery, respectively.

## DISCUSSION

Medial coronoid disease, is seen in middle or large breed dogs, especially younger individuals of these breeds. Clinical manifestations of Medial coronoid disease in dogs

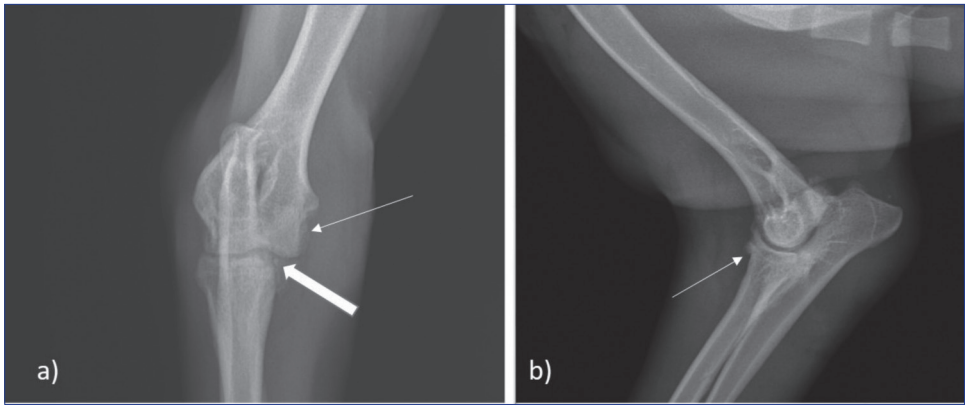
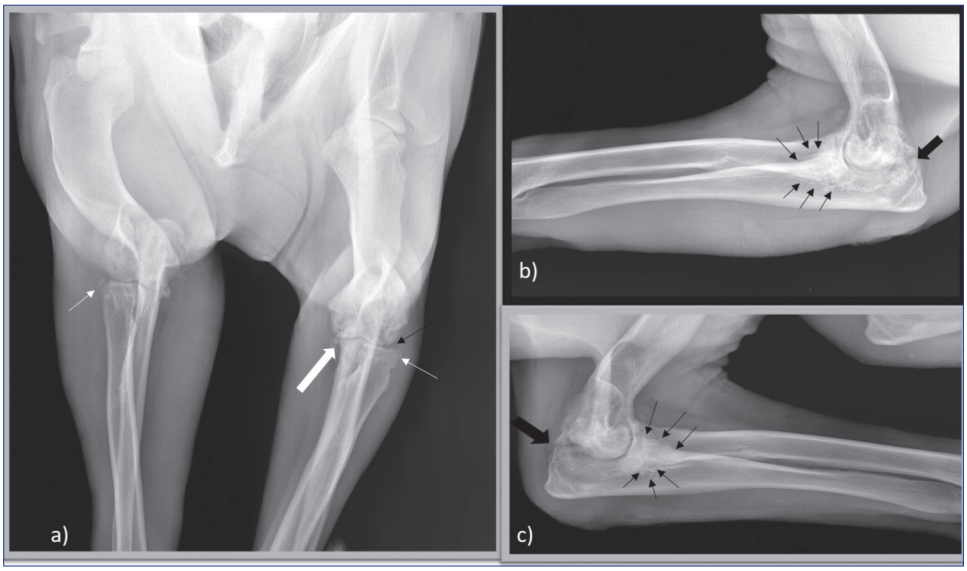



**Fig 5.** Radiographic image of right humeroradial joint of case number 8. a- craniocaudal view, *thick white arrow:* apparent fragmented coronoid process, *thin white arrow:* osteophyte formation on medial epicondylus; b- mediolateral view, *white arrows:* osteophytic proliferations on radial had and anconeus)

**Fig 6.** Radiographic views of case number 17. a- bilateral, *thin white arrows:* sesamoid bones on radial head, *thick white arrow:* distinct fragmentation of medial coronoid process, *black arrow:* osteophyte formation on lateral epicondylus; b- mediolateral right, *thin black arrows:* subtrochlear sclerosis, *thick black arrow:* UAP and ostophyte formation; c- mediolateral left, *thin white arrows:* subtrochlear sclerosis, *black arrow:* UAP and osteophyte formation on left humeroradial joint




most commonly occur at 4-8 months of age and in some occasions the disease was reported to have developed in adult animals (>6 years) [14]. Gender predilection was noted in males as 2 folds of the female individuals [3,9]. In this study, it is seen that all of the cases consisted of large breed dogs. Despite the lack of a study with respect to the prevalence of the disease in Anatolian Sheep Dogs, that the 2 out of 20 patients were of this breed was found to be noteworthy. When the age is evaluated in terms of age, two dogs were older (case number 11: 36 months old; case number 12: 4 months old) than the reported average age range which indicated that clinical signs might have occurred at later periods of life [14]. It was also determined that the incidence of MCD is higher in male dogs than in female dogs. Both breed type and age range at the onset of the disease were found to be consistent with the sources predominantly formed in male dogs [3,9,14].

In dogs with medial coronoid disease, variable lameness are marked by muscular atrophy due to degenerative lesions in the joint, as well as stiff and stilted gait. Reduced range of motion on hyperextension and hyperflexion of the radioulnar joint accompanied by pain [1]. Clinical examination findings of the cases showed that the abnormal gait, muscle atrophy, decreased range of motion of the elbow joint were compatible with the other reports.

Periarticular osteophytosis was detected in all cases based on radiographic findings. Besides, medial coronoid process was morphologically detected however; percentage values of subtrochlear sclerosis and milimetric calculations of osteophyte depth [23,25] were unable to be assessed. Therefore, no correlation could be established between the preoperative subjective radiographic findings and functional recovery period or the postoperative outcome. Furthermore, no correlation was found between the duration of preoperative lameness and functional recovery period or postoperative outcome according to the postoperative patient owner's report.

Despite the surgical treatment of joint diseases, subsequent radiographic evaluation revealed that osteoarthritis was a progressive process and as it was already known that radiography alone was an inconclusive diagnostic tool in detecting the early pathologic changes of the joint, computed tomography and arthroscopy, in contrast, were most likely to have yielded more accurate results [10]. In this regard, however; relatively short-term monitoring of the

patients and the small number of the cases included were considered to be the limiting factors of the current study so as to judge the accuracy of these techniques.

Computed tomography is considered to be a more reliable diagnostic tool than the other imaging techniques. Standard transversal sequences obtained with computed tomography enable the coronoid process to be viewed without the negative impact of the images of superimposed adjacent bone tissue rendering this diagnostic tool a high sensitivity and specificity in the diagnosis of fragmented coronoid process [18,26]. Recently, the use of computed tomography has become increasingly widespread in veterinary practice and recent studies have shown that MCD might be detected as early as 14 weeks of age. In order to obtain the most effective results in the therapeutic approach, the lesions should be detected before severe osteoarthritis developed [18]. Therefore, we are in agreement with the argument that early orthopedic examination should be planned concurrently with the vaccination program being scheduled.

In the study, UAP was detected in 4 out of 20 cases on the same joint. Concurrent occurrence of FCP and UAP was reported to be a sporadic finding in the previous studies [7,19,27]. That UAP was detected in 4 cases in this study was considered to have pointed out the frequency of this entity unlike the reported studies [27]. Nevertheless, it was considered that concurrent occurrence of FCP and UAP might have demonstrated a negative impact on the functional recovery period and the outcome of the disease on the basis of the results obtained. Although UAP was reported to have most commonly encountered in German Shepherd dogs [27,28], that none of the cases with UAP in this study were of this breed was an intriguing finding.

The treatment of Medial Coronoid Disease includes conservative therapy or the surgical removal of the fragment by arthroscopy/arthrotomy. Certain authors suggested the superiority of arthroscopy in terms of the speed of the healing process and improved visualization over arthrotomy [10,29,30]. On the other hand, it was reported in recent studies with respect to objective evaluations by gait analysis (inverse dynamics and peak vertical forces, vertical impulse and goniometry) that arthroscopy revealed no superiority over medical therapy or arthrotomy [31]. In this study, removal of the fragmented or fissured portion and the debridement of the remnants from medial coronoid process were unable to be performed by arthroscopy due to the inadequacy of the technical equipment and the use of the technique was limited only to the full recognition of the lesion in 2 cases. Furthermore, the arthrotomy procedure was performed in almost the same time with minimal arthroscopy.

Recent studies showed that local abnormal loading forces resulted in the pathologic lesions of medial coronoid process. Different methods of ulnar osteotomy were suggested for the treatment [20-23]. Humeroulnar joint nonconformity was detected in none of the cases, therefore proximal ulnar osteotomy or bioblique dynamic ulnar osteotomy was not considered along with the removal of the fragmented portion.

Although periarticular degenerative changes developed in all cases, the lameness score on day 30 was reported to be 0 in 12 cases, which was considered to be associated with the relief of pain in the joint after the removal of the intraarticular fragments. The score of 1 in 8 cases in the early postoperative period was considered to have resulted from the severity of the articular damage.

In conclusion, clinical improvement was achieved in the dogs with the lesions due to medial coronoid disease by the aid of a proper therapeutic approach.

## REFERENCES

**1. Boulay JP:** Fragmented medial coronoid process of the ulna in the dog. *Vet Clin North Am: Small Anim Pract,* 28 (1): 51-74, 1998. DOI: 10.1016/S0195-5616(98)50004-6

**2. Fitzpatrick N, Smith TJ, Evans RB, Yeadon R:** Radiographic and arthroscopic findings in the elbow joints of 263 dogs with medial coronoid disease. *Vet Surg,* 38, 213-223, 2009. DOI: 10.1111/j.1532-950X.2008.00489.x

**3. Morgan JP, Wind A, Davidson AP:** Hereditary bone and joint diseases in the dog: Osteochondroses, hip dysplasia, elbow dysplasia. 77-78, Hannover: Schlütersche GmbH&Co. KG, 2000.

**4. Temwichitr J, Leegwater PAJ, Hazewinkel HAW:** Fragmented coronoid process in the dog: A heritable disease. *Vet J,* 185 (2): 123-129. 2010. DOI: 10.1016/j.tvjl.2009.06.022

**5. Wavreille V, Fitzpatrick N, Drost WT, Russell D, Allen MJ:** Correlation between histopathologic, arthroscopic, and magnetic resonance imaging findings in dogs with medial coronoid disease. *Vet Surg,* 44 (4): 501-510, 2015. DOI: 10.1111/j.1532-950X.2014.12233.x

**6. Gemill TJ, Clements DN:** Fragmented coronoid process in the dog: Is there a role for incongruency? *J Small Anim Pract,* 48, 361-368, 2007. DOI: 10.1111/j.1748-5827.2007.00320.x

**7. Grøndalen J, Grøndalen T:** Arthrosis in the elbow joint of young rapidly growing dogs. V. A pathoanatomical investigation. *Nord Vet Med,* 33 (1): 1-16, 1981.

**8. Lau SF:** Development of medial coronoid disease in Labrador retrievers: Diagnostic and pathologic studies. *PhD Thesis,* Utrecht University, Netherlands, 2013.

**9. Griffon DJ:** Surgical diseases of the Elbow. **In,** Tobias MK, Johnston AS (Eds): Veterinary Surgery Small Animal. 736-749, Saunders, Missouri, 2012.

**10. Coppieters E, Seghers H, Verhoeven G, Gielen I, Samoy Y, De Bakker E, Van Ryssen B:** Arthroscopic, computed tomography, and radiographic findings in 25 dogs with lameness after arthroscopic treatment of medial coronoid disease. *Vet Surg,* 45, 246-253, 2016. DOI: 10.1111/vsu.12443

**11. Hazewinkel HAW, Meij BP, Theyse LFH:** Surgical treatment of elbow dysplasia. *Vet Q,* 20 (Suppl.1): 29-31, 1998.

**12. Michelsen J:** Canine elbow dysplasia: Aetiopathogenesis and current treatment recommendations. *Vet J,* 196 (1): 12-19, 2013. DOI: 10.1016/j.tvjl.2012.11.009

**13. Olsson SE:** The early diagnosis of fragmented coronoid process and osteochondritis dissecans of the canine elbow joint. *J Am Anim Hosp Assoc,* 19, 616-626, 1983.

**14. Vermote KAG, Bergenhuyzen ALR, Gielen I, van Bree H, Duchateau L, Van Ryssen B:** Elbow lameness in dogs of six years and older: Arthroscopic and imaging findings of medial coronoid disease in

51 dogs. *Vet Comp Orthop Traumatol,* 23 (1): 43-50, 2010. DOI: 10.3415/VCOT-09-03-0032

**15. Narojek T, Fiszdon K, Hanyzs E:** Canine elbow dysplasia in diffferent breeds. *Bull Vet Inst Pulowy,* 52, 169-173, 2008.

**16. Cook CR, Cook JL:** Diagnostic imaging of canine elbow dysplasia: A review. *Vet Surg,* 34 (2): 144-153, 2009. DOI: 10.1111/j.1532-950X.2008.00481.x

**17. Franklin SP, Burke EE, Holmes SP:** Utility of MRI for characterizing articular cartilage pathology in dogs with medial coronoid process disease. *Front Vet Sci,* 4 (25): 1-9. 2017. DOI: 10.3389/fvets.2017.00025

**18. Lau SF, Wolschrijn CF, Hazewinkel HAW, Siebelt M, Voorhout G:** The early development of medial coronoid disease in growing Labrador retrievers: Radiographic, computed tomographic, necropsy and micro-computed tomographic findings. *Vet J,* 197, 724-730, 2013. DOI: 10.1016/j.tvjl.2013.04.002

**19. Carpenter LG, Schwarz PD, Lowry JE, Park RD, Steyn PF:** Comparison of radiologic imaging techniques for diagnosis of fragmented medial coronoid process of the cubital joint in dogs. *J Am Vet Med Assoc,* 203 (1): 78-83, 1993.

**20. Caron A, Fitzpatrick N:** Bi-Oblique dynamic proximal ulnar osteotomy: Surgical technique and clinical outcome in 86 dogs. *Vet Surg,* 45 (3): 356-363, 2016. DOI: 10.1111/vsu.12464

**21. Ness MG:** Treatment of fragmented coronoid process in young dogs by proximal ulnar osteotomy. *J Small Anim Pract,* 39, 15-18, 1998. DOI: 10.1111/j.1748-5827.1998.tb03663.x

**22. Puccio M, Marino DJ, Stefanacci JD, McKenna B:** Clinical evaluation and long term follow-up of dogs having coronoidectomy for elbow incongruity. *J Am Anim Hosp Assoc,* 39 (5): 473-478, 2003. DOI: 10.5326/0390473

**23. Fitzpatrick N, Caron A, Solano MA:** Bi-oblique dynamic proximal ulnar osteotomy in dogs: Reconstructed computed tomographic assessment of radioulnar congruence over 12 weeks. *Vet Surg,* 42 (6): 727-738, 2013. DOI: 10.1111/j.1532-950X.2013.12014.x

**24. Cogar SM, Cook CR, Curry, SL, Grandis A, Cook JL:** Prospective evaluation of techniques for differentiating shoulder pathology as a source of forelimb lameness in medium and large breed dogs. *Vet Surg,* 37 (2): 132-141, 2008. DOI: 10.1111/j.1532-950X.2007.00364.x

**25. Hazewinkel HAW:** Elbow dysplasia: Introduction, clinical investigation and force plate evaluation. *International Elbow Working Group Annual Meeting WSAVA Congress,* Bangkok Thailand. 2015. http://www.vet-iewg.org/wp-content/uploads/2017/02/proceedings2015.pdf, *Accessed:* 10.10.2017.

**26. Villamonte-Cevalier A, Van Bree H, Broeckx BJG, Dingemanse W, Soler M, Van Ryssen B, Gielen I:** Assesment of medial coronoid disease in 180 canine lame elbow joints: A sensitivity and specificity comparison of radiographic, computed tomographic and arthroscopic findings. *BMC Vet Res*, 11, 243, 2015. DOI: 10.1186/s12917-015-0556-9

**27. Meyer-Lindenberg A, Staszyk C, Gasse H, Fehr M, Nolte I:** Caudomedial approach for removal of an ununited anconeal process and assessment of the medial coronoid process of the ulna. *J Vet Med A Physiol Pathol Clin Med*, 49 (5): 277-280, 2002. DOI: 10.1046/j.1439-0442.2002.00421.x

**28. Meyer-Lindenberg A, Fehr M, Nolte I:** Co-existence of ununited anconeal process and fragmented medial coronoid process of the ulna in the dog. *J Small Anim Pract,* 47 (2): 61-65, 2006. DOI: 10.1111/j.1748-5827.2006.00051.x

**29. Meyer-Lindenberg A, Langhann A, Fehr M, Nolte I:** Arthrotomy versus arthroscopy in the treatment of the fragmented medial coronoid process of the ulna (FCP) in 421 dogs. *Vet Comp Orthop Traumatol,* 16 (4): 204-210, 2003. DOI: 10.1055/s-0038-1632780

**30. Evans RB, Gordon-Evans WJ, Conzemius MG:** Comparison of three methods for the management of fragmented medial coronoid process in the dog. A systematic review and meta-analysis. *Vet Comp Orthop Traumatol,* 21, 106-109, 2008.

**31. Galindo-Zamora V, Dziallas P, Wolf DC, Kramer S, Abdelhadi J, Lucas K, Nolte I, Wefstaedt P:** Evaluation of thoracic limb loads, elbow movement, and morphology in dogs before and after arthroscopic management of unilateral medial coronoid process disease. *Vet Surg,* 43 (7): 819-828, 2014. DOI: 10.1111/j.1532-950X.2014.12250.x

Kafkas Univ Vet Fak Derg
24 (5): 717-723, 2018
DOI: 10.9775/kvfd.2018.19776

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Development and Evaluation of an ELISA System for Detection of PEDV-Specific IgA Antibodies in Colostrum

Ren-Feng LI [1] Xiang-Qin TIAN [2] Yan YU [1] Jin-Qing JIANG [1] San-Hu WANG [1]
Zi-Liang WANG [1] Jin-You MA [1] Li-Rong WANG [1] Shou-Ping ZHANG [1] Yan-Yan YANG [3]

[1] College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, CHINA
[2] Medical Research Center, Xinxiang Medical College, Xinxiang 453003, CHINA
[3] Key Laboratory of Animal Immunology of the Ministry of Agriculture, Henan Provincial Key Laboratory of Animal Immunology, Henan Academy of Agricultural Sciences, Zhengzhou, 450002, CHINA

**Article Code: KVFD-2018-19776 Received: 22.03.2018 Accepted: 05.07.2018 Published Online: 06.07.2018**

**How to Cite This Article**

**Li RF, Tian XQ, Jiang JQ, Wang SH, Wang ZL, Ma JY, Wang LR, Zhang SP, Yang YY:** Development and evaluation of an ELISA system for detection of PEDV-specific IgA antibodies in colostrum. *Kafkas Univ Vet Fak Derg,* 24 (5): 717-723, 2018. DOI: 10.9775/kvfd.2018.19776

## Abstract

Porcine epidemic diarrhea virus (PEDV) is a devastating swine infectious disease. Development of high-performance methods to diagnose and evaluate viral immune status remains very important to control PEDV. Here, a recombinant S1 protein-based indirect enzyme-linked immunosorbent assay (rpS1-iELISA) was developed to monitor IgA antibody in the colostrum. Optimized cut-off value of the rpS1-iELISA was determined as 0.448. Results yielded a sensitivity of 96.87% and a specificity of 100.0%. Repeatability tests indicated that the coefficients of variation of the colostrum samples within and between runs were both less than 8%. Test results of 523 field colostrum samples showed that the rpS1-iELISA had excellent agreement with immunofluorescence assay (*kappa* = 0.958) and better test performance than a commercial ELISA kit. This test will aid in future diagnostics and assessment of the protective levels of mucosal immune response against PEDV by measuring IgA levels in the colostrum.

***Keywords:*** *ELISA, IgA antibody, Colostrum, Porcine epidemic diarrhea virus, Spike protein*

# Kolostrumda Domuz Epidemik Diare Virus-Spesifik IgA Antikorlarının Belirlenmesinde ELISA Sisteminin Geliştirilmesi ve Değerlendirilmesi

## Öz

Domuz Epidemik Diare Virus (DEDV) enfeksiyonu domuzların oldukça yıkıcı enfeksiyöz bir hastalığıdır. Virusa karşı bağışıklık durumunu değerlendirmek ve tanıyı koyabilmek için yüksek performanslı metotların gelişimi hastalığın kontrol altına alınabilmesi için elzemdir. Bu çalışmada, kolostrumdaki IgA antikorlarını taramak amacıyla rekombinant S1 protein temelli indirekt enzime bağlı immunosorbent metot (rpS1-iELISA) geliştirildi. rpS1-iELISA için optimize edilmiş eşik değeri 0.448 olarak belirlendi. Sonuçlar %96.87 duyarlılık ve %100 özgüllük gösterdi. Tekrarlanabilirlik testleri kolostrum örnekleri ve koşumlar arasında varyasyon katsayısının %8'den daha az olduğunu gösterdi. Toplam 523 adet saha kolostrum örneklerinin test sonuçları rpS1-iELISA'nın immunofloresans testi (*kappa* = 0.958) ile mükemmel uyumlulukta olduğunu ve ticari ELISA kitinden daha iyi performansa sahip olduğunu gösterdi. Mevcut test kolostrumda IgA seviyesini ölçerek DEDV'e karşı mukozal bağışıklık yanıtın koruyuculuk seviyesini tahmin etmede ve tanıyı koymada yardımcı olacaktır.

***Anahtar sözcükler:*** *ELISA, IgA antikoru, Kolostrum, Domuz Epidemik Diare Virus, Spike proteini*

## INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is a member of the coronavirus family which causes vomiting, watery diarrhea, and dehydration in pigs and high mortality in neonatal piglets [1]. The first outbreak of PEDV was recognized in England in 1971 [2]. Since then, especially from 2010, PEDV outbreaks have been documented in many countries and severely affected the swine industry [3-5]. PEDV circulating in the world can be separated into three groups (G1, G2, G3), which have three subgroups (G1-1, G1-2, G1-3) [6]. Recent reports on PEDV recombinant strains in Italy

**İletişim (Correspondence)**
+86 373 3040 718
lirenfeng@sina.com

and China have indicated the new evolution strategy of PEDV [7,8], which increases our insight into PEDV genetic variation and bring new challenge for PEDV control.

After an acute outbreak, PEDV can persist longer in affected herds and cause an enzootic infection [9]. Although several commercial vaccines have been used to control large outbreaks, PEDV remains one of the most important causes of economic loss in many pig farms in China [10]. In addition to effective vaccines, high-throughput and reliable methods to diagnose and monitor the immune levels of PEDV vaccine in herds are urgently needed for PEDV surveillance and control.

Porcine epidemic diarrhea virus is an enveloped coronavirus containing a positive-stranded RNA genome. This virus is approximately 28 kb in length and has a 5′ cap and a 3′ polyadenylated tail. Four structural proteins, including spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins are encoded by the PEDV genome. The S protein, which is a type I membrane glycoprotein, can induce neutralizing antibodies and interacts with cell receptor in the host. The S protein can be divided into S1 (1-789 aa) and S2 (790-1.383 aa) domains. S1, as an immunodominant region, can induce an active immune response of host and has been an attractive target to develop PEDV vaccine and serologic test methods [9].

Given the special features of the porcine mucosal immune system, the presence of serum antibodies against PEDV is not always correlated with protection. Sow immunity plays an important role in preventing viral infection and disease in newborn piglets, and the IgA level in the colostrum is a better marker of protection from PEDV infection than serum neutralizing (SN) titer from serum samples [4,11]. The current vaccination evaluation systems for PEDV that focus mainly on the immune response at the systemic level and mucosal immunity associated with PEDV is relatively insufficient. Therefore, measuring the localized IgA immune responses is essential to evaluate the protection derived either by vaccines or by previous PEDV exposure [12]. In this study, an IgA ELISA method based on the recombinant partial S1 protein of PEDV isolate (CH/HNQX-3/14), which is a novel strain currently circulating in Henan, China, was developed to detect IgA antibody in the colostrum of PEDV-infected and vaccinated sows.

# MATERIAL and METHODS

### *Ethics Approval*

According to the Animals Use in Research Committee (AURC) of Henan Institute of Science and Technology, this study does not require any special approval.

### *Colostrum Samples*

Thirty-six negative control colostrum specimens used in the test were derived from unvaccinated healthy sows, and 32 positive control colostrum specimens were supplied kindly by Prof. Yanyan Yang (Key Laboratory of Animal Immunology of Agriculture Ministry of China) and retested by Western blot and immunofluorescence assay (IFA). The positive control colostrum for transmissible gastroenteritis virus (TGEV), classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), foot and mouth disease virus (FMDV), and porcine pseudorabies virus (PRV) were obtained from the Henan Center for Animal Disease Prevention and Control. These positive control colostrum were further validated with their corresponding ELISA kits and found to be strong positive and had no cross-reactivity with other swine pathogens.

### *Expression and Purification of Recombinant PEDV S1 Protein*

The region encoding the partial S1 domain (aa 492-796) of PEDV variant CH/HNQX-3/14 (GenBank No. KR095279.1) was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) using a forward primer (5′-GGGGATCC TCTTTTGTTACTTTGCC-3′) and a reverse primer (5′-GC GAATTCAGGCGTGTTGTAAAGC-3′). Reverse transcription was performed according to kit protocol (ThermoFisher Scientific, Shanghai, China). In brief, 1 µL of total RNA (1-2 µg) was added to reaction mixture containing 5×reaction buffer, 10 mM dNTP mix, 1 µL Oligo(dT)$_{18}$ Primer, 20 U RNAse inhibitor and 200 U M-MuLV Reverse Transcriptase to 20 µL of final volume. cDNA was synthesized at 42°C for 1 h. Remaining enzymes were heat-inactivated at 70°C for 5 min. PCR of S1 gene was performed as follows: initial denaturing at 95°C for 5 min; followed by 30 cycles at 95°C for 30 s, 57°C for 40 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The amplified products were purified by a QIAquick Gel Extraction kit (Qiagen China Co., Shanghai, China), and then cloned into pET-30a(+) (Invitrogen) between the *Bam*HI and *Eco*RI restriction sites to generate an expression construct designated as pET30a(+)-pS1.

The recombinant plasmid pET30a(+)-pS1 was transformed into *Escherichia coli* BL$_{21}$ (DE3) competent cells and grown at 37°C in Luria-Bertani broth supplemented with 100 µg/mL kanamycin to an optical density (OD) of 0.6 at 600 nm. The expression products were purified using nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Shanghai, China) following the manufacturer's protocol. The final protein products were quantified by micro BCA protein assay kit (ThermoFisher Scientific) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot hybridization.

### *Development of Indirect ELISA Test with Recombinant S1 Protein*

Colostrum samples were tested for IgA antibodies against PEDV by the recombinant partial S1 protein-based indirect enzyme-linked immunosorbent assay (rpS1-iELISA). Briefly, the optimal coating concentration of recombinant pS1

protein and optimum colostrum dilutions for ELISA were determined using a checkerboard titration. The antigen was diluted in coating buffer (50 mM carbonate buffer, pH 9.6) and incubated overnight at 4°C. The following day, plates were washed thrice with phosphate-buffered saline containing 0.05% Tween 20 (PBST) and then blocked with casein/PBST blocking buffer (ThermoFisher Scientific) for 2 h at 37°C, followed by incubation with colostrum samples diluted in PBS for 30 min at 37°C. After a wash step, an optimal diluted horseradish peroxidase (HRP)-conjugated goat anti-porcine IgA secondary antibody (Bethyl Laboratories) was added and incubated at 37°C for 30 min. The reaction was developed using tetramethylbenzidine (TMB) peroxide (ThermoFisher Scientific) as the substrate and stopped by adding 50 µL of 2 M sulfuric acid. ODs were measured at 450 nm using an ELISA plate reader (BioTek). Positive, negative, and blank (sterile water) samples were tested in triplicate and recorded for statistical analysis.

### ELISA Cut-Off Values and Assay Performance

The threshold cut-off value was evaluated by receiver operator characteristic (ROC) analysis (MedCalc software, Mariakerke, Belgium) using 36 PEDV negative colostrum samples and 32 PEDV positive colostrum samples, with IFA as the reference method to classify all samples. Positive colostrum samples from PEDV (n=10), TGEV (n=5), CSFV (n=9), PRRSV (n=7), FMDV (n=5), PRV (n=5), and 10 PEDV-negative colostrum samples were used to evaluate the diagnostic specificity. Each sample was tested in triplicate, and the mean $OD_{450\ nm}$ value and standard deviation (SD) were calculated.

The rpS1-iELISA reproducibility within and between runs was evaluated as previously proposed [13]. Six colostrum samples (3 IFA-positive and 3 IFA-negative samples) were selected for the reproducibility experiments. For intra-assay reproducibility, three replicates of each colostrum sample were assigned to the same plate. For inter-assay (between-run) reproducibility, three replicates of each sample were run on different plates. The mean $OD_{450\ nm}$ values, SD, and coefficient of variation (CV) were calculated.

### Application of The rpS1-iELISA in The Field

The rpS1-iELISA was applied to test 523 field colostrum samples collected from 16 pig farms in Henan province. The colostrum samples were also evaluated for anti-PEDV IgA antibodies using IFA and a commercial ELISA kit (coELISA, BIONOTE) following the manufacturer's instruction. Cohen's kappa (κ) value was introduced as a measure of agreement between these assays. Additionally, the rpS1-iELISA was used to evaluate the immune efficacy of a commercial PEDV vaccine (WEIKE Biotech Company, Harbin, China) by measuring the IgA antibody in the colostrum from two sow herds with or without vaccination. The vaccinated sows were intramuscularly immunized with PEDV vaccine (2 mL per sow) at 6 and 2 (booster) weeks before parturition. The unvaccinated healthy pregnant sows were selected from a farm with no history of PEDV exposure.

### IFA

IFA was performed as described previously [14]. Vero cells were grown in 24-well plates and inoculated with the cell culture-adapted PEDV strain (CH/HNQX-3/14) at a multiplicity of infection of 0.05 with MEM supplemented with 2.5 µg/mL trypsin (Sigma). PBS-treated cells were used as negative control. After incubation at 37°C for 72 h, the cells were washed thrice with PBST and fixed with 80% acetone for 20 min. Cells were then washed thrice with PBST and blocked with casein/PBST blocking buffer at 37°C for 2 h. After three washes with PBST, the colostrum samples were added and incubated at 37°C for 30 min. Next, fluorescein isothiocyanate-conjugated goat anti-porcine IgA (Thermo Scientific™ Pierce) was added at a dilution of 1:100 with PBST to each well. After 30 min of incubation at 37°C, the plates were rinsed thrice with PBST and examined using fluorescent microscopy (Zeiss, Germany).

### Statistical Analysis

ELISA data were collected at $OD_{450\ nm}$ and expressed as the mean±SD. Statistical analyses were performed by one-way ANOVA followed by Bonferroni multiple comparison *post hoc* test (GraphPad Prism 6.0 software), and *P* value of $<0.05$ was considered significant.

## RESULTS

The cloned pS1 gene of PEDV was expressed as a soluble protein with an N-terminal His-tag. SDS-PAGE results showed that, after the cells were induced with 0.8 mM IPTG (30°C for 8 h), the pS1 gene exhibited higher expression level at the position corresponding to the expected molecular mass of approximately 45 kDa *(Fig. 1)*. Expressed PEDV pS1 protein was purified by Ni-NTA, and the protein bound to Ni-NTA was eluted with 200 mM imidazole. SDS-PAGE and Western blot hybridization further demonstrated that purification removed, to a large extent, other proteins, leaving a predominant band at 45 kDa *(Fig. 1)* and achieving a protein yield of over 10 mg/L of culture, with purity greater than 95%.

The rpS1-iELISA was optimized in a checkerboard fashion to maximize signal-to-noise ratios. The optimal concentration of coating antigen was achieved at a concentration of 1.25 µg/mL recombinant pS1 protein. To further determine the optimum colostrum dilution for the testing platform, a well-characterized PEDV "high" positive control colostrum was serially diluted two-fold against antigen-coated ELISA wells at a fixed concentration. Colostrum dilution of 1/100 was found to provide the highest absorbance value and was selected for subsequent assays. The secondary antibody (HRP-conjugated goat anti-porcine IgA) dilutions of 1/10,000 was selected for this assay.

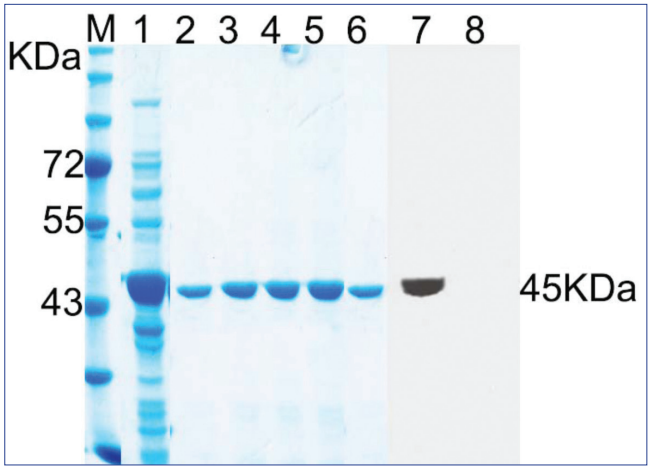



**Fig 1.** Expression and purification analysis of PEDV pS1 protein by SDS-PAGE and Western blot. Lane M: pre-stained protein molecular weight marker (10-180 KDa); Lane 1: SDS-PAGE of unpurified recombination pS1 protein, which was expressed in *Escherichia coli* $BL_{21}$ (DE3) induced by 0.8 mM IPTG for 8 h at 30°C; Lanes 2-6: SDS-PAGE of purified recombination pS1 protein by Ni-NTA; Lane 7: Reaction of pS1 protein with PEDV positive swine sera by Western blot; 8: pET30a(+)/BL21 vector control

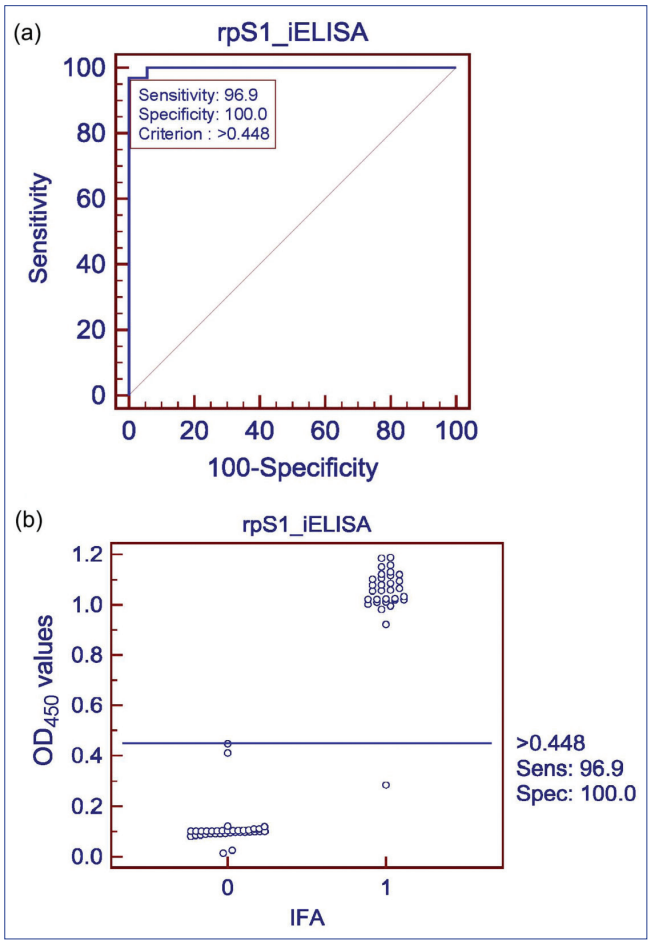



**Fig 2.** Determination of the cut-off value. (a) ROC analysis of the rpS1-iELISA. The area under the ROC curve was 0.998 when the optimal cut-off $OD_{450\ nm}$ value was 0.448. (b) Distribution of the rpS1-iELISA $OD_{450\ nm}$ values according to the classification of the colostrum by IFA results. The horizontal solid line represents the cut-off value

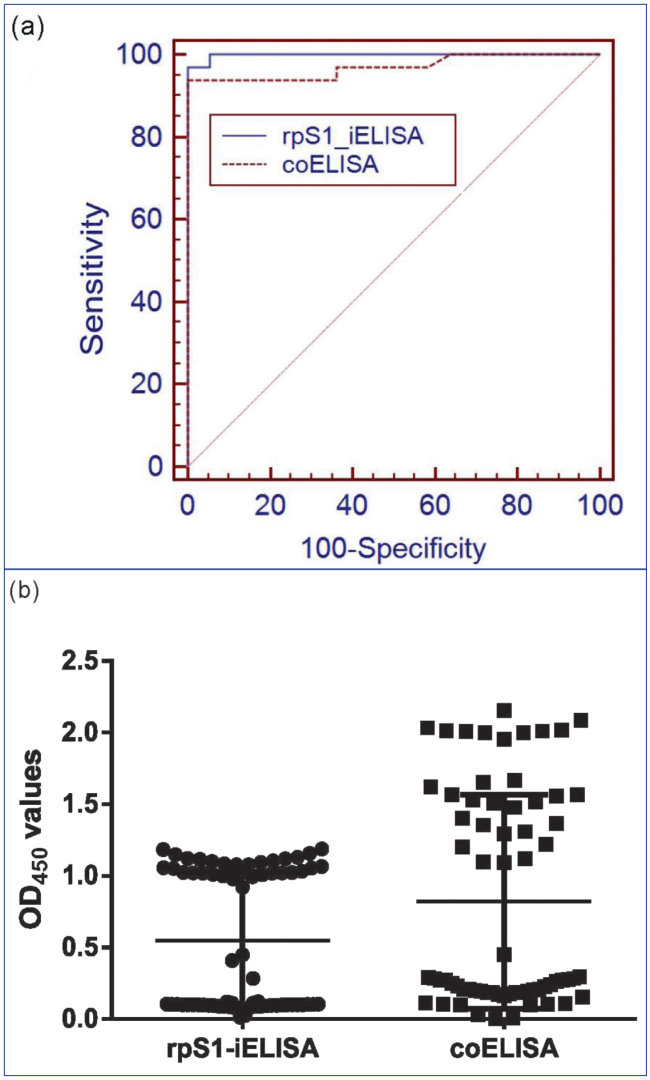



**Fig 3.** Comparison of test performance between rpS1-iELISA and coELISA by ROC analysis using known 32 positive and 36 negative colostrum samples. (a) The area under the ROC curve (AUC) for the rpS1-iELISA and coELISA are 0.998±0.00212 and 0.970±0.00223, respectively; (b) Distribution of the rpS1-iELISA and coELISA $OD_{450\ nm}$ values according to the classification of colostrum by IFA results. The horizontal solid line represents the cut-off value

Based on the ROC curve analysis of the rpS1-iELISA, the $OD_{450\ nm}$ values of the 36 negative colostrum samples varied from a minimum of 0.014 to a maximum of 0.448 and from 0.185 to 1.188 for the 32 positive colostrum samples. The $OD_{450\ nm}$ of 0.448 was selected as the optimal cut-off value, with an area under the curve (AUC) of 0.998±0.00212 and 95% confidence interval (CI) ranging from 0.944 to 1.000, giving sensitivity and specificity of 96.87% (95% CI: 83.8%, 99.9%) and 100.0% (95% CI: 90.3%, 100.0%), respectively *(Fig. 2)*. By contrast, the ROC analysis for the coELISA showed a lower AUC value (0.970±0.00223, 95% CI: 0.896-0.996) and lower sensitivity (93.75%, 95% CI: 79.2%-99.2%) compared with the rpS1-iELISA *(Fig. 3)*.

To evaluate the specificity of the developed rpS1-iELISA, positive colostrum samples for TGEV, CSFV, PRRSV, FMDV,

***Table 1.*** *Assessment of intra-assay and inter-assay repeatability of the rpS1-iELISA using 3 positive- and 3 negative colostrum samples*

| Colostrum Sample | Intra-assay Variability | | Inter-assay Variability | |
|---|---|---|---|---|
| | X±SD | CV | X±SD | CV |
| 1 | 1.043±0.045 | 4.30% | 1.031±0.037 | 3.80% |
| 2 | 1.036±0.033 | 3.20% | 1.101±0.029 | 3.61% |
| 3 | 1.048±0.036 | 4.40% | 1.054±0.056 | 4.38% |
| 4 | 0.087±0.014 | 3.46% | 0.093±0.009 | 3.11% |
| 5 | 0.081±0.005 | 4.72% | 0.090±0.011 | 6.02% |
| 6 | 0.091±0.008 | 7.94% | 0.089±0.007 | 4.32% |

*Note: Data are the mean±standard deviation of three replications; CV, coefficient of variation; 1, 2 and 3 indicate positive samples; 4, 5 and 6 indicate negative samples*

***Table 2.*** *Evaluation of statistical agreement among three tests. Kappa values shown represent a statistical measure of test agreement and were calculated using MedCalc Software*

| Reference test | | rpS1-iELISA | | | coELISA | | |
|---|---|---|---|---|---|---|---|
| | | Positive | Negative | AP[a] | Positive | Negative | AP[a] |
| IFA | Positive | 334 | 9 | 0.656 | 318 | 25 | 0.656 |
| | Negative | 1 | 179 | 0.344 | 2 | 178 | 0.344 |
| AP[a] | | 0.641 | 0.359 | | 0.612 | 0.388 | |
| Kappa value[b] | | 0.958 | | | 0.888 | | |

*[a] Apparent prevalence (AP) of the IFA, rpS1-iELISA or coELISA: positive ≡ positive number/total number; negative ≡ negative number/total number*
*[b] Observed proportional agreement between rpS1-iELISA and IFA: (334+179)/523=0.981; chance proportional agreement: (0.641×0.656)+(0.359×0.344)= 0.545; observed minus chance agreement: (0.981-0.545)=0.436; maximum possible agreement beyond chance level: (1-0.545)=0.455; agreement quotient (Kappa value) between rpS1-iELISA and IFA: (0.436/0.455)=0.958. The same method was used for calculating the agreement between the coELISA and IFA and obstained a Kappa value of 0.888*

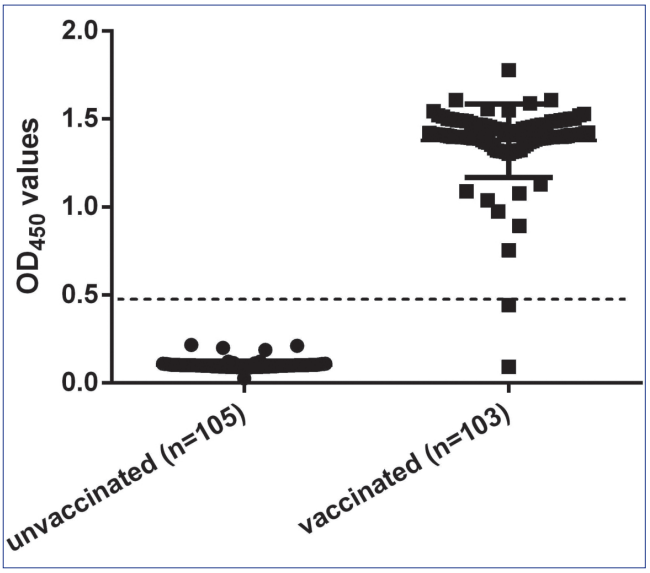



**Fig 4.** Distribution of anti-PEDV IgA antibodies in colostrum obtained from known PEDV vaccinated- and unvaccinated sows. Data presented as ELISA $OD_{450\,nm}$ values ± SD. The assay cut-off ($OD_{450\,nm}$ value of 0.448) is indicated by the dashed line

and PRV were used to analyze the cross-reaction with purified recombinant PEDV pS1 antigen. Three replicates of each sample were run on the same occasion. The OD values (mean±SD) were 0.083±0.008 for TGEV, 0.087±0.011 for CSFV, 0.056±0.019 for PRRSV, 0.091±0.014 for FMDV, and 0.073±0.010 for PRV. These results indicate that the PEDV pS1 protein-based ELISA had high specificity for detecting the anti-PEDV-specific IgA in the colostrum without antigenic cross-reactivity to the antibodies against other porcine viruses.

Reproducibility experiments within and between rpS1-iELISA plates were performed with three positive and three negative colostrum samples. The results showed that the CVs of intra-assay and inter-assay for three positive colostrum samples were both less than 5.0%. The intra-assay and inter-assay CVs of the three negative colostrum samples ranged from 3.46% to 7.94% and from 3.11% to 6.02%, respectively. These data indicated that the rpS1-iELISA was highly repeatable *(Table 1)*.

A total of 523 clinical colostrum samples were collected from 16 commercial pig farms in Henan province which had a history of diarrhea. From the 523 field colostrum samples, 65.58% (343/523) positive and 34.42% (180/523) negative samples were determined by IFA. Further analyses revealed that 334 of 343 positive colostrum samples determined by IFA were also positively analyzed by the rpS1-iELISA, while 179 of 180 negative colostrums were negatively confirmed by the rpS1-iELISA. The

ratios of positive and negative consistency for the two methods were 97.38 (334/334) and 99.44% (179/180), respectively.

To further assess the agreement between rpS1-iELISA and IFA, κ value was calculated using the formula described in *Table 2*. The *p* and *pe* values were calculated as 0.981 and 0.545, respectively. The agreement quotients (κ value) of the rpS1-iELISA was 0.958, which indicates that a satisfactory agreement between the rpS1-iELISA and IFA was obtained. Moreover, the rpS1-iELISA displayed significantly higher sensitivity and greater κ value than the coELISA (positive ratio of 334/523, 63.86% for rpS1-iELISA vs. 318/523, 60.8% for coELISA; κ value of 0.958 for rpS1-iELISA vs. 0.888 for coELISA, $P<0.05$) *(Table 2)*. This result suggested that rpS1-iELISA was preferable to the coELISA when applied to detect PEDV IgA antibody in the colostrum.

The IgA antibody in the colostrum was measured using the rpS1-iELISA to gain further insight into the efficacy of PEDV vaccine and impact of prefarrowing vaccination on maternal transfer of the IgA antibody. A total of 103 colostrum samples from healthy PEDV-vaccinated sows and 105 colostrum samples from unvaccinated healthy sows were examined. Comparing the average post-vaccination titers, the IgA levels of vaccinated sows were significantly higher than those of non-immunized ones ($P<0.05$). However, when the actual distribution values were evaluated within the groups, data from the two herds showed their respective homogeneous profiles, except for two cases in vaccinated sows with $OD_{450}$ nm below the cut-off value of 0.448 *(Fig. 4)*. These data indicated that IgA levels in the two sow herds were reliable, and the vaccine actually increased IgA antibody responses in the colostrum.

# DISCUSSION

Porcine epidemic diarrhea is a highly infectious disease that currently poses a serious threat to the swine industry. Precise diagnostics of PEDV and immune surveillance methods to monitor disease outbreaks and evaluate vaccine efficacy are required to implement control strategies. Previous studies confirmed that the colostral IgA level, but not the serum neutralization titer, induced by immunization against PEDV is correlated with protection of newborn piglets against virulent PEDV challenge [9]. Therefore, the evaluation of IgA level in the colostrum could be very useful to ensure that the piglets receive adequate passive immunity.

ELISA has been applied widely in diagnosis and antibody detection for animal infectious disease [15-21]. Several studies have used recombinant expressed and purified S protein of PEDV for ELISA serodiagnosis [12,22-24]. However, these studies focused mainly on the IgG test, and the numbers of known positive and negative samples used in these assays were relatively smaller than in the present study. Additionally, for mucosal infection, measurement of localized IgA level is critical to evaluate protection derived either by vaccines or by previous pathogen exposure [23]. In this study, a truncated spike gene pS1 domain from a novel PEDV variant (CH/HNQX-3/14) was expressed in *E. coli* and used as coating antigen to develop indirect ELISA to measure IgA antibody in the colostrum. Furthermore, a comparative analysis between rpS1-iELISA and IFA was performed, and the results showed an overall testing agreement of 95.8%. This phenomenon indicated that the rpS1-iELISA had approximately the same reliability as the PEDV whole virus-based IFA, whereas rpS1-iELISA avoided several disadvantages of IFA, such as high cost and relatively subjective interpretation. Moreover, rpS1-iELISA showed better test performance than a commercial IgA ELISA kit, and a higher specificity compared to another S1-protein based ELISA described by Gerber et al.[25]. No cross-reactivity with TGEV, CSFV, PRRSV, FMDV, or PRV was detected with the assay developed in the present study. Nevertheless, further studies are needed to assess whether this assay is suitable for the detection of IgA antibodies in fecal and oral fluid samples, and improve its test performance to distinguish IgA antibodies induced by vaccination from those resulting from PEDV infection.

In addition, the protection ability of IgA levels from immunized sows by artificial infection with PEDV was not evaluated because of the constraints of experimental conditions. However, our tracking survey confirmed that the proportion of piglets showing any abnormal intestinal symptoms at least once at any of the examination time points was lower in vaccinated sow herds than in unvaccinated ones (data not shown). This result suggested that the vaccine was apparently effective for protecting piglets from virulent PEDV challenge. An infectious challenge study is needed to determine whether such IgA levels can protect sows or piglets against PEDV infection, as well as clarifying IgA antibody kinetics in colostrum.

The current study reports the adaptation of a recombinant, highly purified partial S1 protein of PEDV to develop indirect ELISA platform for detecting PEDV IgA antibodies in the colostrum. This assay could be useful for PEDV diagnostics and evaluation of PEDV colostral immunity.

## Acknowledgements

This study was supported by grants from the Key Research Project of Henan Higher Education Institutions (18A230004), the Doctoral Scientific Research Foundation of Henan Institute of Science and Technology (206010617002), the Program for Innovative Research Team (in Science and Technology) in University of Henan Province (18IRTSTHN019), the Excellent Youth Foundation of Henan Scientific Committee (174100510005), and the Program for Science and Technology Innovation Talents in Universities in Henan Province (14HASTIT026).

## REFERENCES

**1. Bridgen A, Duarte M, Tobler K, Laude H, Ackermann M:** Sequence determination of the nucleocapsid protein gene of the porcine epidemic diarrhoea virus confirms that this virus is a coronavirus related to human coronavirus 229E and porcine transmissible gastroenteritis virus. *J Gen Virol,* 74 (9): 1795-1804, 1993. DOI: 10.1099/0022-1317-74-9-1795

**2. Pensaert MB, de Bouck P:** A new coronavirus-like particle associated with diarrhea in swine. *Arch Virol,* 58 (3): 243-247, 1978. DOI: 10.1007/BF01317606

**3. Choudhury B, Dastjerdi A, Doyle N, Frossard JP, Steinbach F:** From the field to the lab-An European view on the global spread of PEDV. *Virus Res,* 226, 40-49, 2016. DOI: 10.1016/j.virusres.2016.09.003

**4. Song D, Park B:** Porcine epidemic diarrhoea virus: A comprehensive review of molecular epidemiology, diagnosis, and vaccines. *Virus Genes,* 44 (2): 167-175, 2012. DOI: 10.1007/s11262-012-0713-1

**5. Trujillo-Ortega ME, Beltrán-Figueroa R, García-Hernández ME, Juárez-Ramírez M, Sotomayor-González A, Hernández-Villegas EN, Becerra-Hernández JF, Sarmiento-Silva RE:** Isolation and characterization of porcine epidemic diarrhea virus associated with the 2014 disease outbreak in Mexico: Case report. *BMC Vet Res,* 12: 132, 2016. DOI: 10.1186/s12917-016-0763-z

**6. Park SJ, Song DS, Ha GW, Park BK:** Cloning and further sequence analysis of the spike gene of attenuated porcine epidemic diarrhea virus DR13. *Virus Genes,* 35 (1): 55-64, 2007. DOI: 10.1007/s11262-006-0036-1

**7. Li R, Qiao S, Yang Y, Guo J, Xie S, Zhou E, Zhang G:** Genome sequencing and analysis of a novel recombinant porcine epidemic diarrhea virus strain from Henan, China. *Virus Genes,* 52 (1): 91-98, 2016. DOI: 10.1007/s11262-015-1254-1

**8. Boniotti MB, Papetti A, Lavazza A, Alborali G, Sozzi E, Chiapponi C, Faccini S, Bonilauri P, Cordioli P, Marthaler D:** Porcine epidemic diarrhea virus and discovery of a recombinant swine enteric coronavirus, Italy. *Emerg Infect Dis,* 22 (1): 83-87, 2016. DOI: 10.3201/eid2201.150544

**9. Carvajal A, Arguello H, Martinez-Lobo FJ, Costillas S, Miranda R, de Nova PJG, Rubio P:** Porcine epidemic diarrhoea: New insights into an old disease. *Porcine Health Manag,* 1, 12, 2015. DOI: 10.1186/s40813-015-0007-9

**10. Fan B, Jiao D, Zhao X, Pang F, Xiao Q, Yu Z, Mao A, Guo R, Yuan W, Zhao P, He K, Li B:** Characterization of chinese porcine epidemic diarrhea virus with novel insertions and deletions in genome. *Sci Rep,* 7, 44209, 2017. DOI: 10.1038/srep44209

**11. Baek P, Choi H, Lee S, Yoon I-J, Lee YJ, Lee DS, Lee S, Lee C:** Efficacy of an inactivated genotype 2b porcine epidemic diarrhea virus vaccine in neonatal piglets. *Vet Immunol Immunopathol,* 174, 45-49, 2016. DOI: 10.1016/j.vetimm.2016.04.009

**12. Wang X, Wang Z, Xu H, Xiang B, Dang R, Yang Z:** Orally administrated whole yeast vaccine against porcine epidemic diarrhea virus induced high levels of iga response in mice and piglets. *Viral Immunol,* 29 (9): 526-531, 2016. DOI: 10.1089/vim.2016.0067

**13. Jacobson RH:** Validation of serological assays for diagnosis of infectious diseases. *Rev Sci Tech Off Int Epiz,* 17 (2): 469-486, 1998. DOI: 10.20506/rst.17.2.1119

**14. Okda F, Liu X, Singrey A, Clement T, Nelson J, Christopher-Hennings J, Nelson EA, Lawson S:** Development of an indirect ELISA, blocking ELISA, fluorescent microsphere immunoassay and fluorescent focus neutralization assay for serologic evaluation of exposure to North American strains of Porcine Epidemic Diarrhea Virus. *BMC Vet Res,* 11:180, 2015. DOI: 10.1186/s12917-015-0500-z

**15. Wang XX, Wang FX, Li ZG, Wen YJ, Wang X, Song N, Wu H:** Development of an indirect enzyme-linked immunosorbent assay (ELISA) to differentiate antibodies against wild-type porcine reproductive and respiratory syndrome from the vaccine strain TJM-F92 based on a recombinant Nsp2 protein. *J Virol Methods,* 251, 151-154, 2018. DOI: 10.1016/j.jviromet.2017.09.001

**16. Jittimanee S, Nuntawan Na Ayudhya S, Kedkovid R, Teankum K, Suradhat S, Thanawongnuwech R:** An indirect enzyme-linked immunosorbent assay using a recombinant truncated capsid protein of *Porcine circovirus-2*. *J Vet Diagn Invest,* 24 (6): 1129-1132, 2012. DOI: 10.1177/1040638712461251

**17. Liu C, Kokuho T, Kubota T, Watanabe S, Inumaru S, Yokomizo Y, Onodera T:** A serodiagnostic ELISA using recombinant antigen of swine transmissible gastroenteritis virus nucleoprotein. *J Vet Med Sci,* 63 (11): 1253-1256, 2001. DOI: 10.1292/jvms.63.1253

**18. Fu Y, Li P, Cao Y, Wang N, Sun P, Shi Q, Ji X, Bao H, Li D, Chen Y, Bai X, Ma X, Zhang J, Lu Z, Liu Z:** Development of a blocking elisa using a monoclonal antibody to a dominant epitope in non-structural protein 3A of foot-and-mouth disease virus, as a matching test for a negative-marker vaccine. *PLos One,* 12 (1): e0170560, 2017. DOI: 10.1371/journal.pone.0170560

**19. Genç O, Büyüktanir Ö, Yurdusev N:** Development of Qualitative and quantitative ELISA models for bovine brucellosis diagnosis. *Kafkas Univ Vet Fak Derg,* 16 (2): 287-291, 2010. DOI: 10.9775/kvfd.2009.692

**20. Kalkanov I, Dinev I, Aleksandrov M, Dimitrov K, Zarkov I:** Morphological and etiological investigations in a rotaviral enteritis outbreak in calves. *Kafkas Univ Vet Fak Derg,* 22 (2): 309-313, 2016. DOI: 10.9775/kvfd.2015.14365

**21. Song L, Zhang H, Hou P, Wang H, Zhao G, Xia X, He H:** Development and preliminary application of an indirect ELISA to detect infectious bovine rhinotracheitis virus using recombinant glycoprotein D of IBRV strain SD. *Kafkas Univ Vet Fak Derg,* 22 (4): 503-509, 2016. DOI: 10.9775/kvfd.2015.14890

**22. Yuan X, Lin H, Li B, He K, Fan H:** Efficacy and immunogenicity of recombinant swinepox virus expressing the truncated S protein of a novel isolate of porcine epidemic diarrhea virus. *Arch Virol,* 162 (12): 3779-3789, 2017. DOI: 10.1007/s00705-017-3548-1

**23. Gerber PF, Opriessnig T:** Detection of immunoglobulin (Ig) A antibodies against porcine epidemic diarrhea virus (PEDV) in fecal and serum samples. *MethodsX,* 2, 368-373, 2015. DOI: 10.1016/j.mex.2015.10.001

**24. Li Y, Zheng F, Fan B, Muhammad HM, Zou Y, Jiang P:** Development of an indirect ELISA based on a truncated S protein of the porcine epidemic diarrhea virus. *Can J Microbiol,* 61 (11): 811-817, 2015. DOI: 10.1139/cjm-2015-0213

**25. Gerber PF, Gong Q, Huang YW, Wang C, Holtkamp D, Opriessnig T:** Detection of antibodies against porcine epidemic diarrhea virus in serum and colostrum by indirect ELISA. *Vet J,* 202 (1): 33-36, 2014. DOI: 10.1016/j.tvjl.2014.07.018

Kafkas Univ Vet Fak Derg
24 (5): 725-733, 2018
DOI: 10.9775/kvfd.2018.19855

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Hepatoprotective Effect of *Punica granatum* Seed Oil Extract on Paracetamol Intoxication in Rat Via Inhibory Effect of CYP2E1 [1][2]

Damla CETIN [1] Pinar AKSU KILICLE [2] Gulname FINDIK GUVENDI [3] Muhammed YAYLA [1]

[1] *This work was supported by Kafkas University Scientific Research Project (grant number: 2016TS31)*
[2] *A part of this study was presented at international congress by 2nd Iternational Science Symposium (Science Festival), 5th-8th September 2017, Tbilisi, Georgia*

[1] Department of Pharmacology, Kafkas University, Faculty of Medicine, TR-36100 Kars - TURKEY
[2] Department of Biology, Kafkas University, Faculty of Science, TR-36100 Kars - TURKEY
[3] Department of Pathology; Recep Tayyip Erdogan University, Faculty of Medicine, TR-53100 Rize - TURKEY

**Article Code: KVFD-2018-19855 Received: 12.01.2018 Accepted: 02.07.2018 Published Online: 08.07.2018**

**How to Cite This Article**

**Cetin D, Aksu Kilicle P, Findik Guvendi G, Yayla M:** Hepatoprotective effect of *Punica granatum* seed oil extract on paracetamol intoxication in rat via inhibory effect of CYP2E1. *Kafkas Univ Vet Fak Derg,* 24 (5): 725-733, 2018. DOI: 10.9775/kvfd.2018.19855

## Abstract

*Punica granatum* seed oil enriched by punikalagins has been shown to have a therapeutic effect against antidiabetic, anticancer, antiinflammatory and some organ toxicities. We aimed to reveal both protective and therapeutic effects of punica granatum seed oil, which has strong antioxidant and anti-inflammatory activity on paracetamol-induced hepatic toxicity via biochemically, molecularly and pathologically in our study. Our study, 64 albino wistar rats were fasted for 24 h and then divided into 8 equal groups. Group 1: Healthy, Group 2: 2 g/kg of paracetamol (2a: 24 h, 2b: 48 h) (orally), Group 3: 140 mg/kg of n-acetylcysteine (orally) + paracetamol, Group 4: 0.32 mg/mL *Punica granatum* (i.p) + paracetamol, Group 5: 0.64 mg/mL *Punica granatum* + paracetamol, Group 6: Paracetamol + 0.32 mg/mL *Punica granatum,* Group 7: Paracetamol + 0.64 mg/mL *Punica granatum,* Group 8: Paracetamol + 140 mg/kg n-acetylcysteine. The study was terminated at 24 and 48 h after paracetamol administration. Serum ALT and AST levels were significantly increased at 24th and 48th h of paracetamol administration according to toxicity. While malondialdehyde, CYP2E1 and TNF-α levels also increased in the liver, superoxide dismutase and glutathione peroxidase levels decreased significantly. Increased ALT, AST levels with malondialdehyde and TNF-α levels significantly decreased by punica granatum seed oil (low doses) application and antioxidant levels were also significantly improved. *Punica granatum* seed oil may be used as a potential therapeutic agent in the future by strengthening the antioxidant system and preventing inflammation, especially liver toxicity due to overdose of paracetamol in suicide- battered individuals.

***Keywords:*** *Rat, Paracetamol, Toxicity, Liver, Punica granatum*

# Sıçanlarda CYP2E1 İnihibisyonu İle Parasetamol İntoksikasyonunda *Punica granatum* Çekirdek Yağı Ektraktının Hepatoprotektif Etkisi

## Öz

Punikalaginlerle zengin olan *Punica granatum* çekirdek yağının, antidiyabetik, antikanser, antiinflamatuar ve bazı organ toksisitelerine karşı terepötik etkileri gösterilmiştir. Çalışmamızda, parasetamol ile indüklenen hepatotoksisite üzerine güçlü antioksidan ve anti-inflamatuvar etkinliğe sahip punica granatum çekirdek yağının biyokimyasal, moleküler ve patolojik olarak koruyucu ve terapötik etkilerini ortaya koymayı amaçladık. Çalışmamızda 64 albino wistar sıçan 24 saat aç bırakıldıktan sonra 8 eşit gruba ayrıldı. Grup 1: Sağlıklı, Grup 2: Parasetamol (2a: 24 saat, 2b: 48 saat) (oral), Grup 3: 140 mg/kg n-asetilsistein (oral) + parasetamol, Grup 4: 0.32 mg/mL *Punica granatum* (i.p.) + parasetamol, Grup 5: 0.64 mg/mL *Punica granatum* + parasetamol, Grup 6: Parasetamol + 0.32 mg/mL *Punica granatum,* Grup 7: Parasetamol + 0.64 mg/mL *Punica granatum,* Grup 8: Parasetamol + 140 mg/kg n-asetilsistein. Çalışma, parasetamol uygulamasından 24 ve 48 saat sonra sonlandırıldı. Parasetamol uygulamasının 24. ve 48. saatlerinde toksisiteye göre serum ALT ve AST düzeyleri anlamlı şekilde arttı. Malondialdehit, CYP2E1 ve TNF-α düzeyleri karaciğerde de yükselirken, süperoksit dismutaz ve glutatyon peroksidaz düzeyleri anlamlı olarak azaldı. *Punica granatum* çekirdek yağı (düşük dozlarda) uygulaması ile artmış ALT, AST düzeyleri, malondialdehit ve TNF-α seviyeleri anlamlı derecede azalmış ve antioksidan düzeyleri önemli derecede düzelmiştir. *Punica granatum* çekirdek yağı, intihar girişiminde bulunan kişilerde parasetamolün aşırı dozundan dolayı oluşan karaciğer toksisitesini önleyerek ve antioksidan sistemi güçlendirerek potansiyel terapötik bir madde olarak kullanılabilir.

***Anahtar sözcükler:*** *Sıçan, Parasetamol, Toksisite, Karaciğer, Punica granatum*

**İletişim (Correspondence)**
+90 555 8339313
damlacetin.erz@gmail.com

# INTRODUCTION

Paracetamol is a drug that has been used safely in baby, children and adults for many years as an analgesic and antipyretic [1]. Intoxication is frequently encountered because paracetamol is easily accessible. Especially in childhood, drug intoxication comes first [2,3]. Upon overdose, paracetamol is metabolized to N-acetylbenzoquinonimine (NAPQI), a intermediate reactive metabolite by the cytochrome p450 enzymes in the liver. NAPQI accumulation in the liver depletes free glutathione deposits leading to oxidative stress in the cells. It also leads to oxidation of sulfhydryl groups leading to apoptosis by increasing $Ca^{++}$ concentration. This developing intoxication causes hepatic necrosis if not treated and liver failure in advanced cases [4-6].

Nowadays, Nacetylcysteine (NAC) is used as an antidote for hepatotoxicity due to paracetamol. NAC, a glutathione precursor, inhibits hepatic necrosis by providing NAPQI detoxification [7,8]. However, both NAC and other antioxidant acting agents can't able completely reverse the liver damage due to paracetamol toxicity. Therefore, it is important to develop drugs with stronger antioxidant and antiinflammatory activity to prevent liver damage due to paracetamol toxicity.

*Punica granatum* has grown up commonly and consume all around the world. It has been known strong antioxidant effects and has been used as a medication in ancient times. Also, *Punica granatum* and its compartments have benefiacial effects on diarrhea, dental problems, erectil dysfunction, protecting radiation, alzheimer, arthritis, obesity [9,10]. The *Punica granatum* fruit has three parts: the kernel, the shell and the water. The *Punica granatum* kernel is rich by its chemical content [11]. It also contains important antioxidants such as ellagic acid and gallic acid [12]. In that way there are number of different and possible biological effects. *Punica granatum* has strong antioxidant and antiinflammatory activity and has been used as an antiparasitic, anticoagulant, aphtha, diarrhea and ulcer healing agent [9,10]. The protective effects of *Punica granatum* juice and *Punica granatum* crust extract at liver toxicity depending to paracetamol has shown on that article [12]. However, *Punica granatum* seed will have different physiological effects due to the presence of many biologically active materials, unlike oil, water and crust extracts. Especially *Punica granatum* seed oil enriched by punikalagins has shown the theapeutic effects against antidiabetic, anticancer, antiinflammatory and some organ toxicities (brain, liver, kidney). However, there are no studies on the effects of *Punica granatum* seed oil or punicalagins on paracetamol toxicity. For this reason, we aimed to reveal on paracetamol-induced hepatic toxicity both protective and therapeutic effects of *Punica granatum* seed oil (PSO) with strong antioxidant and antiinflammatory activity in biochemical, molecular and pathological studies.

# MATERIAL and METHODS

A total of 64 male albino wistar rat were used in the experiments. Each rat weighed 200-250 g, and all were obtained from Ataturk University's Experimental Animal Laboratory at the Medicinal and Experimental Application and Research Centre. The animal experiments and procedures were performed in accordance with national guidelines for the use and care of laboratory animals and approved by Kafkas University's Local Animal Care Committee (KAÜ-HADYEK: 2016/014). The rat was housed in standard plastic cages on sawdust bedding in an air-conditioned room at 22±1°C. Standard rat food and tap water were given *ad libitum*.

### *Chemicals*

All of the chemicals used in our laboratory experiments were purchased from Sigma Chemical Co. (Munich, Germany). *Punica Granatum* spp., known as Hicaz in our country, was purchased from city of Mersin (obtained year: 2016) Thiopental sodium was obtained from IE Ulagay A.S. (Istanbul, Turkey).

### *Preparation of Punica granatum Seed Oil Extract*

Essential oils of the plants were obtained on a Clevenger (Wisd-Wise Therm) device by water vapor distillation. For this purpose, the fruits were dried and then the seeds were separated. 160 g of the plant was pulverized in the shredder. The sample was placed in a glass balloon and 1600 mL of distilled water was added to it, then placed in a Clevenger apparatus and the apparatus was operated. After the evaporation started, it was left to stand for three hours. During this time, the hydrosol accumulated in the Clevenger in collection tube was taken in a sterile separate bottle. After the taken last hydrosol accumulated in the collection tube, the remaining volatile oil was stored in the dark bottles in the refrigerator at +4°C until used in the experiment.

### *Analysis of Ingredients in Punica granatum Seed Oil Extract by HPLC-DAD*

Gallic acid, ellajic acid and punicalajin A and B were detected in extract and quantitative determinations were made on the Agilent 1200 Series High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) (Germany).

### *Sample Preparations*

The extract was dissolved in methanol to a concentration of 1 mg/mL and a stock solution was obtained and centrifuged at 10.000 rpm for 5 min. Workup solutions were prepared by diluting the supernatant with phosphate buffer (pH=2.5, 0.025 M). The working solutions were injected into the system after passing through the injection filter. Each injection was repeated three times *(Table 1)*.

**Table 1.** *Amounts of substance in 1.00 g extract*

| Substance | Amounts (mg/g) |
|---|---|
| Gallic acid | 14.45±0.53 |
| Punikalajin A | 191.56±0.36 |
| Punicalajin B | 189.48±0.62 |
| Ellageic Acid | 68.02±0.42 |

### Experimental Design

Animals fasted for 24 h and were divided into eight equal groups (n=8). Group 1: Control; did not receive any medication. Group 2 received only orally paracetamol 2 g/kg for 24 h (in 1% Carboxymethylcellulose (CMC) in 1X Phosphate Buffered Saline (PBS) [13-15] 2 mL). Group 2b received only orally paracetamol 2 g/kg for 48 h (in 1% CMC in 1X PBS 2 mL). Groups 3; received orally N-acetylcysteine 140 mg/kg 24 h after received paracetamol. Groups 4; received i.p. injection PSO 0.32 mg/kg 24 h after received paracetamol. Groups 5; received i.p. injection PSO 0,64 mg/kg 24 h after received paracetamol. Groups 6; received i.p. injection PSO 0,32 mg/kg before 24 h received paracetamol. Groups 7; received i.p. injection PSO 0,64 mg/kg before 24 h received paracetamol. Groups 8; received orally N-acetylcysteine 140 mg/kg before 24 h received paracetamol. The rats were allowed food post-administration of drugs for the next 24 h until they were sacrificed.

Following the experimental period, Group 1, 2, 6, 7 and 8 were killed on the 24$^{th}$ h after the administration of paracetamol and Group 2b, 3, 4 and 5 were killed on the 48$^{th}$ h after the administration of paracetamol by an overdose of a general anesthetic (thiopental sodium, 50 mg/kg). Blood samples were collected into heparinized bottles by heart puncture. The liver was removed immediately after sacrifice.

### Biochemical Analyses

Serum Measurements of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), total protein serum samples were separated by centrifuging at 4.000 rpm for 10 min at 4°C within 1 h after collection, and were stored in a -86°C freezer before being used for biochemical analysis (including ALT, AST, total protein). To assess hepatic function, ALT and AST from each sample were measured in duplicate with highly sensitive Enzyme Linked Immunosorbent Assay (ELISA) kits specifically designed for rats, according to the manufacturer's instructions (BEN SRL-B0127E and B1253E (ITALY), respectively).

### Liver Measurements of Oxidant, Antioxidants, CYP2E1 and TNF-a

Rat tissues were kept at -86°C. 100 mg of tissues from each rat was firstly perfused with PBS/heparin. All tissue samples from each rat were grinded in liquid nitrogen using a TissueLyser II grinding jar set (Qiagen, Hilden, Germany). Then they were centrifuged according to the manufacturer's instructions. Then, Superoxide Dismutase (SOD) activity, and Malondialdehyde (MDA) level, Glutathione Peroxidase (GSH) level, Cytochrome P450 2E1 (CYP2E1) and Tumor Necrosis Factor Alpha (TNF-α) levels from each supernatant were measured in duplicate with highly sensitive kits (Cayman-706002, 10009055, 703002 (USA)), and CSB-E09782r (China), specifically designed for rat tissue, according to the manufacturer's instructions. The protein concentrations were determined by the Lowry method using commercial protein standards (Sigma Aldrich, Total protein kit-TP0300-1KT-(USA)). All the data was presented as the mean±standard deviation (S.D.) results based on per mg of protein.

### Real-Time PCR Analyses

The samples for molecular analyses were immediately stored at -80°C. Before storage, all of the tissue samples from each rat were first perfused with PBS/heparin and then ground in liquid nitrogen with a TissueLyser II grinding Jar Set.

### RNA Extraction and cDNA Synthesis

The liver tissues (20 mg) were stabilized in an RNA stabilization reagent (RNAlater, Qiagen) and then disrupted with the TissueLyser II (Qiagen) (2x2 min). All of the RNA was purified using an RNeasy Mini Kit (Qiagen) and RNA samples were reverse-transcribed into complementary DNA with a high-capacity cDNA reverse transcription kit (Qiagen) according to our previous study [16,17].

### Gene Expression

Relative rat TNF-α (PP-RA-300 taqman probe (Qiagen)) mRNA expression analyses were performed with StepOnePlus Real-Time PCR System technology (Applied Biosystems) using cDNA synthesized from RNA of ovary tissue described as previously [16].

### Histopathological Procedures

The tissues reserved for histopathological examination were rapidly fixed in 10% buffered formalin for 24 h. After fixation, each tissue sample was routinely processed described as previously [16]. In haematoxylin Eosin stain (H&E) apoptotic cells are evaluated according to nucleus morphology because it stains chromatin. Observable changes include: Cell shrinkage or cytoplasmic shrinkage, condensation of chromatin and collection at the periphery of the nucleus membrane, shrinking or fragmentation of the nucleus.

### Statistical Analyses

IBM SPSS statistical software Version 20.0 was used for the biochemical analysis. The results are presented as the means±standard deviation (SD). The between-group

comparisons for biochemical and molecular analyses were performed with one-way ANOVA and Duncan's multiple comparison tests. Significance was accepted at P<0.05.

# RESULTS

### *Serum ALT and AST Levels*

Primer indicators of liver toxicity are serum ALT and AST levels were measured as gold standart. Serum AST and ALT levels were significantly increased at 24$^{th}$ and 48$^{th}$ h of paracetamol according to toxicity. At our study Serum AST and ALT levels decreased in paracetamol toxicity after *Punica granatum* oil was added (P<0.05). Low dose PSO administration has lower AST and ALT levels more than high dose PSO administration (P<0.05). There was no significant difference between our study and NAC and low dose PSO administration. Finally, in our study, we found that low dose PSO administration significantly reduced AST and ALT levels compared to high dose PSO administration in the groups in which we wanted to show a therapeutic response to toxicity (P<0.05). Again, no significant difference was found between NAC and low dose PSO in our study. Improvement of ALT and AST levels, which improved with PSO administration, suggests that PSO prevents liver toxicity *(Fig. 1)*.

### *Tissue Oxidative Marker*

The primary mechanism of injury in paracetamol toxicity is increase of oxidative stress. The MDA levels of membrane phospholipids, the ultimate degradation product, are evaluated to show the tissue damage caused by the oxidative stress. Tissue MDA levels were significantly increased according to the duration of exposure at the 24$^{th}$ and 48$^{th}$ h of paracetamol administration exposed to the control group (P<0.05). In our study, *Punica granatum* reduced the MDA level due to the protective effect of the seed oil (P<0.05). Low dose PSO application has reduced MDA level more than high dose PSO application

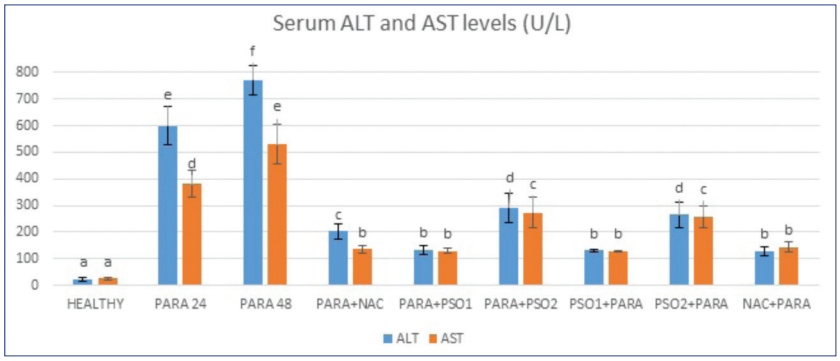
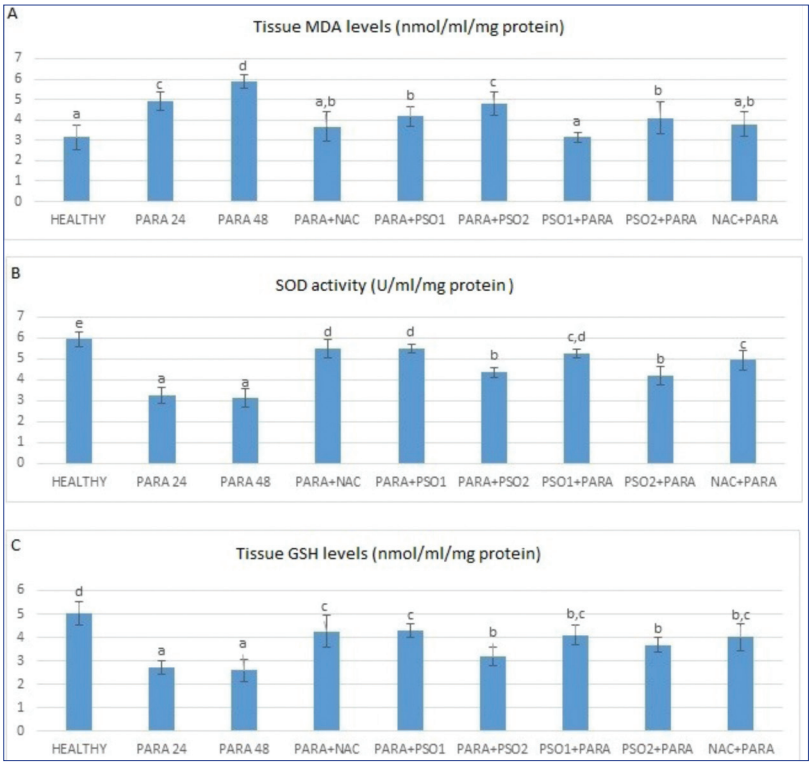



**Fig 1.** Effects of PSO on changes in the ALT and AST level in paracetamol toxicity. Means in the same column with the same letter are not significantly different; means in the same column with different letters indicate significant differences between the groups. P<0.05 (PSO: *Punica granatum* seed oil, PARA: Paracetamol)

**Fig 2.** Effects of PSO on changes in the SOD, MDA activity and GSH level in paracetamol toxicity. Means in the same column with the same letter are not significantly different; means in the same column with different letters indicate significant differences between the groups. P<0.05 (PSO: *Punica granatum* seed oil, PARA: Paracetamol)



(P<0.05). MDA level decreased with NAC application and no significant difference was observed with low dose PSO. MDA levels has decreased more at PSO threatment groups than paracetamol toxicity groups. Comparing both protective and therapeutic groups of PSO in our study, PSO has a strong protective effect and reduce damage in the treatment process *(Fig. 2)*.

### *Tissue CYP2E1 Levels*

NAPQI which is produced from paracetamol by CYP2E1 in the liver. Active metabolite is responsible for the development of paracetamol toxicity. Increased CYP2E1 activity due to damage causes to increase of damage. Therefore, pharmacological agents with antioxidant properties can also correct the increased CYP2E1 enzyme activity at the same time, changing the style o the damage. In our study, groups with paracetamol treated showed an increase CYP2E1 activity in liver, depending on the duration of exposure (P<0.05). In the treated groups, However, low dose PSO application caused a further decrease in CYP2E1 activity more than high dose PSO application (P<0.05). CYP2E1 activities in the NAC treatment group was found lower than low dose PSO treatment group *(Fig. 3)*.

### *Tissue Antioxidant Markers*

One of the most important defense systems of our body is antioxidants. Deficiency of antioxidants leads to increased oxidative stress and to necrosis of the liver. In

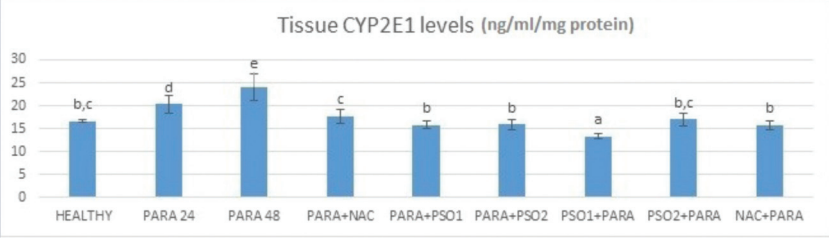



**Fig 3.** Effects of PSO on changes in the CYP2E1enzym levels in paracetamol toxicity. Means in the same column with the same letter are not significantly different; means in the same column with different letters indicate significant differences between the groups. P<0.05 (PSO: *Punica granatum* seed oil, PARA: Paracetamol)

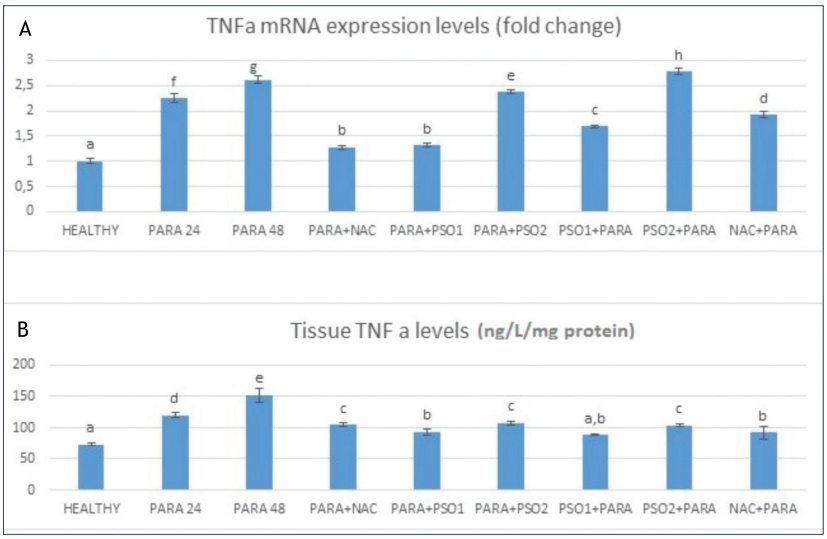



**Fig 4.** Effects of PSO on changes in the TNF-α mRNA expression levels (A) and tissue TNF-α levels (B) in paracetamol toxicity. Means in the same column with the same letter are not significantly different; means in the same column with different letters indicate significant differences between the groups. P<0.05 (PSO: *Punica granatum* seed oil, PARA: Paracetamol)

***Table 2.*** *Pathological scoring*

| Groups | Periportal Inflammation | Congestion | Cellular Damage Severity | Comman Cellular Damage | Necrosis |
|---|---|---|---|---|---|
| Healthy | minimal | ++ | none | none | none |
| PARA24 | ++ | + | minimal | minimal | ++ |
| PARA48 | ++ | + | minimal | minimal | none |
| PARA + NAC | + | + | none | none | none |
| PARA + PSO1 | + | ++ | none | none | ++ |
| PARA + PSO2 | + | + | none | none | none |
| PSO1 + PARA | + | + | none | none | none |
| PSO2 + PARA | + | + | none | none | none |
| NAC + PARA | + | + | none | none | local |

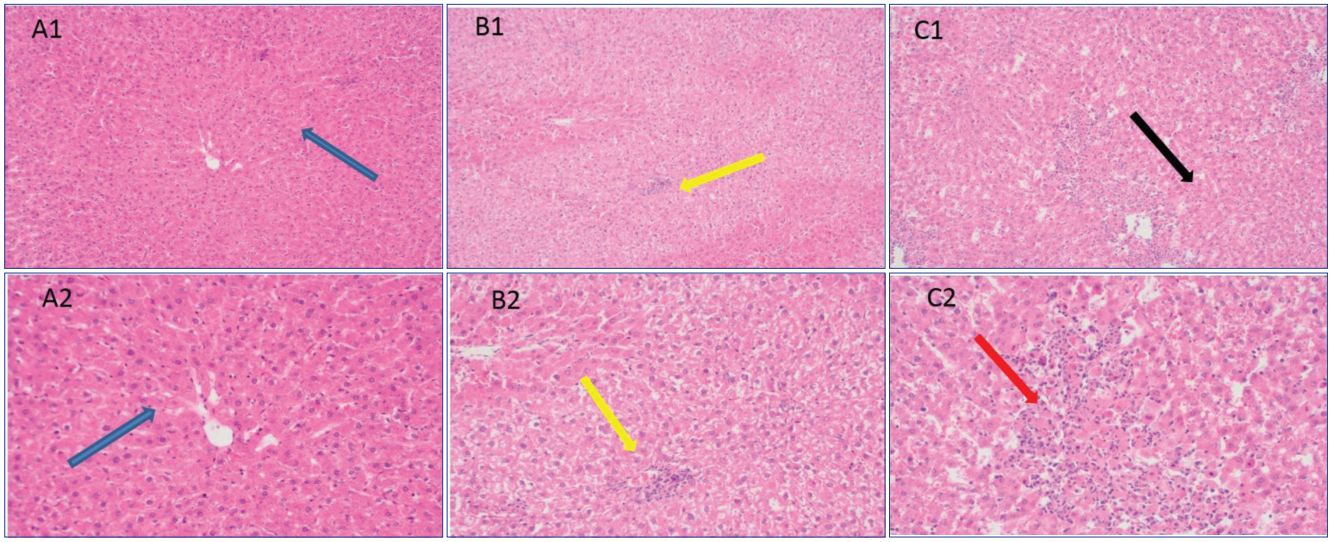



**Fig 5.** A) 1-40x, 2-100x, H-E staining results of healthy, B) 1-40x, 2-100x, H-E staining results of paracetamol toxicity 24 h, C) 1-40x, 2-100x, H-E staining results of paracetamol toxicity 48 h. *blue arrow:* cellular damage minimal, *yellow arrow:* periportal inflammation, *red arrow:* necrosis, *black arrow:* apoptosis

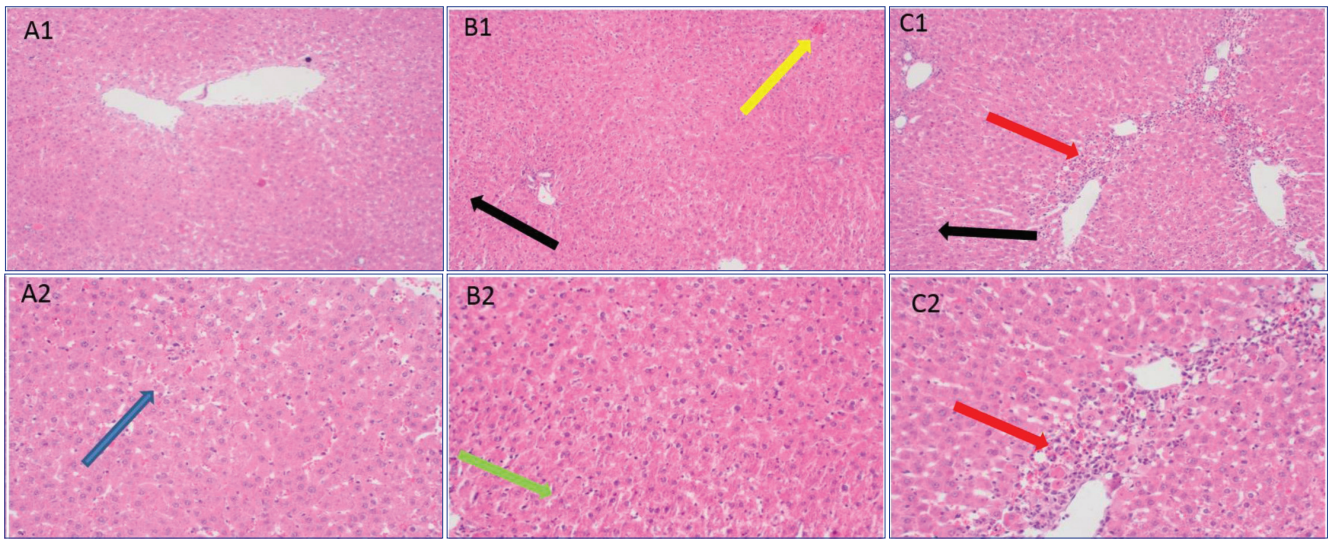



**Fig 6.** A) 1-40x, 2-100x, H-E staining results of PARA+NAC, B) 1-40x, 2-100x, H-E staining results of PARA+PSO1, C) 1-40x, 2-100x, H-E staining results of PARA+PSO2. *blue arrow:* minimal necrosis, *yellow and green arrow:* congestion, *red arrow:* portal inflammation, *black arrow:* mitotic cells

our study, liver SOD activities decreased significantly in the paracetamol group by the exposure of duration. In the PSO groups which is given protectively, SOD activities increased significantly ($P<0.05$). And again low dose PSO application increased the SOD activity more than the high dose. The protective effect of NAC increased SOD activity at the same level as low dose PSO administration ($P<0.05$). And again we observed that, administration of low dose PSO increased the activity of SOD more than that of the NAC group. In treatment groups, SOD activities were found to increase in the same amount in low dose PSO and NAC groups. In high dose PSO treatment, SOD activity is increased, but this increase was not as high as in the low dose treatment. there is a significant decrease in paracetamol toxicity in tissue GSH levels compared to control. In the treatment groups, NAC and PSO treatment significantly increased GSH levels. High dose PSO therapy increased GSH levels, but this increase was not as high as other treatment groups. By the protective effect, low dose PSO and NAC application significantly increased GSH levels ($P<0.05$). High dose PSO administration increased GSH levels but did not increase as much as other anti toxic groups *(Fig. 2)*.

### *Tissue TNF-a Levels and mRNA Expression Analyses*

Paracetamol-induced toxicity is accompanied by oxidative stress and inflammation leading to increased exacerbation. The most important cytokines that causes inflammation is TNF-α. TNF-α, a proinflammatory cytokine, is significantly

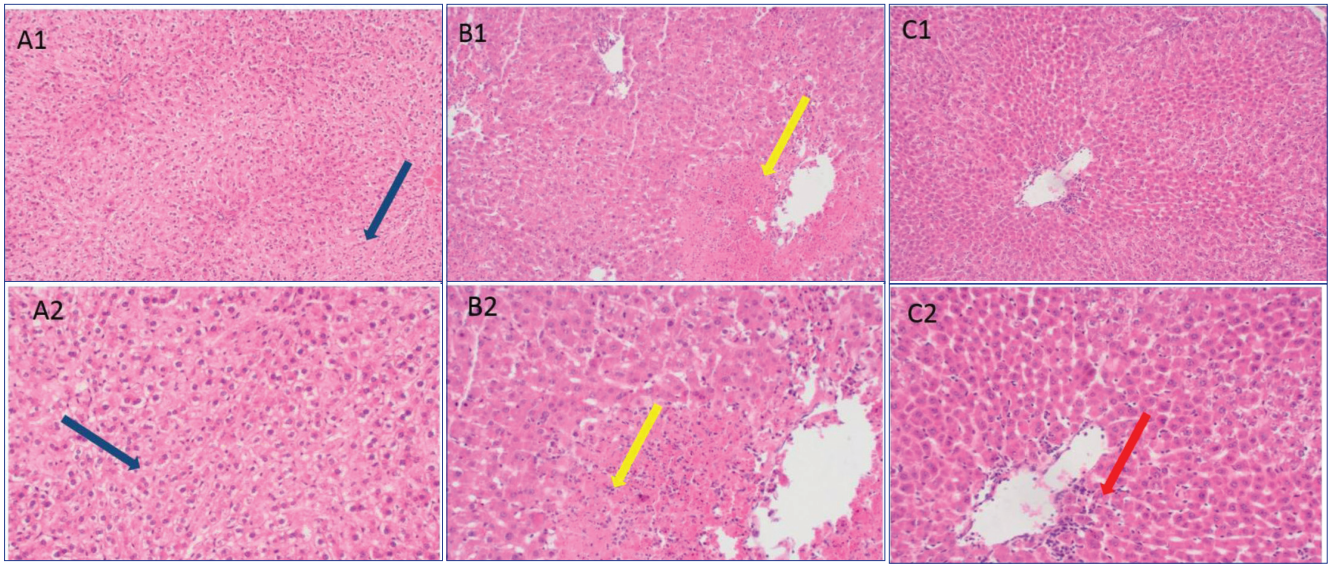



**Fig 7.** A) 1-40x, 2-100x, H-E staining results of PSO1+PARA, B) 1-40x, 2-100x, H-E staining results of PSO2+PARA, C) 1-40x, 2-100x, H-E staining results of NAC+PARA. *blue arrow:* cellular damage, *yellow arrow:* necrosis, *red arrow:* portal inflammation

increased due to damage in paracetamol toxicity. In our study, tissue TNF-α level significantly increased with time depending on the application of paracetamol (P<0.05). It has been shown that low dose PSO treatment reduces tissue TNF-α level more than NAC and high dose PSO treatment. Tissue TNF-α levels in all treatment groups were significantly decreased more than the toxicity groups (P<0.05). Tissue TNF-α levels significantly decreased in PSO and NAC application. Difference of Increase in tissue TNF-α levels causes to be the low dose PSO and NAC inhibitory effect is higher than that of the high dose PSO.

TNF-α mRNA expression levels significantly increased in paracetamol applicated groups in directly proportional to the duration of exposure relative to control. Low dose PSO therapy significantly reduced TNF-α mRNA expression levels caused by paracetamol (P<0.05). High dose PSO treatment did not showed any significant effect on TNF-α mRNA expression levels. NAC treatment significantly reversed the increase in TNF-α mRNA expression levels. Low doses of PSO given as protective against paracetamol toxicity significantly reduced TNF-α mRNA expression levels, while high-dose PSO administration was ineffective. NAC significantly reduced the levels of TNF-α. MRNA expression. Decreased inflammation due to PSO shows the antiinflammatory activity of the PSO and has an important place in the prevention and treatment of damage *(Fig. 4)*.

### *Histopathological Analyses*

No pathology was observed in the liver tissues of the control group. At the 24$^{th}$ h of paracetamol administration, chronic inflammation, hemorrhage, and focal lithic necrosis were seen around portal areas. At the 48$^{th}$ h of paracetamol application, moderate to severe chronic inflammation, apoptotic cells, and double nucleus formation were observed around portal areas *(Table 2)*. İn treated groups, Chronic inflammation and focal lithic necrosis observed. In some portal areas in the treated groups with NAC in the treatment groups, while mild inflammation, focal necrosis areas, necrotic areas were observed around the portal area in low dose PSO treatment. However, in high-dose PSO treatment, regenerative findings (increased mitotic activity) in hepatocytes, showed chronic inflammation around the portal areas, and dilatation of the sinusoids. While administration of low dose PSO resulted in mild regenerative atypia in hepatocytes against paracetamol toxicity, chronic inflammation around the portal areas were seen with high dose PSO administration. In the group given NAC, inflammation around the portal areas and a few focal lytic necrosis centers were observed *(Fig. 5,6,7)*.

## DISCUSSION

In our study, the possible protective and therapeutic effects of PSO in liver toxicity caused by paracetamol were demonstrated. However, the therapeutic effect was seen especially in low dose PSO administration, at high dose all parameters showed lower activity.

Paracetamol when it converts to NAPQI by the CYP2E1 enzyme in the liver can be tolerated by the endogenous antioxidants in our body when taken in therapeutic doses. However, in the case of high dose paracetamol ingestion, the active metabolite NAPQI, which cannot be tolerated due to insufficient endogenous antioxidant deposits, accumulates in the liver leading to damage. The CYP2E1 enzyme level plays an important role in the formation of the amount of active metabolite responsible for paracetamol toxicity and in indirectly evolved liver damage. The high level enzyme paracetamol purifies the active and harmful metabolites [18]. In our study, the CYP2E1 enzyme induced

by paracetamol toxicity, causes more NAPQI formation. For reverse paracetamol toxicity, the PSO reduced the level of CYP2E1 enzyme and prevented further NAPQI formation. ALT and AST are serum markers of the liver damage.

After 12 h of paracetamol poisoning ALT and AST levels increases due to severity of damage [19]. İn our study showed that when paracetamol given ALT and AST levels increased proportional with time. our protective and treatment groups showed that ALT and AST levels decreased significantly, especially when low dose PSO was given. At the same time at paracetamol toxicity teatment there is no difference between currently used NAC and PSO application. Previous studies showed that increasing ALT and AST levels due to toxicity are significantly reduced by application of *Punica granatum* juice extract [12].

Oxidative stress is primer mechanism and responsible from resulting damage. When Oxidative stress depended peroxidation starts it spreads leading to membrane damage [20]. The MDA production is the result of the peroxidation of the membrane fatty acids and is used to determine the degree of lipid peroxidation [21]. Our study showed that Increased MDA levels due to paracetamole have been shown to significantly decrease with PSO application. Again in study, it has been shown that PSO reduces oxidative stress in parallel with our findings [22].

There are many antioxidants in our body that compensate for the harmful effects of increasing free radicals. Free oxygen radicals and antioxidant defense systems are normally in balance. Antioxidants are activated in response to increased oxidative stress in pathological conditions like as toxicity and infection. As we know GSH and SOD are the most powerful antioxidans. GSH detoxifies paracetamol by conjugating with the active metabolite NAPQI. However, increased NAPQI level causes damage by consuming glutathione deposits. Free $O^{\cdot}$ radicals were increased by NAPQI are scavenging by SOD. Because of the inadequate antioxidant response in the developing toxicity situations, agents that strengthen the antioxidant defense system are needed exogenously.

Today, *Punica granatum* juice is one of the most important antioxidant sources. Antidiarrheic [23], antifungal [24], anti-ulcer [25], antitumoral [26], antibacterial [27] effects have been shown in studies with *Punica granatum*. The core part of the *Punica granatum* fruit strengthen these antioxidants and antiinflammatory systems are rich in Punikalagins and ellagic acids and taken place at etnobotanic studies for many years. In our study decreased antioxidant defense systems due to high dose paracetamol increased significantly with PSO application. Here we describe the therapeutic and prophylactic efficacy of PSO, both by reducing the level of CYP2E1 and by exhibiting increased oxidative stress and reduced antioxidant response. Previous studies have shown that PSO inhibits damage by strengthening antioxidant defense mechanisms in organ toxins like liver and kidney.

Another important parameter which lead to paracetamol induced hepatotoxicity is inflammation. TNF-α, a pro-inflammatory cytokine, stimulates the acute phase reaction. İt induces Apoptotic cell death and inflammation during toxicity [28]. TNF-α regulates the initiation and maintenance of events related to inflammatory reactions by increasing the release of inflammatory cytokines, especially interleukin 1 beta, 6 and 8 [29,30]. In previous studies, It has been demonstrated that increased TNF-α levels correlate with paracetamol induced hepatotoxicity. Also, Ferah et al.[31] demonstrated protective effect of TNF-α inhibitors in paracetamol induced hepatotoxicity. Therefore, TNF-α is an another important parameter developing paracetamol induced toxicity. In our study, levels of TNF-α, which is associated with oxidative stress and inflammation increased in paralelly with paracetamol administration. At the same time, TNF-α expression up regulated in liver tissues via paracetamol administration. In this study, Increased TNF-α levels reduced with a strong antiinflammatory effect of PSO. Low dose PSO administration has strong inhibitory effect according to NAC and high dose PSO administration. In previous studies also have shown that PSO has TNF-α mediated antiinflammatory activity in various organ toxicities. Based all of these information, PSO has exert protective and therapotic effect by antiinflammtory activity

*Punica granatum* seed oil with strong antiinflammatory and antioxidant activity was statitistically significant improved hepatic necrosis due to toxicity with serum ALT, AST and tissue MDA levels by decrasing CYP2E1 enzyme, which is the primary role in the development of paracetamol toxicity. At the same time *Punica granatum* seed oil prevented the severity of damage by strengthening the antioxidant defense system and preventing inflammation and demonstrated protective effect. It has been demonstated that therapotic effects of *Punica granatum* seed oil is promising in the treatment of paracetamol intoxication when compared to NAC used routinly in paracetamol intoxications.

## Conflict of Interest

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work.

## REFERENCES

**1. Burke A, Smyth EM, Fitzgerald GA:** Analgesic-antipretic agents: Pharmacothherapy of Gout. **In,** Brunton LL, Lazo JS, Parker K (Eds): Goodman and Gilman's Pharmacological Basis of Therapeutics. McGraw-Hill, NewYork. 671-716. 2006.

**2. Peltek Kendirci HN, Yağlı Çolakoğlu E, Hızlı Ş, Koçak M, Saylam E, Polat E, Yılmaz G:** Evaluation of intoxication cases who referred to

pediatric emergency room in our hospital. *Turk J Pediatr Dis*, 5 (1): 29-35, 2011.

**3. Kahveci M, Celtik C, Karasalihoğlu S, Acunaş GB:** Bir üniversite hastanesi acil servisine başvuran çocukluk çağı zehirlenmelerin değerlendirilmesi. *Sted*, 13 (1): 19-21, 2004.

**4. Dahlin DC, Miwa GT, Lu AYH, Nelson SD:** N-Acetyl-P-Benzoquinone Imine: A cytochrome P-450-mediated oxidation product of acetaminophen. *Proc Natl Acad Sci*, 81 (5): 1327-1331, 1984.

**5. Jaeschke H, Bajt ML:** Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicol Sci*, 89 (1): 31-41, 2006. DOI: 10.1093/toxsci/kfi336

**6. Ritter JM, Flower R, Henderson G, Rang H:** Rang&Dale's Pharmacology. 7th ed., Elsevier Churchill Livingston, 698-709, 2011.

**7. American Academy of Pediatrics Committee on Drug:** Acetaminophen Toxicity in Children. *Pediatrics*, 108 (4): 1020-1024, 2001. DOI: 10.1542/peds.108.4.1020

**8. Lai MW, Klein-Schwartz W, Rodgers GC, Abrams YJ, Haber DA, Bronstein AC, Wruk KM:** 2005 Annual report of the American Association of Poison Control Centers' national poisoning and exposure database. *Clin Toxicol*, 44 (6-7): 803-932, 2006. DOI: 10.1080/15563650600907165

**9. Jurenka J:** Therapeutic applications of pomegranate *(Punica granatum L.)*: A Review. *Altern Med Rev*, 13 (2): 128-144, 2008.

**10. Lansky EP, Newman RA:** *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. *J Ethnopharmacol*, 109 (2): 177-206, 2007. DOI: 10.1016/j.jep.2006.09.006

**11. Kaufman M, Weisman Z:** Pomegranate oil analysis with emphasis on MALDI- TOF/MS triacylglycerol fingerprinting. *J Agric Food Chem*, 55, 10405-10413, 2007. DOI: 10.1021/jf072741q

**12. Pirinççioğlu M, Kızıl G, Kızıl M, Kanay Z, Ketani A:** The protective role of pomegranate juice against carbon tetrachloride-induced oxidative stress in rats. *Toxicol Ind Healthy*, 30 (10): 910-918, 2014. DOI: 10.1177/0748233712464809

**13. Palabiyik SS, Karakus E, Akpinar E, Halici Z, Bayır Y, Yayla M, Kose D:** The role of urotensin receptors in the paracetamol-induced hepatotoxicity model in mice: Ameliorative potential of urotensin II antagonist. *Basic Clin Pharmacol Toxicol*, 118 (2): 150-159, 2016. DOI: 10.1111/bcpt.12447

**14. Yayla M, Halici Z, Unal B, Bayir Y, Akpinar E, Gocer F:** Protective effect of Et-1 receptor antagonist bosentan on paracetamol induced acute liver toxicity in rats. *Eur J Pharmacol*, 726, 87-95, 2014. DOI: 10.1016/j.ejphar.2014.01.022

**15. Karcioglu SS, Palabiyik SS, Bayir Y, Karakus E, Mercantepe T, Halici Z, Albayrak A:** The role of RAAS ınhibition by aliskiren on paracetamol-ınduced hepatotoxicity model in rats. *J Cell Biochem*, 117, 638-646, 2016. DOI: 10.1002/jcb.25313

**16. Canayakin D, Bayir Y, Baygutalp Kilic N, Sezen Karaoğlan E, Atmaca HT, Kocak Ozgeris FB, Keles MS, Halici Z:** Paracetamol-induced nephrotoxicity and oxidative stress in rats: The protective role of *Nigella sativa*. *Pharm Biol*, 54 (10): 2082-2091, 2016. DOI: 10.3109/13880209.2016.1145701

**17. Tatar A, Yayla M, Kose D, Halici Z, Yoruk O, Polat E:** The role of endothelin 1 and endothelin receptor antagonists in allergic rhinitis inflammation: Ovalbumin induced rat model. *Rhinology*, 54 (3): 266-272, 2016. DOI: 10.4193/Rhin15.059

**18. Manyike PT, Kharasch ED, Kalhorn TF, Slattery JT:** Contribution of CYP2E1 and CYP3A to acetaminophen reactive metabolite formation. *Clin Pharmacol Ther*, 67 (3): 275-282, 2000. DOI: 10.1067/mcp.2000.104736

**19. Akther N, Andrabi K, Nissar A, Ganaie S, Chandan BK, Gupta AP, Khuswant M, Sultana S, Shawl AS:** Hepatoprotective activity of LC-ESI-MS standardized *Iris spuria* rhizome extract on its main bioactive constituents. *Phytomedicine*, 21 (10): 1202-1207, 2014. DOI: 10.1016/j.phymed.2014.04.007

**20. Slater TF:** Free-radical mechanisms in tissue injury. *Biochem J*, 222 (1): 1-15, 1984.

**21. Valenzuela A:** The biological significance of malondialdehyde determination in the assessment of tissue oxidative stress. *Life Sci*, 48 (4): 301-309, 1991.

**22. Faria A, Monteir R, Mateus N, Azevedo I, Calhau C:** Effect of pomegranat *(Punica granatum)* juice intake on hepatic oxidative stress, *Eur J Nutr*, 46 (5): 271-278, 2007. DOI: 10.1007/s00394-007-0661-z

**23. Das AK, Mandal SC, Banerjee SK, Sinha S, Das J, Saha BP, Pal M:** Studies on antidiarrhoeal activity of *Punica granatum* seed extract in rats. *J Ethnopharmacol*, 68 (1-3): 205-208, 1999. DOI: 10.1016/S0378-8741(99)00102-6

**24. Dutta BK, Rahman I, Das TK:** Antifungal activity of Indian plant extracts. *Mycoses*, 41 (11-12): 535-536, 1998. DOI**:** 10.1111/j.1439-0507.1998.tb00718.x

**25. Gharzouli K, Khennouf S, Amira S, Gharzouli A:** Effects of aqueous extracts from *Quercus ilex* l. root bark, *Punica granatum* l. fruit peel and *Artemisia herba-alba* Asso leaves on ethanol-induced gastric damage in rats. *Phytother Res*, 13 (1): 42-45, 1999. DOI: 10.1002/(SICI)1099-1573(199902)13:1<42::AID-PTR383>3.0.CO;2-2

**26. Afaq F, Saleem M, Krueger CG, Reed JD, Mukhtar H:** Anthocyanin- and hydrolyz tannin-rich P*unica granatum* fruit extract modulates MAPK and NF-kappaB pathways and inhibits skin tumorigenesis in CD-1 mice. *Int J Cancer*, 113 (3): 423-433, 2005. DOI: 10.1002/ijc.20587

**27. Prashanth D, Asha MK, Amit A:** Antibacterial activity of *Punica granatum*. *Fitoterapia*, 72 (2): 171-173, 2001. DOI :10.1016/S0367-326X(00)00270-7

**28. Kalfa M, Aksu K:** Treatment with tumor necrosis factor-alpha antagonists and infections. *READ Derg*, 3 (3-4): 49-56, 2011. DOI: 10.2399/raed.11.009

**29. Del Campo JA, Gallego P, Grande L:** Role of inflammatory response in liver diseases: Therapeutic strategies. *World J Hepatol,* 10 (1):1-7, 2018. DOI: 10.4254/wjh.v10.i1.1

**30. Kandır S, Keskin E:** Serum IL-1β, IL-6, IL-10 and TNF-α levels in thyroidectomized rats. *Kafkas Univ Vet Fak Derg,* 22 (2): 297-300, 2016. DOI: 10.9775/kvfd.2015.14371

**31. Ferah I, Halici Z, Bayir Y, Demirci E, Unal B, Cadirci E:** The role of infliximab on paracetamol-induced hepatotoxicity in rats. *Immunopharmacol Immunotoxicol*, 35 (3): 373-381, 2013. DOI: 10.3109/08923973.2013.775589

Kafkas Univ Vet Fak Derg
24 (5): 735-742, 2018
DOI: 10.9775/kvfd.2018.19884

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Optimization of Culture Conditions for High Cell-Density Fermentation of Bovine *Escherichia coli*

Jishan LIU [1,2,†] Shuguang LI [1,2,†] Xiaojing XIA [3,†,a] Hongmei HU [4] Qiang FU [1]
Yueqiang XIAO [1] Guanggang QU [1] Zhiqiang SHEN [1,2] Likun CHENG [1,2]

[†] *Those authors contributed equally to this work*
[1] Post-doctoral Scientific Research Workstation, Shandong Binzhou Animal Science and Veterinary Medicine Academy, Binzhou 256600, P.R. CHINA
[2] Shandong Lvdu Bio-science and Technology Co. Ltd., Binzhou 256600, P.R. CHINA
[3] College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, P.R. CHINA
[4] Institution of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Science, Jinan 250000, P.R. CHINA
[a] ORCID: 0000-0001-7717-8411

**Article Code: KVFD-2018-19884 Received: 31.03.2018 Accepted: 01.07.2018 Published Online: 02.07.2018**

**How to Cite This Article**

**Liu J, Li S, Xia X, Hu H, Fu Q, Xiao Y, Qu G, Shen Z, Cheng L:** Optimization of culture conditions for high cell-density fermentation of bovine *Escherichia coli*. *Kafkas Univ Vet Fak Derg*, 24 (5): 735-742, 2018. DOI: 10.9775/kvfd.2018.19884

## Abstract

Bovine *Escherichia coli* infection, which causes major economic losses to the cattle industry each year, can be prevented by administering formalin-inactivated vaccine. However, to enhance the application of this vaccine, the cell density of the formalin-inactivated *E. coli* should be boosted. This can be achieved by reducing the accumulation of acetate, a primary inhibitory metabolite in *E. coli* fermentation. To this end, the present study investigated the effect of pH, dissolved oxygen (DO) levels, and feeding methods on bovine *E. coli* fermentation, and developed two-stage pH and DO control strategies and a combined pH- and DO-mediated feeding strategy for the fermentation. The optimized conditions for Bovine *E. coli* were pH 7.0 at 0-10 h, 6.5 at 10-24 h; DO 50% at 0-10 h, 30% at 10–24 h; pH and DO feedback feeding at 0-10 h and 10-24 h, respectively. With Bovine E. coli fermentation under the optimized conditions, the acetate accumulation was 1.12 g/L and the cell density was 36.47 (OD600), which were 59.12% lower and 77.29% higher than these with the original conditions (pH 7.0; DO 20%; residual glucose concentration maintained at 2.0 g/L). After analyzing the main nodes of acetate synthesis, it was found that the lower carbon flux enters the Embden-Meyerhof pathway. Under the optimized conditions, the pyruvate flux and acetyl-CoA synthesis were low, and much of the acetyl-CoA participated in the tricarboxylic acid cycle. The extracellular acetate flux was 8.3%, which was 65.13% lower than in the original conditions.

***Keywords:*** *Escherichia coli, Acetate, Dissolved oxygen, Feeding strategy, Metabolic flux distribution*

# Sığırlarda *Escherchia coli*'nin Yüksek Hücre Yoğunluğunda Fermentasyonu İçin En Uygun Kültür Koşullarının Sağlanması

## Öz

Sığırlardaki *Escherchia coli* enfeksiyonları her yıl sığırcılık sektörünü büyük çaplı maddi zarara uğratmakla birlikte, formalinle etkisizleştirilmiş bakteriden yapılan aşıyla önlenebilmektedir. Ancak, bu aşının etkisini artırmak için formalinde etkisizleştirmiş *E. coli* hücre yoğunluğunun artırılması gerekmektedir. Bunu elde etmek için *E. coli* fermentasyonunu baskılayan birincil bir metabolik ürün olan asetatın birikimi azaltılmalıdır. Bu amaçla, mevcut çalışmada pH, çözünmüş oksijen (ÇO) düzeyleri ve beslenme şeklinin sığırlardaki *E. coli* fermentasyonuna etkileri araştırılmış ve iki aşamalı bir pH ve ÇO kontrolü stratejisi geliştirilerek pH ve ÇO düzeylerini birlikte değerlendiren bir beslenme planı ile fermentasyonun artırılması yoluna gidilmiştir. Sığır *E. coli* için optimize edimiş koşullar pH için 0-10 saat aralığında 7.0, 10-24 saat aralığında 6.5; ÇO için 0-10 saat aralığında %50, 10-24 saat aralığında %30, pH ve ÇO temelinde besleme için sırasıyla 0-10 saat ve 10-24 saat olarak belirlendi. Optimize koşullar altında sığır *E. coli* fermentasyonu ile birlikte asetat birikimi 1.12 g/L olup hücre yoğunluğu 36.47 (OD600) olarak tespit edildi. Bu değerler orjinal koşullardan (pH 7.0; ÇO %20; kalıntı glukoz derişimi 2.0 g/L'de tutulmuş) sırasıyla %59.12 daha düşük ve %77.29 daha yüksek bulunmuştur. Asetat sentezinin temel unsurları incelendiğinde, daha az olan karbon akışının Embden-Meyerhof yolağına kaydığı saptanmıştır. En uygun koşullarda pirüvat akımı ve asetil CoA sentezi düşük iken, asetil CoA'nın büyük çoğunluğu trikarboksilik asit döngüsüne katılmıştır. Hücre dışı asetat akımı %8.3 olup, özgün koşullara göre %63.13 daha düşük çıkmıştır.

***Anahtar sözcükler:*** *Escherichia coli, Asetat, pH, Çözünmüş oksijen, Besleme stratejisi, Metabolik akım dağılımı*

**İletişim (Correspondence)**
+86-543-3252652 (Z. Shen); +86-13854359698 (L. Cheng) Fax: +86-543-3252652 (Z. Shen); +86-543-3418279 (L. Cheng)
m15169958288@163.com (Z. Shen); clksd@126.com (L. Cheng)

# INTRODUCTION

Bovine *Escherichia coli*, which is present in most cattle producing countries, is the causative agent of septicemia, enterotoxemia and meningitis, leading to major economic losses to the cattle industry each year [1]. Protection against bovine *E. coli* infection is provided by formalin-inactivated bacterins [2], but the low cell density of bovine *E. coli* inhibits the application of this vaccine. Improving the cell density of bovine *E. coli* would enhance the applicability of the vaccine to the cattle industry. The cell density is limited by acetate, a primary inhibitory metabolite that accumulates in *E. coli* cultures, which was caused of acetate inhibition for DNA transcription, RNA translation, protein stability and compound synthesis [3]. The acetate accumulation can be reduced by optimizing the culture conditions and genetically modifying the cells [4-6]. In *E. coli*, acetate is formed via two pathways: the phosphotransacetylase-acetate kinase (Pta-AckA) pathway and the pyruvate oxidase B (PoxB) pathway, which use acetyl-CoA and pyruvate as substrate, respectively [7,8]. Conversely, reducing the carbon flux in the Embden-Meyerhof pathway (EMP) reducing of the acetate excretion by lowering the pyruvate production [3,9].

Acetate synthesis and its metabolic flux distribution are affected from the values of the culture parameters [8,10]. At the pH extremes of *E. coli* growth, (4.4 and 9.2; note the wide range), the *E. coli* cells release several enzymes related to acetate formation, including the proteins encoded by pta, sucB, and sucC [11]. Acetate secretion by *E. coli* is 6 g/L at pH 6.0 and 6.5, increasing to 12 g/L at pH 7.5 [12]. The dissolved oxygen (DO) level critically determines the acetate formation and cell density in *E. coli* cultivations. Increasing the DO level decreases the acetate accumulation by enhancing the TCA cycle activity and altering the transcription levels of genes associated with glucose and acetate metabolism, whereas low DO levels increase the acetate accumulation [5]. However, increasing the DO concentration during *E. coli* growth stimulates the intracellular accumulation of reactive oxygen species (ROS). High ROS levels are known causes of stress in *E. coli*, causing irreversible damage to their cellular components [13]. By adjusting the glucose-feeding rate in a fed-batch fermentation process, the glucose concentration can be maintained below the critical value that triggers acetate formation [14,15]. The pH-stat activates the addition of a nutrient when the pH increases, maintaining the growth rate and glucose concentration significantly below the threshold for acetate production [16,17] An appropriate feeding method is important for reducing the acetate accumulation and increasing the cell density. The present study investigated the effect of pH, DO, and feeding methods on bovine *E. coli* fermentation, then developed two-stage pH and DO control strategies and a combined pH- and DO-mediated feeding strategy in bovine *E. coli* fermentation. Finally, to elucidate the driving mechanisms of low acetate accumulation and high cell density, it analyzed the flux distribution of the important nodes of acetate synthesis under the original and optimized culture conditions.

# MATERIAL and METHODS

### *Microorganism and Medium*

The bovine *E. coli* strain used in this study was obtained in an earlier study in our laboratory, and stored at the Culture Collection of Shandong Binzhou Animal Science and Veterinary Medicine Academy (Collection number: TCCC17023). During storage, the organism was maintained on Luria-Bertani agar.

The seed medium contains the following ingredients: glucose 5.0 g/L, yeast extract 10.0 g/L, $(NH_4)_2SO_4$ 4.0 g/L, sodium citrate 2.0 g/L, $MgSO_4{\cdot}7H_2O$ 1.5 g/L, $KH_2PO_4$ 2.0 g/L, $FeSO_4{\cdot}7H2O$ 0.15 g/L, vitamin $B_1$ 0.05 g/L. The bovine *E. coli* was cultivated in fermentation medium with the following constituents: glucose 5.0 g/L, yeast extract 2.0 g/L, $(NH_4)_2SO_4$ 3.0 g/L, sodium citrate 1.5 g/L, $MgSO_4{\cdot}7H_2O$ 1.5 g/L, $KH_2PO_4$ 2.0 g/L, $FeSO_4{\cdot}7H_2O$ 0.1 g/L. The pH of both seed and fermentation media was adjusted to 7.0 using 4 mol/L NaOH.

### *Culture Conditions*

Fermentations were performed in 10-L fermenters (Biotech -2015 Bioprecess controller, Bailun, Shanghai, China). A 500-mL baffled flask (Shuniu, Chengdu, China) containing 100 mL seed medium was inoculated with a single colony of bovine *E. coli*, then cultivated at 35°C shaking at 200 rpm for 12 h. A 100-mL inoculum of this culture was inoculated aseptically (2% v/v) into 5-L of fermentation medium in a 10-L fermenter. The temperature was maintained at 35°C during the cultivation period. Unless otherwise specified, the pH was adjusted to 7.0 with 25% ammonium hydroxide (w/w), and the DO level was maintained at 20%. When the initial glucose supply was depleted, the residual glucose concentration was maintained at 2.0 g/L by adding glucose solution (50% w/v) to the fermenters.

### *pH Control Strategy*

The pH levels in the fermentation process were measured automatically by pH electrodes attached to the fermenters, and were controlled at their designated values (6.0, 6.5, 6.8, 7.0, 7.2, and 7.5) using 25% ammonium hydroxide. By maintaining the pH at different levels, we could investigate the effects of pH on bovine *E. coli* fermentation. From the results, a two-stage pH control was developed for the bovine *E. coli* fermentation.

### *DO Control Strategy*

The DO concentrations in the fermentation process were measured automatically by DO electrodes attached to

the fermenters, and were controlled at their designated values (5%, 20%, 30%, 50%, and 100%) by adjusting the agitation and aeration rates. By maintaining the DO at different levels, we could investigate the effects of DO on bovine *E. coli* fermentation. From the results, a two-stage DO control strategy was developed for the bovine *E. coli* cultivation.

### Feeding Strategies

The feeding strategy in the bovine *E. coli* fermentation was varied as intermittent feeding, pH feedback feeding, DO feedback feeding and a glucose-stat feeding strategy. During intermittent feeding, glucose (3 g/L) was added to the fermenter every 2 h. In the pH and DO feedback feeding strategies, the glucose solution was fed when the pH or DO level rose above its set value. The glucose solution automatically entered the fermenter through a peristaltic pump. In the glucose feeding strategy, the glucose concentration was controlled at 0.5 g/L by adjusting the feeding rate of the glucose solution.

### Analysis of Fermentation Products

The DO, pH and temperature were measured automatically by electrodes attached to the fermenters. The cell optical density (OD) was monitored at 600 nm in a spectrophotometer (722N, INESA, China) [18]. The glucose concentration was monitored using an SBA-40E biosensor analyzer (Biology Institute of Shandong Academy of Sciences, Jinan, China). The acetate concentration was determined by high-performance liquid chromatography using an Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA) equipped with an Aminex HPX-87H (Bio-Rad Laboratories, Inc, USA) [19].

### Analysis of Metabolic Flux

The metabolic flux distribution of acetate synthesis during the later cultivation period of the bovine *E. coli* fermentation under different culture conditions was calculated by MATLAB 7.0. The results were based on the analysis of metabolic flux balance and stoichiometry [3,20].

### Statistical Analysis

All experiments were conducted in triplicate, and the data were averaged and presented as the mean±standard deviation. One-way analysis of variance followed by Dunnett's multiple comparison test were used to determine significant difference [21]. Statistical significance was defined as $P<0.05$.

## RESULTS

### pH Control Strategy of Bovine E. coli Fermentation

*Effect of pH: Fig. 1* compares the cell densities and accumulated acetate concentrations at different pH levels in the bovine *E. coli* fermentation. The acetate concentration increased with increasing pH, reaching 2.14 g/L at pH 6.5 and 3.53 g/L at pH 7.5. The cell density increased with pH up to 7.0, then decreased at higher pH. At pH 7.0, the acetate concentration was 2.74 g/L. The cell density was highest at pH 7.0 (20.57), 35.06% higher than at pH 6.5 (15.23), and 44.15% higher than at pH 7.5 (14.27).

*Two-stage pH Control Strategies:* Based on the varying pH results, we applied four two-stage pH control strategies in the bovine *E. coli* fermentation: strategy I (6.5 at 0-10 h, 7.0 at 10-24 h), strategy II (6.5 at 0-10 h, 7.2 at 10-24 h), strategy III (7.0 at 0-10 h, 6.5 at 10-24 h), and strategy IV (7.2 at 0-10 h, 6.5 at 10-24 h). *Fig. 2* shows the cell densities and acetate concentrations under the different pH controls in the bovine *E. coli* fermentation. When the pH was controlled at a higher level during the early fermentation stage than during the late stage, the cell growth rate was improved and the cell density was increased, but the acetate accumulation was high. The final cell densities (acetate accumulations) in strategies III and IV were 23.47 (2.45 g/L) and 20.58 (2.66 g/L), respectively. When the pH was lower during the early cultivation stage than during the late stage, less acetate was excreted, but the growth rate and cell density were comparatively low. The cell densities (acetate

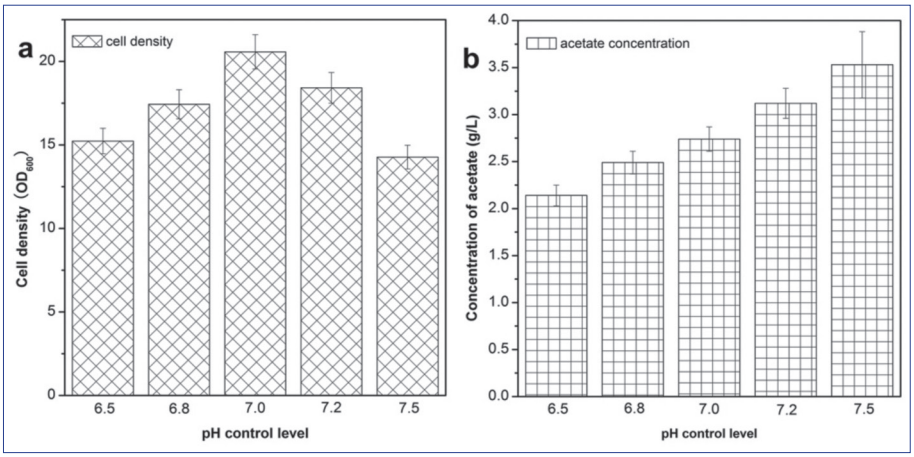



**Fig 1.** Effect of pH on cell density (a) and acetate accumulation (b) in bovine *E. coli* fermentation ($P<0.05$)

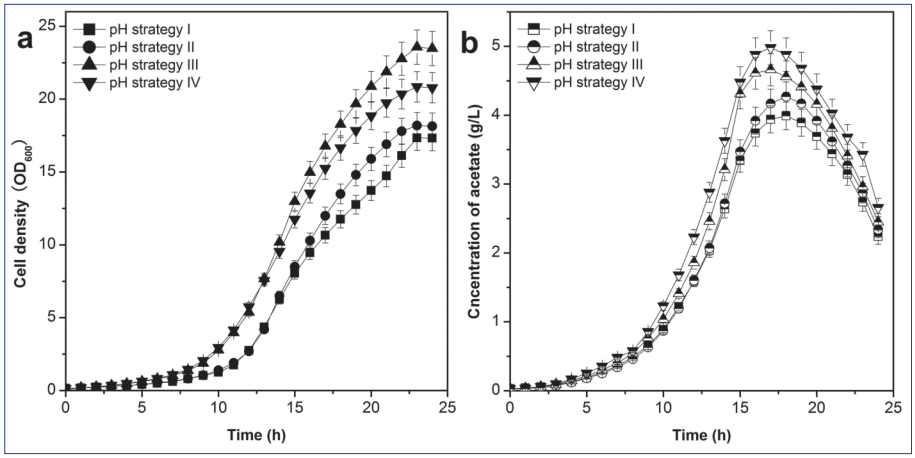
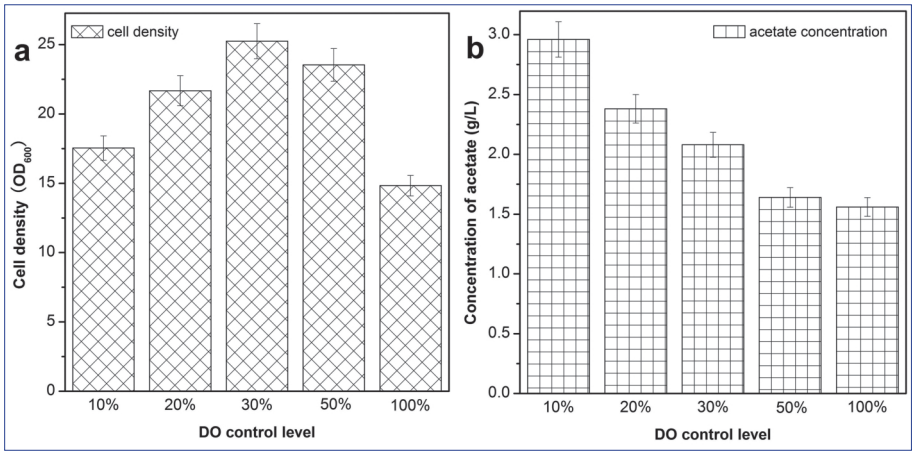



**Fig 2.** Effect of two-stage pH control strategy on cell density (a) and acetate accumulation (b) in bovine *E. coli* fermentation ($P$<0.05)

**Fig 3.** Effect of DO level on cell density (a) and acetate accumulation (b) in bovine *E. coli* fermentation ($P$<0.05)



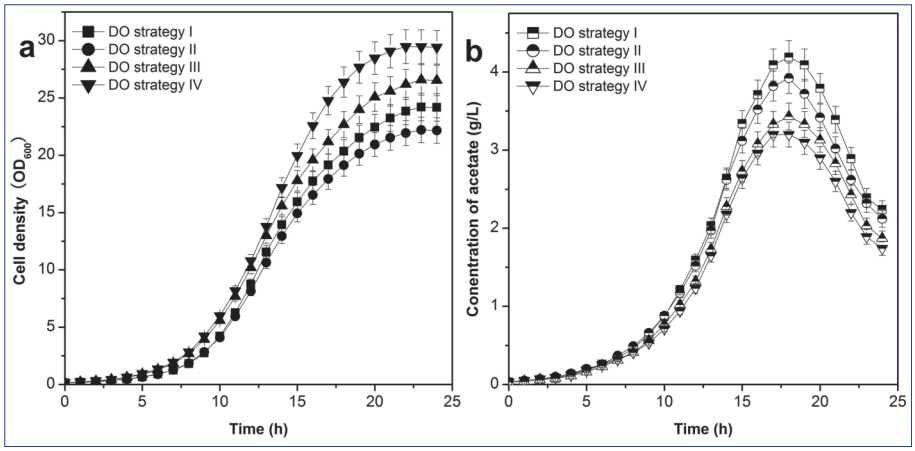




**Fig 4.** Effect of two-stage DO control strategy on cell density (a) and acetate accumulation (b) in bovine *E. coli* fermentation ($P$<0.05)

accumulations) in strategies I and II were 17.34 (2.96 g/L) and 18.14 (2.34 g/L), respectively. Strategy III achieved the highest cell density, but accumulated more acetate than strategies I and II.

### *DO Control Strategy of Bovine E. coli Fermentation*

*Effect of DO Level:* The cell densities and acetate accumulations at different DO levels in the bovine *E. coli* fermentation are displayed in *Fig. 3*. The acetate accumulation decreased with increasing DO level, and the cell density was maximized at 30% DO. Comparatively, the cell density at 30% DO (25.24) was 43.89% and 70.19% higher than that at 10% DO (17.54) and 100% DO (14.83), respectively. The acetate accumulation at 30% DO was 2.08 g/L, 42.31% lower and 33.33% higher than that at 10% DO (2.96 g/L) and 100% DO (1.56 g/L), respectively. A relatively high DO level boosted the cell density while also reducing the acetate excretion.

*Two-stage DO Control Strategies:* Based on the above results, we trialed four two-stage control strategies in the

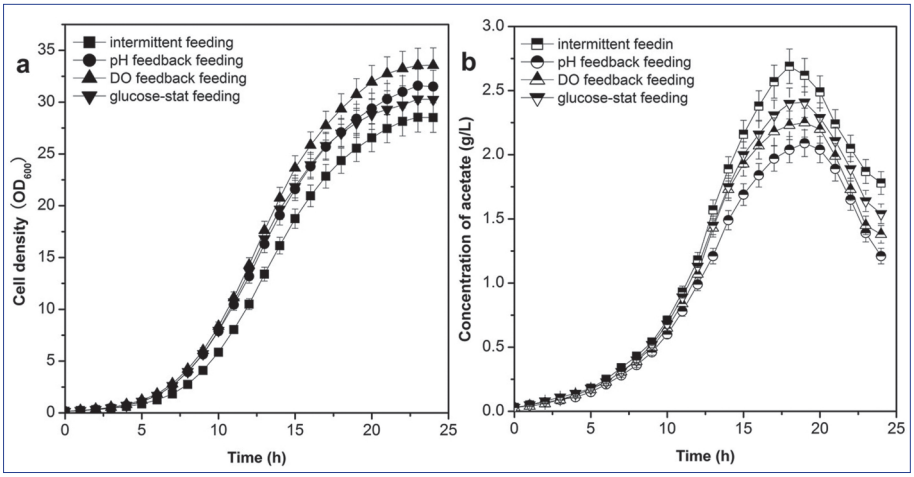



**Fig 5.** Effect of feeding strategy on cell density (a) and acetate concentration (b) in bovine *E. coli* fermentation ($P<0.05$)

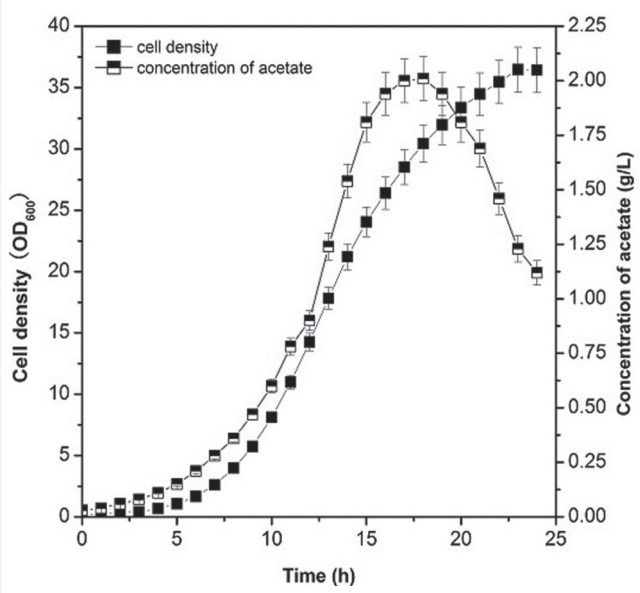



**Fig 6.** Application of combined pH and DO feedback feeding in the bovine *E. coli* fermentation ($P<0.05$)

in *Fig. 4*. When the DO level was higher during the early fermentation stage than during the later stage, the cell density was increased and the acetate accumulation was reduced. DO strategy IV achieved the highest cell density (29.47, 10.89% higher than in Strategy III) and lowest acetate accumulation (1.74 g/L, 6.95% lower than in strategy III). The cell densities (acetate accumulations) in DO strategies I and II were 24.21 (2.24 g/L) and 22.21 (2.12 g/L), respectively.

### *Feeding Strategy of Bovine E. coli Fermentation*

*Effect of Feeding Strategy: Fig. 5* shows the cell densities and acetate concentrations in bovine *E. coli* fermentation under four feeding strategies: intermittent feeding, pH feedback feeding, DO feedback feeding and glucose-stat feeding. Both the growth and acetate excretion depended on the feeding strategy. DO feedback feeding maximized the cell density (33.56), but did not minimize the acetate accumulation (at the end of the fermentation, the acetate concentration was 1.38 g/L). The pH feedback strategy minimized the acetate excretion (1.21 g/L), but reduced

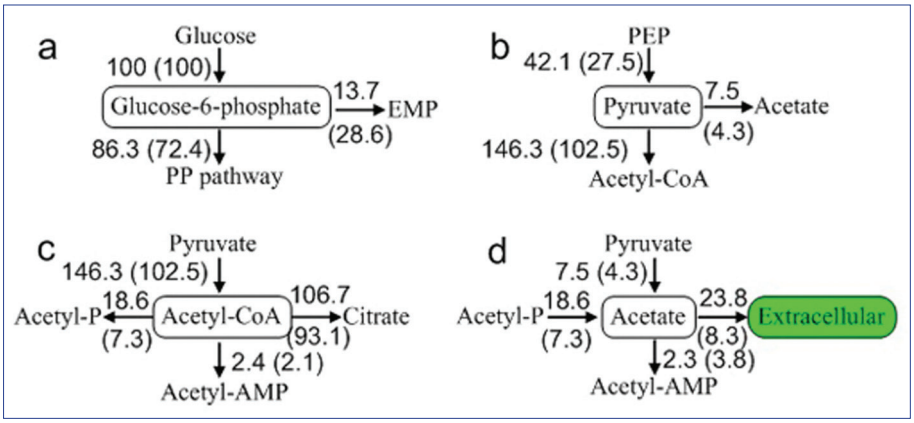



**Fig 7.** Metabolic flux distributions of important nodes for acetate synthesis under the original and optimized conditions during the later fermentation phase (20–24 h) of bovine *E. coli* fermentation

bovine *E. coli* fermentation: strategy I (20% at 0-10 h, 30% at 10-24 h), strategy II (20% at 0-10 h, 50% at 10-24 h), strategy III (50% at 0-10 h, 20% at 10-24 h), and strategy IV (50% at 0-10 h, 30% at 10-24 h). The results are presented

the cell density to 31.59. The cell densities (acetate accumulations) in the intermittent and glucose-stat feeding strategies were 28.54 (1.78 g/L) and 30.29 (1.54 g/L), respectively.

*Application of a Combined Feeding Strategy:* Based on the analysis of the fermentation process and the results of different feeding strategies, we applied a combined feeding strategy of pH (0-10 h) and DO (10-24 h) feedback feeding in the bovine *E. coli* fermentation. The time-dependent cell density and acetate concentration in this combined feeding strategy are presented in *Fig. 6*. The combined feeding reduced the acetate accumulation to 1.12 g/L and increased the cell density to 36.47.

*Metabolic Flux Distribution of Main Nodes for Acetate Synthesis:* After analyzing the effect of pH, DO and feeding strategy on bovine *E. coli* fermentation, the optimized fermentation conditions were obtained as follows: pH 7.0 at 0-10 h and 6.5 at 10-24 h; DO 50% at 0-10 h and 30% at 10-24 h; pH feedback feeding at 0-10 h and DO feedback feeding at 10-24 h. *Fig. 7* shows the flux distributions of the important nodes for acetate synthesis under the original conditions (pH 7.0; DO 20%; residual glucose concentration maintained at 2.0 g/L) and the optimized conditions. Under the optimized conditions, less carbon flux entered the EMP pathway, so the pyruvate flux (27.5%) was 34.68% lower than under the original conditions. Under the optimized (original) conditions, the flux of acetyl-CoA from pyruvate was 102.5% (146.3%), and 90.74% (72.93%) of the acetyl-CoA entered the TCA cycle. The flux of acetate synthesis from pyruvate and acetyl-CoA was 11.6% under the optimized conditions, 55.56% lower than under the original conditions; consequently, the acetate accumulation was lowered. The extracellular acetate flux under the optimized conditions was 8.3%, 65.13% lower than under the original conditions.

## DISCUSSION

pH exerts complex effects because it influences the solubility of nutrients and trace elements, and the cellular metabolism in general [22]. pH homeostasis is important for the function and stability of all cellular enzymes [23]. The main regulator of pH is the *pta* gene, which is involved in acetate synthesis. At high pH, this gene is induced [11], and its product accumulates to high acetate concentrations. Components of the TCA cycle, such as *suc*B and *suc*C, are induced at low pH in culture, encouraging exploitation of the high proton potential and increasing the capacity of the TCA cycle. Consequently, the concentrations of acetyl-CoA and acetate decrease [10,11]. In the present study, the acetate concentration was lower in strategies I and II than in strategies III and IV because the pH was maintained at low levels during the early cultivation period, but the cell density was also low. High $H^+$ concentration inhibits the activity of phosphofructokinase, interdicting the EMP pathway and ultimately disrupting the cell growth [24]. Controlling the pH at 7.0 (0-10 h) and 6.5 (10-24 h) increased the cell density of bovine *E. coli*.

Raising the DO level reduced the acetate accumulation in the bovine *E. coli* fermentation. The transcription levels of the gluconeogenesis (*pck*A, *pps*A) and anaplerotic pathway (*ppc*, *sfc*A) genes are reportedly lower at low DO levels than at high DO levels, favoring the accumulation of pyruvate and acetyl-CoA, and increasing the acetate accumulation through the Pta-AckA and PoxB pathways [5]. At high DO levels, the high transcription levels of gluconeogenic genes increase the conversion of pyruvate to glucose by gluconeogenesis, reducing the pyruvate concentration and consequently reducing the acetate accumulation [4]. The acetate concentration was minimized at 100% DO, but the cell density was lowest under this condition, possibly because the high ROS contents at 100% DO caused irreversible damage to the cellular components [13]. Maintaining a moderately high DO level can both decrease the acetate accumulation and increase the cell density. Among the two-stage DO control strategies, the acetate excretion was suppressed by maintaining the DO level at 50% during the early fermentation phase, whereas DO levels of 20% and 30% avoided the formation of ROS. The highest cell density was obtained in strategy IV (50% at 0-10 h, 30% at 10-24 h).

Acetate excretion can be prevented by maintaining the glucose concentration below the critical level for acetate synthesis, and the acetate accumulation decreases as the glucose concentration lowers [25]. An intermittent feeding and glucose-stat feeding strategy increases the acetate accumulation by maintaining a high glucose concentration, and also reduces the cell density [15]. For reducing acetate formation during a fed-batch process, the changes in DO or pH are easily monitored online [17]. In a previous study, a pH-based or DO-based feeding strategy was found to maintain the glucose concentration and thereby reduce the acetate excretion [26]. The pH and DO feedback feeding strategies also lowered the acetate concentration and raised the cell density in the present study of bovine *E. coli* fermentation. Applying the DO feedback feeding in the later fermentation period satisfies the oxygen requirements of the cells, enhancing the balance between growth rate and oxygen consumption, and inhibiting the formation of acetate [3,27]. Consequently, when the pH and DO feedback feeding strategies were combined, the cell density increased to 36.47 while the acetate accumulation decreased to 1.12 g/L.

The metabolic flux distribution of acetate synthesis depends on the culture conditions [8]. Under the optimized conditions, less carbon flux enters the EMP pathway, avoiding "overflow" of the central metabolic pathway and decreasing the formations of pyruvate and acetyl-CoA, thereby reducing the acetate excretion [3]. The acetate-synthesis flux from pyruvate and acetyl-CoA was lowered by DO feedback feeding at 30% DO and pH 6.5. At pH 6.5, the acetyl-CoA flux mainly participates in the TCA cycle because the TCA cycle capacity is enhanced at this level [11]. The higher transcription levels of acetyl-CoA

synthetase (Acs) in *E. coli* significantly decrease the acetate accumulation and improve the efficiency of acetate assimilation. High DO level increases the expression of Acs and the optimal conditions enhance the flux from acetate to acetyl-AMP [28]. In the present study, the optimized conditions lowered the acetate-synthesis flux and raised the acetate-assimilation flux. Consequently, the flux of extracellular acetate was 8.3%, which was 65.13% lower than under the original conditions.

In this study, the acetate accumulation was decreased by optimizing the pH and DO levels and the feeding strategy in a bovine *E. coli* fermentation, achieving a high cell-density cultivation of bovine *E. coli*. Under the optimized conditions (pH 7.0 at 0-10 h, 6.5 at 10-24 h; DO 50% at 0-10 h, 30% at 10-24 h; pH and DO feedback feeding at 0-10 h and 10-24 h, respectively), the cell density reached 36.47 ($OD_{600}$) and the acetate accumulation decreased to 1.12 g/L. These values were 77.29% higher and 59.12% lower, respectively, than under the original conditions (pH 7.0; DO 20%; residual glucose concentration maintained at 2.0 g/L). In addition, the optimized conditions reduced the carbon flux entering the EMP pathway and minimized the fluxes of pyruvate and acetyl-CoA synthesis. The extracellular acetate flux (8.3%) was 65.13% lower than under the original conditions. By reducing the acetate accumulation and boosting the cell growth, we can improve the applicability of the bovine *E. coli* vaccine, and better protect the cattle industry from *E. coli* infection. The developed approach also provides a theoretical foundation for decreasing the acetate accumulation in high-cell density cultivations of other bacteria.

## Acknowledgments

This work was supported by the <Development of Science and Technology Plan Program of Binzhou> under Grant <2015ZC0107>, and <Innovation Program of Modern Agricultural Industry System of Shandong> under Grant <SDAIT-09-12>. We also thank Leonie Z. Pipe, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

## Disclosure Statement

No potential conflict of interest was reported by the authors.

## Funding

Development of Science and Technology Plan Program of Binzhou (No. 2015ZC0107), Innovation Program of Modern Agricultural Industry System of Shandong (No. SDAIT-09-12), the National Key Research and Development Program of China (No. 2016YFD0500708), the National Natural Science Foundation of China (No. 31702263), and the China Postdoctoral Science Foundation (No. 2017M622346).

## REFERENCES

**1. Zhao J:** The study of prevention and cure for calf *Escherichia coli* infection. *China Qinghai J Ani Veter Sci*, 45 (4): 59-60, 2015.

**2. Miniats OP, Smart NL, Ewert E:** Vaccination of gnotobiotic primary specific pathogen-free pigs against *Haemophilus parasuis*. *Can J Vet Res*, 55, 33-36, 1991.

**3. Castaño-Cerezo S, Pastor JM, Renilla S, Bernal V, Iborra JL, Cánovas M:** An insight into the role of phosphotransacetylase (pta) and the acetate/acetyl-CoA node in *Escherichia coli*. *Microb Cell Fact*, 8, 54, 2009. DOI: 10.1186/1475-2859-8-54

**4. Eiteman MA, Altman E:** Overcoming acetate in *Escherichia coli* recombinant protein fermentations. *Trends Biotechnol*, 24 (11): 530-536, 2006. DOI: 10.1016/j.tibtech.2006.09.001

**5. Phue JN, Shiloach J:** Impact of dissolved oxygen concentration on acetate accumulation and physiology of *E. coli* BL21, evaluating transcription levels of key genes at different dissolved oxygen conditions. *Metab Eng*, 7, 353-363, 2005. DOI: 10.1016/j.ymben.2005.06.003

**6. Lu N, Zhang B, Cheng LK, Wang JS, Zhang SL, Fu SJ, Xiao YQ, Liu HY:** Gene modification of *Escherichia coli* and incorporation of process control to decrease acetate accumulation and increase L-tryptophan production. *Ann Microbiol*, 67, 567-576, 2017. DOI: 10.1007/s13213-017-1289-8

**7. De Anda R, Lara AR, Hernández V, Hernández-Montalvo V, Gosset G, Bolívar F, Ramírez OT:** Replacement of the glucose phosphotransferase transport system by galactose permease reduces acetate accumulation and improves process performance of *Escherichia coli* for recombinant protein production without impairment of growth rate. *Metab Eng*, 8 (3): 281-290, 2006. DOI: 10.1016/j.ymben.2006.01.002

**8. Zhao CG, Cheng LK, Xu QY, Wang J, Shen S, Chen N:** Improvement of the production of L-tryptophan in *Escherichia coli* by application of a dissolved oxygen stage control strategy. *Ann Microbiol*, 66, 843-854, 2016. DOI: 10.1007/s13213-015-1172-4

**9. Chang DE, Shin S, Rhee JS, Pan JG:** Acetate metabolism in a pta mutant of *Escherichia coli* W3110: Importance of maintaining acetyl coenzyme a flux for growth and survival. *J Bacteriol*, 181 (21): 6656-6663, 1999.

**10. Cheng LK, Wang J, Xu QY, Zhao CG, Shen ZQ, Xie XX, Chen N:** Strategy for pH control and pH feedback-controlled substrate feeding for high-level production of L-tryptophan by *Escherichia coli*. *World J Microbiol Biotechnol*, 29, 883-890, 2013. DOI: 10.1007/s11274-012-1243-7

**11. Stancik LM, Stancik DM, Schmidt B, Barnhart DM, Yoncheva YN, Slonczewski JL:** pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. *J Bacteriol*, 184 (15): 4246-4258, 2002. DOI: 10.1128/JB.184.15.4246-4258.2002

**12. Kleman GL, Strohl WR:** Acetate metabolism by *Escherichia coli* in high-cell-density fermentation. *Appl Environ Microbiol*, 60 (11): 3952-3958, 1994.

**13. Baez A, Shiloach J:** *Escherichia coli* avoids high dissolved oxygen stress by activation of SoxRS and manganese superoxide dismutase. *Microb Cell Fact*, 12, 23, 2013. DOI: 10.1186/1475-2859-12-23

**14. Cheng HC, Wang FS:** Optimal process solvent design for ethanol extractive fermentation with cell recycling. *Biochem Eng J*, 41, 258-265, 2008. DOI: 10.1016/j.bej.2008.05.004

**15. Cheng LK, Wang J, Xu QY, Xie XX, Zhang YJ, Zhao CG, Chen N:** Effect of feeding strategy on L-tryptophan production by recombinant *Escherichia coli*. *Ann Microbiol*, 62, 1625-1634, 2012. DOI: 10.1007/s13213-012-0419-6

**16. Johnston W, Cord-Ruwisch R, Cooney M:** Industrial control of recombinant *E. coli* fed-batch culture: New perspectives on traditional controlled variables. *Bioprocess Biosyst Eng*, 25 (2): 111-120, 2002. DOI: 10.1007/s00449-002-0287-8

**17. Shen YL, Zhang Y, Sun AY, Xia XX, Wei DZ, Yang SL:** High-level production of soluble tumor necrosis factor-related apoptosis-inducing ligand (Apo21/TRAIL) in high-density cultivation of recombinant *Escherichia coli* using a combined feeding strategy. *Biotechnol Lett*, 26, 981-984, 2004.

**18. Cheng LK, Wang J, Fu Q, Miao LZ, Yang XY, Li SG, Li F, Shen ZQ:**

Optimization of carbon and nitrogen sources and substrate feeding strategy to increase the cell density of *Streptococcus suis*. *Biotechnol Biotechnol Equip*, 29 (4): 779-785, 2015. DOI: 10.1080/13102818.2015.1039465

**19 Lin CW, Cheng LK, Wang J, Zhang SL, Fu Q, Li SG, Li F, Han WY, Shen ZQ:** Optimization of culture conditions to improve the expression level of beta1-epstion toxin of *Clostridium perfringens* type B in *Escherichia coli*. *Biotechnol Biotechnol Equip*, 30 (2): 324-331, 2016. DOI: 10.1080/13102818.2015.1126201

**20. Rabinowitz JD, Kimball E:** Acidic acetonitrile for cellular metabolome extraction from *Escherichia coli*. *Anal Chem*, 79, 6167-6173, 2007. DOI: 10.1021/ac070470c

**21. Wang J, Cheng LK, Wang J, Liu Q, Shen T, Chen N:** Genetic engineering of *Escherichia coli* to enhance production of L-tryptophan. *Appl Microbiol Biotechnol*, 97, 7587-7596, 2013. DOI: 10.1007/s00253-013-5026-3

**22. Scherholz ML, Curtis WR:** Achieving pH control in microalgal cultures through fed-batch addition of stoichiometrically-balanced growth media. *BMC Biotechnol*, 13, 39, 2013. DOI: 10.1186/1472-6750-13-39

**23. Marrannes R:** Computer model of unstirred layer and intracellular pH changes: Determinants of unstirred layer pH. *J Biol Phys*, 39, 515-564, 2013. DOI: 10.1007/s10867-013-9309-9

**24. Bobulescu IA, Dubree M, Zhang J, McLeroy P, Moe OW:** Effect of renal lipid accumulation on proximal tubule $Na^+/H^+$ exchange and ammonium secretion. *Am J Physiol Renal Physiol*, 294 (6): 1315-1322, 2008. DOI: 10.1152/ajprenal.00550.2007

**25. Suarez DC, Kilikian BV:** Acetic acid accumulation in aerobic growth of recombinant *Escherichia coli*. *Process Biochem*, 35 (9): 1051-1055, 2000. DOI: 10.1016/S0032-9592(00)00140-0

**26. Zhang Y, Cong W, Shi SY:** Application of a pH feedback controlled substrate feeding method in lactic acid production. *Appl Biochem Biotechnol*, 162, 2149-2156, 2010. DOI: 10.1007/s12010-010-8989-x

**27. Konstantinov K, Kishimoto M, Seki T, Yoshida T:** A balanced DO-stat and its application to the control of acetic acid excretion by recombinant *E. coli*. *Biotechnol Bioeng*, 36 (7): 750-758, 1990. DOI: 10.1002/bit.260360714

**28. Báez-Viveros JL, Flores N, Juárez K, Castillo-España P, Bolivar F, Gosset G:** Metabolic transcription analysis of engineered *Escherichia coli* strains that overproduce L-phenylalanine. *Microb Cell Fact*, 6, 30, 2007. DOI: 10.1186/1475-2859-6-30

Kafkas Univ Vet Fak Derg
24 (5): 743-750, 2018
DOI: 10.9775/kvfd.2018.19913

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# A Historical Research on the First (1927) and Second (1929) Balkan Veterinary Congress on Infectious Animal Diseases [1]

Şule SANAL [1,a] Ayşe MENTEŞ GÜRLER [2,b] Mehmet Yavuz ERLER [3]

[1] *An earlier version of this study was presented at the 10th International Congress of the Balkan History, 3-4 December 2016, Samsun, Turkey*

[1] Department of Veterinary History and Deontology, Faculty of Veterinary Medicine, Ondokuz Mayis University, TR-55139 Samsun - TURKEY
[2] Department of Veterinary History and Deontology, Faculty of Veterinary Medicine Harran University, TR-63200 Şanlıurfa - TURKEY
[3] Department of History, Faculty of Science and Letters, Ondokuz Mayis University, TR-55139 Samsun - TURKEY

[a] ORCID: 0000-0002-5703-5752; [b] ORCID: 0000-0001-8402-7291

**Article Code: KVFD-2018-19913 Received: 11.04.2018 Accepted: 27.07.2018 Published Online: 28.07.2018**

**How to Cite This Article**

**Sanal S, Menteş Gürler A, Erler MY:** A historical research on the First (1927) and Second (1929) Balkan Veterinary Congress on infectious animal diseases. *Kafkas Univ Vet Fak Derg,* 24 (5): 743-750, 2018. DOI: 10.9775/kvfd.2018.19913

## Abstract

The first initiative aimed at international cooperation in the prevention of epizootic diseases was launched by Professor John Gamgee, and the first international veterinary meeting was held in Hamburg, Germany on July 14-18, 1863. At that time, efforts were made to fight and ensure protection against animal diseases in Turkey, but it was not until the early years of the Republic that intensive and multifaceted efforts at a national and international level came into effect. Experienced veterinarians from various countries were invited to Turkey to carry out studies here, and Turkish veterinarians were dispatched to international congresses and meetings on animal health and improvement. At the same time, attempts were made to establish a common border health authority with neighboring countries, and efforts were made to develop health legislation. Close cooperation with the Balkan states on matters of policy following the proclamation of the Republic became obligatory, particularly with respect to animal diseases, and led to the Balkan Governments Infectious Animal Diseases Congress-I being held in Istanbul on October 4-12, 1927, followed by the Balkan Veterinary Congress II, held in Bucharest on September 21, 1929. The present study found that the senior civil servants from Turkey, Greece, Bulgaria, Romania and Yugoslavia that had attended the two congresses discussed the fight against epizootic diseases and the establishment of a veterinary organization on the borders. In conclusion, these congresses may be said to have laid the groundwork for the *"Geneva International Veterinary Convention (1935)"* and the bilateral veterinary conventions concluded between participatory countries that followed.

***Keywords:*** *Balkan Countries, Veterinary congress, Animal diseases, Veterinary history*

# Birinci (1927) ve İkinci (1929) Balkan Veteriner Bulaşıcı Hayvan Hastalıkları Kongreleri Üzerine Tarihsel Bir İnceleme

## Öz

Salgın hayvan hastalıklarının önlenmesinde uluslararası işbirliği yapılması için ilk girişim Profesör John Gamgee tarafından başlatılmış ve 14-18 Temmuz 1863'te Almanya'nın Hamburgh kentinde ilk uluslararası veteriner hekimler toplantısı gerçekleştirilmiştir. Aynı dönemde Türkiye'de de hayvan hastalıkları ile mücadele ve koruma çalışmaları yürütülmekle birlikte, ancak Cumhuriyetin ilk yıllarında ulusal ve uluslararası düzeyde gerçekleştirilen yoğun ve çok yönlü çalışmalar etkili olabilmiştir. Bu dönemde bir taraftan çeşitli ülkelerden veteriner hekimlik alanında deneyimli uzmanlar Türkiye'ye davet edilerek ülkede çalışmaları sağlanmış; diğer taraftan Türk veteriner hekimler hayvan ıslahı ve sağlığı ile ilgili olarak düzenlenen uluslararası kongre ve toplantılara gönderilmiştir. Aynı zamanda, komşu ülkelerle sınır noktalarında ortak sağlık zabıtası kurulması girişimlerinde bulunulmuş ve sağlık mevzuatının geliştirilmesine yönelik çalışmalar yapılmıştır. Cumhuriyet'in ilanından sonra izlenmeye başlanan Balkan devletleri ile yakın iş birliği politikası, özellikle hayvan hastalıkları konusunda zorunlu hale gelmiştir. Bu amaçla, 4-12 Ekim 1927 tarihleri arasında İstanbul'da I. Balkan Hükümetleri Emraz-ı Sariye-i Hayvaniye Kongresi ve 21 Eylül 1929'da da Bükreş'te II. Balkan Baytar Kongresi düzenlenmiştir. Bu çalışmada Türkiye, Yunanistan, Bulgaristan, Romanya ve Yugoslavya'dan üst düzey bürokratların katılımıyla gerçekleşen bu iki kongrede bulaşıcı hayvan hastalıklarıyla mücadele ile sınırlarda veteriner hekimliği örgütünün kurulması konularının görüşüldüğü tespit edilmiştir. Sonuç olarak bu kongrelerin daha sonra yapılacak olan *"Uluslararası Cenevre Veteriner Anlaşması (1935)"* ile katılımcı ülkeler arasında farklı zamanlarda yapılan ikili veteriner anlaşmalarına temel oluşturduğu ileri sürülebilir.

***Anahtar sözcükler:*** *Balkan ülkeleri, Veteriner kongreleri, Hayvan hastalıkları, Veteriner tarihi*

**İletişim (Correspondence)**
+90 414 3183917
mentesgurler@gmail.com

## INTRODUCTION

Animals have, throughout history, always been a means of economic production. While their purpose of use diversified as they became domesticated, diseases and death in animals resulted in starvation, sadness, illness and even death [1].

The first initiative to bolster international cooperation in the prevention of epizootic diseases was taken by Professor John Gamgee, and the first global veterinary meeting was held in Hamburg, Germany on July 14-18, 1863 [2]. Due to presence of important infectious animal diseases in the Ottoman Empire, as the point of entry of these diseases into Europe, delegates attended the second meeting which was held in Vienna. The first measures were taken and arrangements were made to both fight and prevent spread of animal diseases in these years but these attempts could not be effective until the Republican period [3].

At the subsequent international veterinary meetings held, the prevention of epizootic diseases was discussed [4], and the "Office International des Epizooties (OIE)" (The World Organization for Animal Health) was established in Paris on January 25, 1924 with the participation of 28 countries [5]. The animal diseases that first wreaked havoc on the animal population and devastated public health and the economy were particularly rinderpest and glanders, which spread quickly during World War I and the Turkish War of Independence as epidemics [6,7]. In order to regulate veterinary services, particularly due to the epizootic diseases that had become more important under the conditions of war, the Department of Veterinary Affairs was established in Ankara in 1920. Following the proclamation of the Republic, a five-year program was drafted that included border inspections, in an attempt to regulate the provision of veterinary services in line with the decisions taken at the Economic Congress held in Izmir in 1923, and this program entered into practice in 1925 [6]. Rinderpest was eradicated in the interior parts of Turkey in 1925, but the disease continued to enter the country through the movements of animals across the eastern and southern borders, leading to the "border veterinary authority" to be established in 1926 [8,9]. Article 4 of Section II of Law no. 1234 on the Animal Health and Inspection Authority, adopted on May 3, 1928, states: "*Any animal or animal product to be imported to and exported from Turkey in locations where there are customs administrations and other stations and ports allocated by the Ministry of Economy for processes related to the entry and exit of animals and animal products shall be subjected to the processes in such locations. The provisions of the agreements concluded with neighboring counties shall be reserved. Such locations shall be equipped with a quarantine station administered by the border Veterinarians, as well as tools, medicine and guardians as required.*"

In order to prevent the entry of epizootic diseases into the country across the borders and the spread of disease in the country, as well as to ensure a more effective fight against infectious animal diseases in the country, the Council of Ministers decided on August 12, 1928 that Turkey would join the OIE [10].

The Republic of Turkey's efforts to build a "national economy" accelerated, particularly following the conclusion of the Treaty of Lausanne, and great emphasis was placed on the establishment of new institutions and the enhancement of their effectiveness. To this end, legal efforts were made to both fight and prevent spread of animal diseases and to ensure improvements in animals, and institutions that would enable the attainment of such goals were established [11].

In the program to fight against rinderpest, it was decided that the cattle seized while being transported to Thrace would be sent back, and those that could not be sent back would be slaughtered at the location where they were captured. Animals brought into the country by immigrants from Bulgaria and Romania were regularly inspected and vaccinated by veterinarians at the border checkpoints and ports, and animal marketplaces were prohibited in the regions where rinderpest was spreading [12].

With a view of supporting the protection and treatment applications made in the country, discussions were made regarding cooperation with neighboring countries and the development of common prevention and protection strategies. A communiqué of the Ministry of Agriculture in 1925 stated that there was a need to establish a common border health administration in Turkey, Syria and Iraq to eradicate the rinderpest that was thriving around the Mardin-Iraq border [2,11,12].

At the same time, agreements were concluded, and congresses were held with neighboring countries, particularly the Balkan states, to discuss the exports of animals and animal products produced by Turkish farmers in accordance with the requirements for combatting animal disease [11,12].

## MATERIAL and METHODS

Methods commonly used in the analysis and synthesis of medical history were employed in this study. The study subject was dealt using a chronological order. During the study, documents from Turkish Prime Ministry Archives of the Ottoman and Republican Periods were reviewed. In addition, references were also made to books and other documents and literature related to the subject matter.

## RESULTS

Within the framework of the policy of close cooperation with the Balkan States that Turkey began to pursue following the proclamation of the Republic, the first

ambassador of the Republic of Turkey was dispatched to Bucharest in March 1924 [2,13]. The Balkan countries with which Turkey wanted to increase the trade of livestock and animal products voiced their satisfaction at the efforts made to fight against animal diseases in Turkey and raised concerns about the spread of rinderpest in their own countries. To report upon the efforts and measures taken in Turkey, and to ensure countries in the region were taking common action in relation to animal diseases, a Balkan veterinary meeting was planned in Istanbul with the participation of veterinarians from Turkey, Greece, Bulgaria, Romania and Yugoslavia [2,14]. The official name of the congress that convened upon the request of Bulgaria was the "Balkan Governments Infectious Animal Diseases Congress-I", where the languages of correspondence were Turkish and French [15]. An official letter sent by the Turkish Minister of Foreign Affairs to the relevant countries to communicate the names of the Turkish delegates who would be attending the congress was written in the two languages [15,16] *(Fig. 1)*, and the names of the delegates attending the congress from Bulgaria were communicated in an official letter dated October 13, 1927 [17,18] *(Fig. 2)*.

Although Balkan Governments Infectious Animal Diseases Congress-I was planned to convene on October 1, 1927 [15], it was delayed by three days as the Romanian delegates could not arrive on time [19]. In the end, the congress took place in Istanbul on October 4-12, 1927 with the participation of Turkey, Greece, Bulgaria, Romania and Yugoslavia [19,20].

Turkey was represented by Tevfik Kamil (Istanbul Deputy) as the Congress Chairman; Prof. Refik Güran (Bursa Deputy), a Bacteriologist at Istanbul University Faculty of Medicine; Ahmed Şefik Kolaylı, Manager and Bacteriologist at the Pendik Bacteriology Institute; Forgeot, expert bacteriologist at the Pendik Bacteriology Institute; Ali Rasim, Istanbul Veterinary Manager; Rıza İsmail Sezginer, Academician specializing in Bacteriology and Infectious Diseases at Istanbul Veterinary Academy; and Esad Evsen, Istanbul Veterinary Manager [2,15,16].

Bulgaria was represented by Dr. G. Pavloff, Chief of Veterinary Services at the Ministry of Agriculture; Prof. St. Angheloff, Veterinarian; Prof. Bitcheff, Veterinarian; Prof. Gheorghieff, general inspector of Veterinary Services at the Ministry of Agriculture; Prof. Goudeff, general inspector of Veterinary Services at the Ministry of Agriculture; and Dr. Dimitri Douchcoff, inspector of Veterinary Services at the Ministry of Agriculture [2,17,18].

Greece was represented by Dr. Alexandra Haralambo Pavlos, veterinarian; and Dr. C. Melanidi, Manager of the Veterinary Bacteriology Laboratory of the Ministry of Agriculture [2].

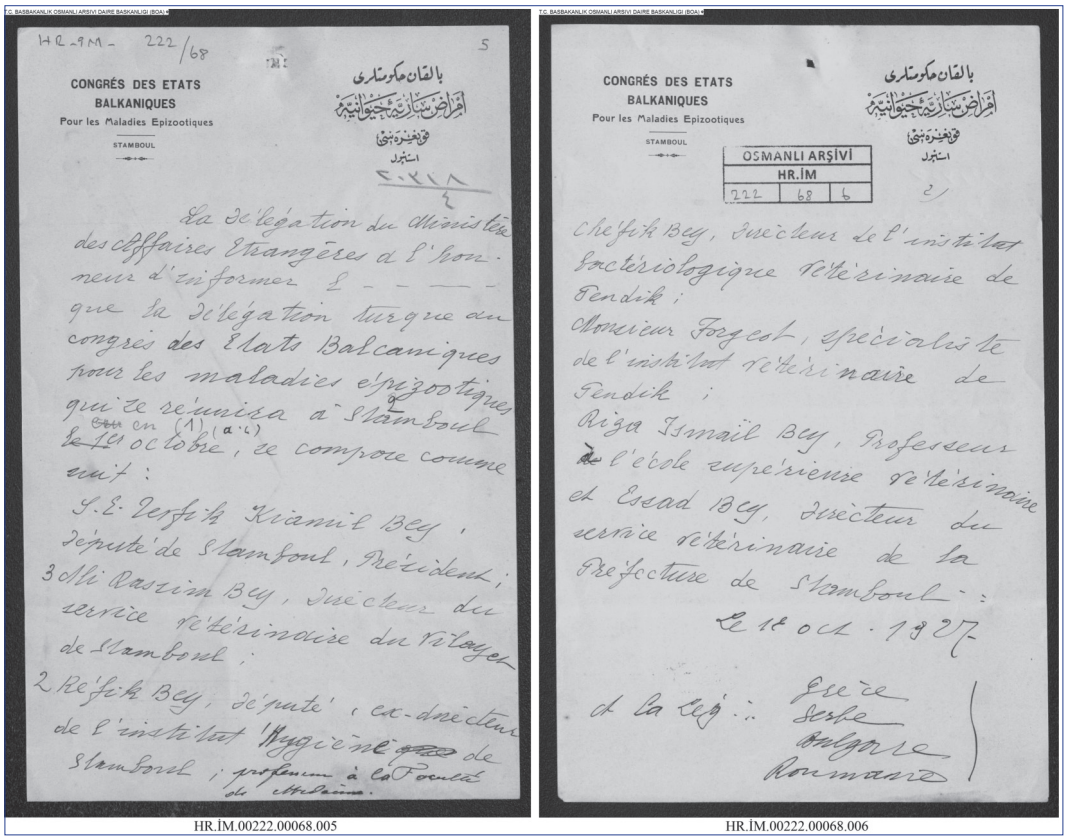



**Fig 1.** Official letter informing the names of Turkish delegates attending the congress

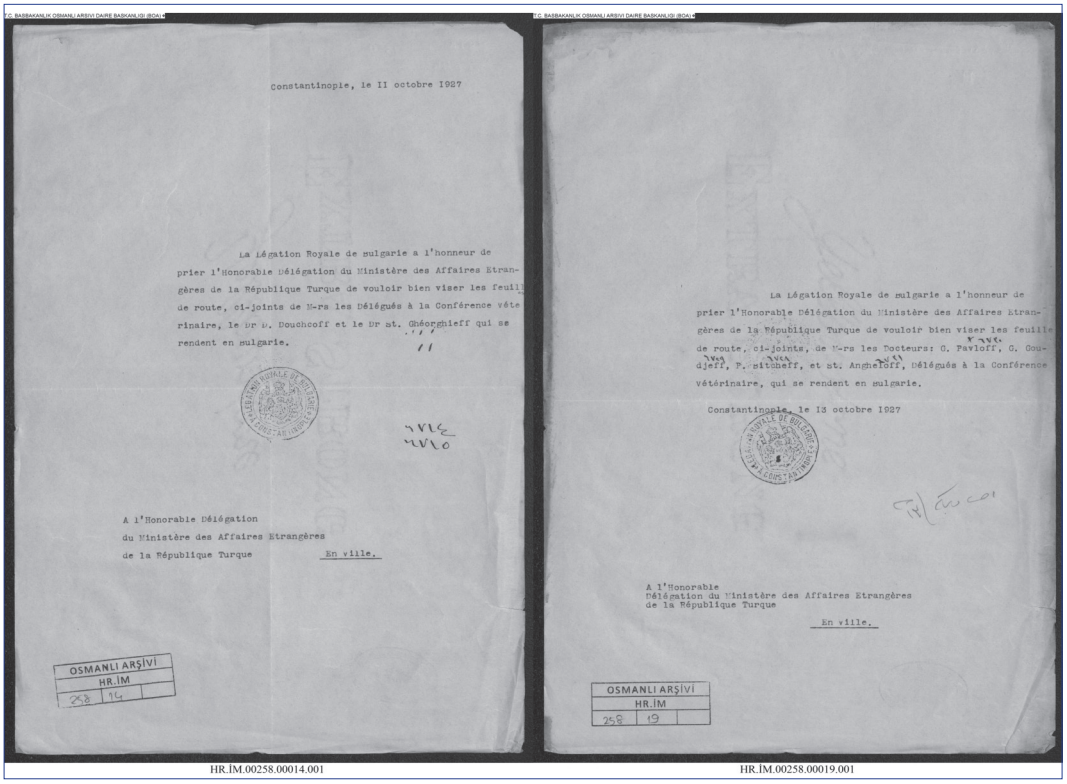



**Fig 2.** Official letters informing the names of Bulgarian delegates attending the congress

Romania was represented by Prof. Constantin S. Motas, lecturer at the Faculty of Veterinary Medicine; Dr. M.C.Z. Papazoğlu, advisor at the Veterinary Service of the Ministry of Agriculture; M. Georges Manolescu, general inspector at the Veterinary Service of the Ministry of Agriculture; and M. Alexsandr Bacleseocnu, general inspector at the Veterinary Service of the Ministry of Agriculture [2].

Yugoslavia was represented by Dr. Bogolov Sievatchitach, chief of the Veterinary Service of the Ministry of Agriculture; Prof. Stjepanplasaj, lecturer at Zagreb University Faculty of Veterinary Medicine; Dr. Kosto Konditch, inspector at the Veterinary Service of the Ministry of Agriculture; and Dr. Antonie Vonkovitch, agricultural inspector at the Ministry of Agriculture [2].

The general meeting at the Congress was chaired by Turkish Minister of Agriculture Sabri Toprak. The delegates of the Balkan states that attended the Congress requested the inclusion of the existing border inspections and four major diseases, as well as some other diseases, in the measures to be implemented. Before commencing the meeting, the delegates provided information on the number of livestock and veterinarians and veterinary institutions in their respective countries [19]. Held at Yildiz Palace, the Congress lasted for eight days that included six sessions, during which the participants discussed the fight against infectious animal diseases and the establishment of a veterinary organization on the borders [2,19] *(Fig. 3)*.

After setting up scientific sub-commissions, the following 22 articles were adopted at the meeting [19,20].

### *Article 1*

a) All countries attending the Congress shall design their own veterinary services in the best manner possible, and draft laws on animal health in the light of the most recent scientific data.

b) A common list of notifiable diseases shall be generated.

### *Article 2*

a) Medical journals shall be published every 15 days and in two languages (countries' own language and French) and be sent to the veterinary services of the countries.

b) The laws and regulation of the various countries governing their veterinary services shall be discussed by the participant countries in French.

c) Abstracts of veterinary medicine publications on infectious diseases shall be mutually exchanged between the participant countries in French.

### *Article 3*

a) The import of animals from countries infected with rinderpest should be prohibited, and the import of livestock for slaughter from such countries shall be permitted only when required, provided that strict measures are taken.


**Fig 3.** Delegates of Balkan Governments Infectious Animal Diseases Congress-I

Front Line 1) Antoniev Onkovitch 2) Dr. Refik 3) P. Bitcheff 4) Prof. Motas 5) Tevfik Kamil 6) Pavloff 7) Petrovich 8) Ali Rasim 9) Alexandr Haralambos

Second Line 1) Şefik Kolaylı 2) Rıza İsmail Sezginer 3) Papazoğlu 4) Hilmi Dilgimen 5) Haralambu Melanidis 6) Esat Esen 7) Nedim 8) M. Alexandr Bacleseocnu 9) Forgeot 10) Kosto Konditch 11) Prof. Goudjeff 12) Prof. Angheloff 13) Mehmet Halit Civelekoğlu 14) Stjepan Plasaj

b) In the case of a rinderpest outbreak in any of the participant countries, it shall notify the other countries via telegram and may request assistance from them.

c) The country in which the disease has broken out shall allow the other participant countries to send experts to examine the circumstances. This shall apply only to the Thrace region of Turkey.

*Article 4:* When an infectious disease breaks out within 15 km of any border, the veterinary organizations of the neighboring countries shall be informed as a matter of urgency. The same shall apply to infectious diseases not listed in the Medical Authority Law.

*Article 5:* Necessary measures shall be taken when making inspections of livestock and animal products at the borders.

*Article 6:* Detailed reports on the origin and health of livestock to be exported in accordance with the veterinary agreements concluded shall be drawn up by a veterinarian who has been authorized by the government. This report should indicate that the animal in question is healthy, that no infectious disease has been seen in the district from which the animal was brought or in the surrounding districts for 40 days, and that rinderpest has not been seen within an area of 40 km for 3 months.

*Article 7:* Inspection of foods of animal origin shall be carried out only by authorized veterinarians.

*Article 8:* Canned meats, sealed in accordance with rules, wool packaged in sealed bags, melted tallow, eggs, milk and dairy products shall be able to be transported without the permission of the veterinary service. Permission shall be required for all other animal products. This permission document should indicate the destination of the product and the border checkpoint through which they will pass, and the number, brand and content of the products. The document shall contain a statement that the product has been produced from healthy animals, and that no infectious disease has been seen in the district from which they were brought or in the surrounding districts for 30 days.

In the report of the examinations conducted by the government veterinarian, or by a veterinarian authorized by the government, prior to and after the slaughter for meat products prepared by salting, drying and smoking, it should be certified that the animal was healthy at the time of slaughter.

*Article 9*

a) Freight cars and boats used for the transport of livestock and animal products should be cleaned and disinfected under the supervision of a veterinarian.

b) Freight cars should not be overloaded and should be suitable for the transportation of animals.

*Article 10*

a) Expert veterinarians should be consulted to terminate the veterinary agreements between the various Balkan governments.

b) It is in the interest of governments to appoint veterinarians at their political offices.

**Fig 4.** Delegates of Balkan Veterinary Congress-II
Front Line 1) P. Bitcheff 2) Mehmet Nuri Ural 3) General Inspector of Romania Veterinary Services 4) Moutas 5) Pavloff 6) Prof. Angeloff
Second Line 1) St. Tuleff 2) Naki Cevat Akkerman 3) Dr. Al. Cicua 4) Prof. St Plasage 5) Ahmet Şefik Kolaylı

*Article 11:* Livestock passing through border checkpoints for trade shall be inspected by the veterinarians of the exporter country.

*Article 12:* The Congress recommends that the countries that have not assigned veterinarians to the management of animal production and improvement do so.

*Article 13:* Issues concerning the fight against rinderpest

*Article 14:* Issues concerning the fight against glanders

*Article 15:* In relation to pox disease, the most recent protection procedures should be applied, rather than conventional techniques.

*Article 16:* Issues concerning the fight against the foot-and-mouth disease

*Article 17:* Issues concerning the fight against swine plague

*Article 18:* Epizootic diseases of bees and silkworms should be included in the veterinary health authority law of all countries.

*Article 19:* Diseases caused by piroplasms and insect vectors should be kept track of regularly, and information should be provided.

*Article 20:* It is recommended that all countries research whether rinderpest is spread by carriers, and if so, the duration of carriage, and should share their experience.

*Article 21:* It is requested that these decisions taken in the meetings be conveyed to the relevant ministers of the participant Governments, hoping that they are put into practice.

*Article 22:* It has been decided that a Balkan countries veterinary meeting is to be held every three years, with the next meeting being held in Bucharest.

Balkan Veterinary Congress II convened at the Faculty of Veterinary Medicine in Bucharest on September 21, 1929 *(Fig. 4)*.

Turkey was represented by İhsan Abidin Akıncı, Undersecretariat of the Ministry of Agriculture and veterinary zootechnician; Mehmet Nuri Ural (Mardin Deputy), veterinarian; Naki Cevat Akkerman, Manager of the Department of Epizootic Diseases; and Ahmet Şefik Kolaylı, Bacteriologist and Manager of the Pendik Bacteriology Institute [2].

Bulgaria was represented by Prof. Angheloff from the Sofia Faculty of Veterinary Medicine; P. Bitcheff and D. St. Tuleff, veterinarian in Varna [2].

Romania was represented by Prof. P. Riegler from the Bucharest Faculty of Veterinary Medicine; Moutas and Dr. Al. Cicua from the Bucharest Faculty of Veterinary Medicine [2].

Yugoslavia was represented by Prof. St. Plasage from the Zagreb University Faculty of Veterinary Medicine [2].

Greece did not attend the meeting [2].

At the meeting, the Animal Health Authority and two subcommissions were set up to examine infectious animal diseases, and decisions were taken. The decisions taken were adopted also in the general meeting [2].

Some of these decisions taken were as follows:

Regarding the discussions at the previous meeting as to whether rinderpest is spread by carriers, Muzaffer Bekman, the manager of the Erzincan Serum Laboratory in Turkey, prepared a report following his studies on the subject. This report indicated that the secretions and entrails of calves that had this disease, or are in the incubation period and do not have the symptoms,

were not carriers of the disease.

It was stated that a fly species found around the Danube (Tuna) in May and June caused *Hemorrhagic septicaemia* in animals, and studies on the biology of this fly were requested to be conducted. Even though there were deaths caused by a similar disease in Kırklareli, Çorlu and Lüleburgaz provinces of Turkey, it was decided that this fly be researched, as there was no information concerning its presence [2].

Following the Balkan Veterinary Congress II, *"Geneva International Veterinary Convention"*, a multilateral agreement was concluded for the first time in the field of veterinary medicine on February 20, 1935 and various bilateral agreements were concluded between the participant countries of the Balkan Congress in the sub-sequent years [21].

## DISCUSSION

Following the Balkan Veterinary Congresses, the participant countries concluded a multilateral agreement and various bilateral agreements in the subsequent years. It may be argued that the Balkan Veterinary Congresses addressed in this study laid the groundwork for agreements at an international level.

In 1929, Turkish President Mustafa Kemal Atatürk signed a decree stating that epizootic and infectious animal diseases that break out in the villages within 15 km of the Turkish-Bulgarian border would be notified to the other country by the veterinarians as per Article 4 of the decisions taken at Balkan Veterinary Congress I, that this had been requested by Bulgaria, and that the implementation of this decision was approved [22]. It may be argued that this decree led to the parties implementing the decisions taken in the Balkan Congresses at the highest level.

It may also be argued that the preparation of the "*Five-year Program*" for combatting infectious diseases [6], the establishment of the "*Border Veterinary Organization*" in 1926 [8,9], and the enactment of the Law on the Animal Health Authority in 1928 were all consistent with the decision taken at the First Balkan Veterinary Congress regarding establishment of a veterinary organization on the borders, and that this had a positive impact on the establishment of Quarantine Stations and Customs Veterinary Administrations that would operate as border checkpoints in 1935.

It may be claimed further that the attendance by senior civil servants of the countries at both Congresses demonstrated the importance placed in the issue by the participant countries.

The publication of detailed information on Veterinary Congress I and the decisions taken in Baytarî Mecmua [19,20], the first veterinary medicine journal of the Republican period [23], shows the special emphasis placed on the subject by Turkish veterinarians.

While it was decided that congresses be held every three years in the first meeting, there is no information on any meeting taking place after the second meeting.

In conclusion, it may be said that these Congresses set a good example of cooperation between neighboring countries in relation to the prevention of epizootic and infectious animal diseases. It may be argued that governments have to take into consideration the veterinary congresses and their resolutions related with infectious animal diseases today also.

## REFERENCES

**1. Durant W:** The Story of Civilization, I. Our Oriental Heritage. Simon and Schuster, New York, 1954.

**2. Bekman M:** Veteriner Kongreleri ve İtilâfnameleri - Lescongrés et Lesconventions Vvétérinaires. Marifet Basımevi, İstanbul, 1945.

**3. Yiğit A, Yaşar A, Menteş Gürler A:** International Veterinary Congresses in Ottoman Era Archives. *32nd World Veterinary Congress.* İstanbul, Abstract Book, p.101. September 13-17, 2015.

**4. Aalbers E:** Short History of The World Veterinary Association 1863-1994, Madrid, p. 30, 1994.

**5. The World Organization for Animal Health:** http://www.oie.int/about-us/history/; Accessed: 07.03.2018.

**6. Menteş Gürler A:** "Beş Senelik Umûr-u Baytâriye Programı" üzerine bir inceleme. *Vet Bil Derg,* 25 (1-2): 9-13, 2009.

**7. Osmanağaoğlu Ş, Melikoğlu B:** Türkiye'de ruam hastalığı eradikasyon çalışmalarına tarihsel bir bakış açısı. *Kafkas Univ Vet Fak Derg*, 15 (3): 331-337, 2009. DOI: 10.9775/kvfd.2008.97-A

**8. T.C. Başbakanlık Cumhuriyet Arşivi:** Cumhuriyet döneminde yapılan haralar, ahırlar ve hayvan hastalıkları ile mücadele merkezleri hakkında hazırlanan raporlar, grafikler ve fotoğraflar. Belge No: 030.10.187.285.1, 110 s. 1946.

**9. Osmanağaoğlu Ş, Melikoğlu B:** Tahaffuzhane ve gümrük veteriner müdürlüklerinin yapılandırılmasına ilişkin tarihsel değerlendirme. *Türk Veteriner Hekimleri Birliği Dergisi,* 11 (1-2): 79-87, 2011.

**10. T.C. Başbakanlık Cumhuriyet Arşivi:** Paris'te kurulan Milletlerarası Bulaşıcı Hayvan Hastalıkları Komitesi'ne hükümetimizin iştiraki ve bu hususta ki anlaşmanın kabulü. Belge No: 30-18-1-1 / 30 - 52 – 3, 1928.

**11. Menteş Gürler A, Osmanağaoğlu Ş:** Cumhuriyet'in İlk 10 yılının Veteriner Hekimlik Açısından Değerlendirilmesi: II. Kurumlar. *II. Ulusal Veteriner Hekimliği Tarihi ve Mesleki Etik Sempozyumu Bildiriler Kitabı*, 127-139, 24-26 Nisan, Konya, 2008.

**12. Temel M:** Atatürk dönemi hayvancılık politikası. *Muğla Üniversitesi Sosyal Bilimler Enstitüsü Dergisi* (İLKE), 24, 201-234, 2010.

**13. Barlas D:** Atatürk Döneminde Türkiye'nin Balkan Politikası. İç: Atatürk Dönemi Türk Dış Politikası. Atatürk Araştırma Merkezi. 275-280, (reprint of the article presented at The Third International Symposium on Atatürk), Ankara, 2000

**14. Osmanağaoğlu Ş, Menteş Gürler A:** Cumhuriyet'in İlk 10 yılının Veteriner Hekimlik Açısından Değerlendirilmesi I: Hayvan Hastalıkları ile Mücadele ve Diğer Önemli Konular. *II. Ulusal Veteriner Hekimliği Tarihi ve Mesleki Etik Sempozyumu Bildiriler Kitabı*, 119-126, 24-26 Nisan, Konya, 2008.

**15. T.C. Başbakanlık Osmanlı Arşivi:** İstanbul'da yapılacak Balkan ülkeleri Bulaşıcı Hayvan Hastalıkları Kongresi'ne iştirak edecek Türk murahhasların listesi ve ilgili ülkelere tebliği. (Osm., Fr.) Belge No: HR-İM-00222-00068-005-001, 1927.

**16. T.C. Başbakanlık Osmanlı Arşivi:** İstanbul'da yapılacak Balkan

ülkeleri Bulaşıcı Hayvan Hastalıkları Kongresi'ne iştirak edecek Türk murahhasların listesi ve ilgili ülkelere tebliği. (Osm., Fr.) Belge No: HR-İM-00222-00068-006-001, 1927.

**17. T.C. Başbakanlık Osmanlı Arşivi:** Veteriner Konferansı Bulgar delegelerine yol tezkiresi talebi. (Fr.) Belge No: HR-İM-00258-00019-001-001, 1927.

**18. T.C. Başbakanlık Osmanlı Arşivi:** Bulgaristan'a gidecekleri bildirilen Veteriner Konferansı Bulgar delegelerinden Dr. D. Douchcoff ile Dr. St. Gheorghieff'e yol tezkiresi verilmesi talebi. (Fr.) Belge No: HR-İM-00258-00014-001-001, 1927.

**19. Anonymous:** İstanbul'da Balkan Hükümetleri Emraz-ı Sariye-i Hayvaniye Kongresi. *Baytarî Mecmua*, 5 (4): 184-190, 1927.

**20. Anonymous:** Sari hayvan hastalıkları hakkında 12 Teşrin-i evvel 927 tarihinde İstanbul'da inikad eden Balkan Hükümetleri Kongresi mukarreratı. *Baytarî Mecmua*, 5 (6): 184-190, 1928.

**21. Özkul T:** Türkiye'de Cumhuriyet Döneminde Yapılan Uluslararası Veteriner Hekimliği Anlaşmaları Üzerine Araştırmalar. *Turkiye Klinikleri J Med Ethics,* 17 (1): 23-32, 2009.

**22. T.C. Başbakanlık Cumhuriyet Arşivi:** 1927 yılında İstanbul'da yapılan Balkan Baytari Kongresi kararlarının 4. maddesinin tetkiki. Belge No: 30.18.1.2.5.45.12(1), 1929.

**23. Melikoğlu Gölcü B, Yüksel Ö, Yüksel, E:** Baytar Mecmuası/Baytarî Mecmua (1923-1933) Üzerine Bir İnceleme. *Osmanlı Bilimi Araştırmaları*, XIX (1): 75-134, 2017.

Kafkas Univ Vet Fak Derg
24 (5): 751-760, 2018
DOI: 10.9775/kvfd.2018.19941

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Evaluation of Se, Cr and Zn-enriched Yeast Culture in Improving *in vitro* Fermentation Characteristics of Cereal Straws

Liang CHEN [1,2] Bin LI [3] Ao REN [1,2] Zhiwei KONG [1] Zhiliang TAN [1,4] Mahmoud ALAGAWANY [5,a]
Mohamed EZZAT ABD EL-HACK [5,b] Chuanshe ZHOU [1,6]

[1] Key Laboratory for Agro-Ecological Processes in Subtropical Region, and Hunan Research center of Livestock & Poultry Sciences, and South-Central Experimental Station of Animal Nutrition and Feed Science in Ministry of Agriculture, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, Hunan 410125, P.R. CHINA
[2] College of Animal Science and Technology, Hunan Agricultural University, Changsha, Hunan 410125, P.R. CHINA
[3] Institute of Animal Science of Tibet Academy of Agricultural and Animal Husbandry Sciences, Lhasa 850000, CHINA
[4] Hunan Co-Innovation Center of Animal Production Safety, CICAPS, Changsha, Hunan 410125, P.R. CHINA
[5] Department of Poultry, Faculty of Agriculture, Zagazig University, Zagazig 44511, EGYPT
[6] Postdoctoral Science Research Workstation, New Hope Group, Chengdu, Sichuan 610041, P.R. CHINA
[a] ORCID: 0971-8020-0002-0000; [b] ORCID: 0000-0002-2831-8534

**Article Code: KVFD-2018-19941 Received: 14.04.2018 Accepted: 22.07.2018 Published Online: 23.07.2018**

**How to Cite This Article**

**Chen L, Li B, Ren A, Kong Z, Tan Z, Alagawany M, Ezzat Abd El-Hack M, Zhou C:** Evaluation of Se, Cr and Zn-enriched yeast culture in improving *in vitro* fermentation characteristics of cereal straws. *Kafkas Univ Vet Fak Derg,* 24 (5): 751-760, 2018. DOI: 10.9775/kvfd.2018.19941

## Abstract

The object of this study was to evaluate the effect of different sources and supplementation levels of yeast culture on *in vitro* fermentation characteristics of goats. The present study was performed in a 3×4 factorial design to examine the impacts of inclusion of three kinds of yeast culture (Se-enriched, Cr-enriched, Zn-enriched yeast culture) at four dose (0, 0.1%, 0.25%, 0.40% and 0.55%) on the *in vitro* fermentation characteristics of cereal straw. For maize stover, the results shown that the average values of *in vitro* gas production, DM and NDF disappearance, pH and the ratio of acetate to propionate were increased ($P<0.05$), while the concentration of ammonia nitrogen ($NH_3$-N) and butyrate were decreased ($P<0.05$) by the supplementation of Se-enriched yeast culture (YC-Se) compared with that of Cr-enriched yeast culture (YC-Cr) and Zn-enriched yeast culture (YC-Zn). For rice straw, the in vitro gas production, DM disappearance and pH were increased ($P<0.05$), and the concentration of ammonia nitrogen was decreased by the supplementation of YC-Se, while the concentration of acetate, propionate, butyrate and total VFA (TVFA) were increased ($P<0.05$) by the supplementation of YC-Zn. The current results indicate that YC-Se is more preferred as yeast culture supplements, and its optimal dose should be 0.25% substrates for maize stover and 0.10% substrates for rice straw *in vitro*. The present positive *in vitro* results should be tested using *in vivo* experiments in future.

***Keywords:*** *Yeast culture, in vitro fermentation, Maize stover, Rice straw*

# Tahıl Samanının *in vitro* Fermantasyon Özelliklerini Geliştirmede Se, Cr ve Zn İle Zenginleştirilmiş Maya Kültürünün Değerlendirilmesi

## Öz

Bu çalışmanın amacı, farklı kaynak ve katkı düzeylerindeki maya kültürünün *in vitro* fermantasyon özelliklerine etkilerini değerlendirmektir. Çalışma üç çeşit maya kültürünün (Se ile zenginleştirilmiş, Cr ile zenginleştirilmiş ve Zn ile zenginleştirilmiş) dört farklı dozda (%0.01, %0.25, %0.40 ve %0.55) dahil edilmesinin mısır samanının *in vitro* fermantasyon özellikleri üzerine etkilerini incelemek için 3 × 4 faktöriyel tasarımda gerçekleştirilmiştir. Mısır samanı için, Cr ile zenginleştirilmiş maya kültürü ve Zn ile zenginleştirilmiş maya kültürü ile karşılaştırıldığında Se ile zenginleştirilmiş maya kültüründe in vitro gaz üretimi, DM ve NDF kaybolması, pH ortalama değerleri ile asetat propiyonat oranı artarken ($P<0.05$), amonyum nitrojen ($NH_3$-N) ve bütrat konsantrasyonlarında düşme ($P<0.05$) şekillendi. Pirinç samanı için, Se ile zenginleştirilmiş maya kültüründe *in vitro* gaz üretimi, DM kaybolması ve pH düşerken ($P<0.05$), amonyum nitrojen konsantrasyonu azaldı. Zn ile zenginleştirilmiş maya kültüründe asetat, propiyonat, bütrat ve total VFA (TVFA) arttı ($P<0.05$). Elde edilen sonuçlar, maya kültürü katkı maddesi olarak Se ile zenginleştirmenin daha ziyadesi ile tercih edilmesi gerektiğini ve *in vitro* optimal dozun mısır samanı için %0.25 ve pirinç samanı için %0.10 olması gerektiğini göstermiştir. Elde edilen *in vitro* sonuçlar *in vivo* çalışmalar ile test edilmelidir.

***Anahtar sözcükler:*** *Maya kültürü, in vitro fermantasyon, Mısır posası, Pirinç posası*

**İletişim (Correspondence)**
+86 731 84615236; Fax: +86 731 84612685
zcs@isa.ac.cn

# INTRODUCTION

During recent years, yeast culture has been used to enhance the utilization efficiency and nutritive value of low-quality roughages [1]. The supplementation of yeast culture to ruminant diets can increase dry matter intake (DMI), nutrient digestibility, cellulose degradation and production performance [1-4]. Many *in vitro* studies showed that yeast culture significantly altered the fermentation of mixed ruminal microorganism and activated digestion of cellulose through pure cultures of predominant ruminal bacteria [1,5,6].

Again, yeast has been a favorite cultivation medium which allows the incorporation of Zn, Cr and Se and other metals, such as Fe, Mn and Cu into biomass, in addition to other unicellular organisms such as Spirulina *(Arthrospira platensis)* and *Lactobacilli plantarum* [7]. Cr-, Zn-, and Se-enriched yeast cultures were produced by growing specific strains of yeast in Cr-, Zn-, and Se-enriched media. Ortman and Pehrson [8] reported that organic Se from Se-enriched yeast is an ideal supplement because animals retain and absorb it more efficient than the inorganic form of Se. Zn-enriched yeast is naturally integrated by the growing yeast into its own structure to improve the bioavailability of Zn and reduce the sides effects of Zn [9]. In mammals, chromium (Cr) has been recognized to be a biologically essential trace element. Researchers have reported that Cr played an essential role in carbohydrates and fats metabolism because of increasing the action of insulin when given to people [10,11]. In recent years, most reports focused on the effects of Se-enriched, Cr-enriched and Zn-enriched yeast supplementation on growth performance, physiology and biochemistry in animals [12-16]. While there is little available information about the use of Se-enriched, Cr-enriched and Zn-enriched yeast in *in vitro* or *in vivo* digestibility in goats or other ruminants. Therefore, the objective of the present study was to evaluate the effect of different sources and supplementation levels of yeast culture on *in vitro* fermentation characteristics of goats.

# MATERIAL and METHODS

The present experiment was approved by the Animal Care Committee, Institute of Subtropical Agriculture (ISA), Chinese Academy of Sciences, Changsha, China. The experiment was performed during 2016.

### *Crop Straws, Yeast Culture and Experimental Design*

Two kinds of crop straws, i.e., rice straw from Xiang 125s (a popular local breed) maize and stover from Kexiangtian 1 (bred by ISA), were selected as *in vitro* fermentation substrates. Straws were dried at 65ºC for 24 h, ground through a 1 mm sieve and stored in plastic bag until assay. Maize stover and rice straw contained (DM basis): 5.3% and 6.2% crude protein (CP), 63.6% and 63.2% neutral detergent fiber (NDF), and 38.6% and 43.4% acid detergent fiber (ADF), respectively.

Yeast cultures were purchased from Angel Yeast Co., Ltd (Yichang city, Hubei Province, China). The indexes of the three kinds of yeast cultures were as following: Zn-enriched yeast (YC-Zn): 2000 ppm, content of Zn element was 2479.52 mg/kg and water content was 3.81%. Se-enriched yeast (YC-Se): 2000 ppm, content of Se element was 0.2% and water content was less than 6.0%. Cr-enriched yeast (YC-Cr): 2000 ppm, content of Cr element was 2091.1 mg/kg and water content was less than 4.0%.

The experiment followed a blocked experimental design; each species of yeast culture was supplemented at five doses: 0%, 0.1%, 0.25%, 0.40% and 0.55% of fermentation substrates respectively, no addition of yeast culture (0%) was taken as control group.

### *In Vitro Gas Production and Sampling*

Culture solutions, i.e., macro-element, buffered and reducing solutions used for *in vitro* fermentation, were prepared to form artificial saliva according to the procedures modified by Tang et al.[17]. The artificial saliva was maintained in an anaerobic environment by continuously pumping $CO_2$ around it for 2 h. Rumen fluids were collected before the morning feeding, from three rumen-cannulated *Xiangdong* black goats (a popular local goat, fed a rice straw based total mixed ration, the ingredients and chemical composition of diets were presented in *Table 1)*, and immediately transported to the laboratory. Rumen contents were strained through four layers of cheesecloth under a continuous flow of $CO_2$. Rumen fluids (5 mL) and artificial saliva (45 mL) were placed in pre-warmed (39°C) 145 mL fermentation bottles.

A sample of 500±5 mg of each straw type was placed in the 145 mL fermentation bottles. Each sample was measured three times at each incubation time point. Every species of yeast culture was added to the straw substrates based on experimental design when the *in vitro* fermentation was started.

All fermentation bottles were connected to pressure sensors and incubated at 39°C. Fermentation bottle pressure was recorded at 0, 1, 2, 4, 6, 12, 24, 36 and 48 h during the *in vitro* fermentation process. After 12, 24 or 48 h of incubation, fermentation was interrupted. Undegraded residues were filtered through 2 layers of nylon cloth (40-um pore size). The incubation solution from each treatment was sampled to determine $NH_3$-N and VFA concentrations at 12, 24 and 48 h, respectively.

### *Chemical Analysis*

The DM (method 930.15) was analyzed using procedures from the Association of Official Analytical Chemists [18]. The NDF content was determined using a Fibretherm Fiber Analyzer (Gerhardt, Bonn, Germany) following Van Soest et al.[19] with the addition of sodium sulfite and alpha-amylase in the NDF analysis. The filtered residue was dried

**Table 1.** *Ingredients and chemical composition of the basal diets offered to goats (g/kg DM)*

| Items | Diet |
|---|---|
| **Dietary ingredient (g/kg DM)** | |
| Forage | |
| Rice straw | 300 |
| Concentrate | |
| Soybeans | 60 |
| Maize | 298 |
| Wheat bran | 280 |
| $CaCO_3$ | 10 |
| Fat | 8 |
| NaCl | 10 |
| Urea | 14 |
| Premix | 20 |
| **Chemical composition (g/kg DM)** | |
| DM | 965 |
| Organic matter | 918 |
| Ash | 86.3 |
| Crude protein | 161 |
| Starch | 252 |
| Neutral detergent fiber | 332 |
| Acid detergent fiber | 118 |
| Gross energy (MJ/kg) | 17.2 |

*Premix was formulated to provide the following (per kg of premix): 400 g of $NaHCO_3$, 2 g of Fe, 1 g of Cu, 0·01 g of Co, 0·05 g of I, 6·6 g of Mn, 4·4 g of Zn, 0·003 g of Se, 333 mg of retinol, 5 mg of cholecalciferol, 838 mg of α-tocopherol*

at 105°C for 12 h and weighed to determine *in vitro* dry matter disappearance (IVDMD). The NDF content in the dried residues was determined to calculate *in vitro* NDF disappearance (IVNDFD).

VFA was measured as Wang et al.[20] described. Total molar concentration was calculated by taking the sum of individual VFA as 1. $NH_3$-N concentration was measured as Wang et al.[21].

### Calculation and Statistical Analysis

The correlation between fermentation bottle pressure and gas volume was measured at 39°C, 20 bottles were used to determine the content in the equation**,** and the following regression equation was established:

$y=1.506x$ (n=20, $R^2=0.999$, $P<0.0001$) (1)

Where *y* represents gas volume (mL), *x* is bottle pressure (kPa), and 1.506 is a constant. Pressure measurements were then converted to gas production (mL). The following Logistic-Exponential equation [22] was fitted to *in vitro* gas production at 0, 1, 2, 4, 6, 12, 24, and 48 h:

$$GP = Vf * \frac{1-\exp(d-t*k)}{1+\exp(b-k*t)} \quad (2)$$

Where GP represents gas production at *t* time, *Vf* is the maximum gas production (ml), *k* is the gas production fraction (/h), *b* and *d* is the shape of the gas production curve. The following equation was used to calculate the elapsed time ($T_{0.5}$, h) until half of the maximum gas production was achieved [22].

$$T_{0.5} = \frac{In\left(\exp(b)+2\exp(d)\right)}{k} \quad (3)$$

$FRD_0$ was used to calculate the initial fractional rate of degradation (/h) as follows [22]:

$$FRD_0 = \frac{k}{1+\exp(b)} \quad (4)$$

Gas production (GP), IVDMD and IVNDFD were corrected by subtracting values obtained for the blanks.

### Statistical Analysis

Data were analyzed by straw substrate using the PROC MIXED procedure in SAS software. For the statistical analyses of gas production parameters, the model included species, supplementation level and their interaction as fixed effects. For the analyses of pH, $NH_3$-N, VFAs, IVDMD and IVNDFD, the model included species, supplementation level and their interaction as fixed effects with incubation time as a repeated factor. Linear and quadratic effects of supplementation level were analyzed using orthogonal polynomial contrasts. Cubic effects of supplementation level were not examined due to inexplicability in a biological context. Least squares means are reported throughout the text, and significance was declared at $P<0.05$.

## RESULTS

### In Vitro Gas Production Parameters

Influences of different yeast culture supplementation on *in vitro* gas production parameters of maize stover and rice straw were listed in *Table 2a* and *Table 2b*, respectively. When selected maize stover as fermentation substrates, the average $V_f$ value of YC-Se was significantly higher ($P<0.05$) compared to YC-Zn group. The largest $V_f$ of YC-Cr was 82.41 mL, which was obtained at the supplementation dose of 0.25%, it is 13.23% higher than that of control group, and presented cubic effect with the dose increased ($P<0.01$). For YC-Zn group, the largest $V_f$ was obtained when the supplemental dose was 0.10%, it is higher compared to that of control group, and there was cubic effect when the dose improved ($P<0.01$). The largest $V_f$ was for the YC-Se group at the supplementation dose of 0.25%, and there was linear effect ($P<0.05$) with the dose improved. The average $T_{0.5}$ values of YC-Cr and YC-Zn group were significantly lower ($P<0.0001$) than that of the YC-Se group. When YC-Se supplementation dose improved,

**Table 2a.** *Effects of three kinds of yeast cultures supplementation on in vitro gas production parameters of maize stover*

| Items[1] | Species[2] | Dose (%) | | | | | | SEM[‡] | Significance (*P*<)[§] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean[†] | 0 | 0.10 | 0.25 | 0.40 | 0.55 | | Species | Dose | Species × Dose |
| $V_f$ (mL) | YC-Cr | 76.58$^{ef}$ | 72.78$^{bc}$ | 69.35$^{c}$ | 82.41$^{a}$ | 80.25$^{a}$ | 78.12$^{a}$ | 1.54 | <0.05 | C(<0.01) | <0.01 |
| | YC-Se | 77.81$^{e}$ | 72.78$^{b}$ | 77.86$^{ab}$ | 80.43$^{a}$ | 79.20$^{a}$ | 78.83$^{b}$ | | | L(<0.05) | |
| | YC-Zn | 74.94$^{f}$ | 72.78$^{b}$ | 76.77$^{a}$ | 74.16$^{ab}$ | 75.33$^{ab}$ | 75.68$^{ab}$ | | | C(<0.01) | |
| | SEM[¶] | 0.69 | | | | | | | | | |
| $T_{0.5}$ (h) | YC-Cr | 14.58$^{f}$ | 14.21 | 15.57 | 12.52 | 13.99 | 16.59 | 0.70 | <0.0001 | NS | <0.05 |
| | YC-Se | 16.67$^{e}$ | 14.21$^{b}$ | 16.60$^{a}$ | 16.78$^{a}$ | 18.09$^{a}$ | 17.65$^{a}$ | | | L(<0.0001) | |
| | YC-Zn | 14.46$^{f}$ | 14.21 | 14.37 | 14.39 | 14.67 | 14.65 | | | NS | |
| | SEM[¶] | 0.31 | | | | | | | | | |
| $FRD_0$ ($\times 10^{-2}$) mL/h | YC-Cr | 3.82$^{e}$ | 2.83$^{b}$ | 3.69$^{b}$ | 4.91$^{a}$ | 4.33$^{a}$ | 3.32$^{b}$ | 0.19 | <0.0001 | Q(<0.05) | <0.0001 |
| | YC-Se | 2.83$^{f}$ | 2.83 | 2.77 | 2.69 | 2.71 | 3.13 | | | NS | |
| | YC-Zn | 2.88$^{f}$ | 2.83 | 2.86 | 2.79 | 2.92 | 3.00 | | | NS | |
| | SEM[¶] | 0.09 | | | | | | | | | |

*[1] Vf, maximum gas production (mL); $FDR_0$, initial fractional rate of degradation at t-value=0; $T_{0.5}$, the elapsed time until half of the maximum gas production was achieved; [2] YC-Cr=Zn-enriched yeast culture; YC-Se=Se-enriched yeast culture; YC-Zn=Zn-enriched yeast culture; [ac] Means within a row for doses that do not have a common superscript differ (P<0.05); [e,f] Means within a column for species that do not have a common superscript differ (P<0.05); [†] Mean=mean for individual species across doses including the dose of 0; [‡] SEM for yeast culture dose; [§] NS, not significant (P>0.05); L, linear effect of dose, Q, quadratic effect of dose, C, cubic effect of dose; [¶] SEM for pooled mean of species including the dose of 0*

**Table 2b.** *Effects of three kinds of yeast cultures supplementation on in vitro gas production parameters of rice straw*

| Items[1] | Species[2] | Dose (%) | | | | | | SEM[‡] | Significance (*P*<)[§] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean[†] | 0 | 0.10 | 0.25 | 0.40 | 0.55 | | Species | Dose | Species × Dose |
| $V_f$ (mL) | YC-Cr | 71.77$^{e}$ | 72.68 | 69.76 | 72.36 | 72.47 | 72.60 | 2.35 | <0.01 | NS | NS |
| | YC-Se | 72.58$^{e}$ | 72.78$^{ab}$ | 75.66$^{a}$ | 74.91$^{ab}$ | 70.86$^{ab}$ | 68.72$^{b}$ | | | Q(<0.05) | |
| | YC-Zn | 67.78$^{f}$ | 72.68 | 70.16 | 62.30 | 65.68 | 68.06 | | | NS | |
| | SEM[¶] | 1.05 | | | | | | | | | |
| $T_{0.5}$ (h) | YC-Cr | 17.97 | 22.08$^{a}$ | 16.78$^{b}$ | 16.45$^{b}$ | 16.07$^{b}$ | 18.46$^{ab}$ | 1.13 | <0.0001 | L(<0.001) | NS |
| | YC-Se | 21.93 | 22.08 | 23.03 | 22.56 | 21.64 | 20.34 | | | NS | |
| | YC-Zn | 19.01 | 22.08$^{a}$ | 21.06$^{ab}$ | 17.25$^{b}$ | 17.15$^{b}$ | 17.50$^{b}$ | | | L(<0.05) | |
| | SEM[¶] | 0.51 | | | | | | | | | |
| $FRD_0$ ($\times 10^{-2}$) mL/h | YC-Cr | 2.35$^{e}$ | 1.79$^{b}$ | 1.93$^{b}$ | 2.79$^{a}$ | 2.84$^{a}$ | 2.41$^{b}$ | 0.21 | <0.001 | L(<0.001) | NS |
| | YC-Se | 1.74$^{f}$ | 1.79 | 1.70 | 1.86 | 1.69 | 1.65 | | | NS | |
| | YC-Zn | 1.94$^{f}$ | 1.79 | 1.59 | 2.43 | 1.94 | 1.93 | | | NS | |
| | SEM[¶] | 0.09 | | | | | | | | | |

*[1] Vf, maximum gas production (mL); $FDR_0$, initial fractional rate of degradation at t-value=0; $T_{0.5}$, the elapsed time until half of the maximum gas production was achieved; [2] YC-Cr=Zn-enriched yeast culture; YC-Se=Se-enriched yeast culture; YC-Zn=Zn-enriched yeast culture; [ab] Means within a row for doses that do not have a common superscript differ (P<0.05); [e,f] Means within a column for species that do not have a common superscript differ (P<0.05); [†] Mean = mean for individual species across doses including the dose of 0; [‡] SEM for yeast culture dose; [§] NS, not significant (P>0.05); L, linear effect of dose, Q, quadratic effect of dose; [¶] SEM for pooled mean of species including the dose of 0*

the $T_{0.5}$ presented linear increasing effect (P<0.0001). For YC-Cr group, the greater value was obtained at the levels of 0.55%, and higher (P<0.05) than that of 0.25%. No significant difference (P>*0.05*) on the value of $T_{0.5}$ when YC-Zn supplementation dose increased. For $FRD_0$ of *in vitro* gas production, the $FRD_0$ value of YC-Cr group was 34.98% and 32.64% higher than that of YC-Se and YC-Zn, respectively (P<0.0001). With the supplemental level of YC-Cr, the $FRD_0$ presented quadratic effect (P<0.05). There were no significant difference in the value of $FRD_0$ when YC-Se and YC-Zn supplementation dose increased (P>0.05). Besides, there were interactive effects on $V_f$ $T_{0.5}$ and $FRD_0$ for the three yeast culture (P<0.05).

For rice straw, the average $V_f$ of YC-Cr and YC-Se was 5.89% and 7.08% higher than that of YC-Zn group, respectively (P<0.01). The $V_f$ of YC-Se increased when the dose of YC-Se increased (quadratic, P<0.05), and the largest value was 75.66 mL, which was obtained at the supplementation level of 0.10%. While there were no significant difference on $V_f$ when YC-Cr and YC-Zn supplementation level increased (P>0.05). There was no significant difference in $T_{0.5}$ when supplemented with three sources of yeast culture (P>0.05). $T_{0.5}$ increased when the dose of YC-Cr supplementation level increased (linear, P<0.01), while $T_{0.5}$ decreased when the dose of YC-Zn supplementation level increased (linear, P<0.01). Additionally, no significant

**Table 3.** *Effects of three kinds of yeast cultures on in vitro IVNDFD and IVDMD of maize stover and rice straw*

| Items[1] | Species[2] | Dose (%) | | | | | | SEM[‡] | Significance (*P*)[§] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean[†] | 0 | 0.10 | 0.25 | 0.40 | 0.55 | | Species | Dose | Species × Dose |
| **Maize stover** | | | | | | | | | | | |
| IVNDFD (%) | YC-Cr | 36.44$^{f}$ | 37.55 | 36.35 | 37.15 | 36.48 | 34.64 | 1.02 | <0.01 | NS | NS |
| | YC-Se | 38.86$^{e}$ | 38.66 | 39.34 | 38.70 | 39.11 | 38.46 | | | NS | |
| | YC-Zn | 37.80$^{ef}$ | 38.66 | 36.88 | 38.66 | 37.63 | 37.17 | | | NS | |
| | SEM[¶] | 0.46 | | | | | | | | | |
| IVDMD (%) | YC-Cr | 51.79$^{f}$ | 51.60 | 52.27 | 52.80 | 48.70 | 53.59 | 1.25 | <0.05 | NS | NS |
| | YC-Se | 53.86$^{e}$ | 51.62$^{b}$ | 54.64$^{a}$ | 54.75$^{a}$ | 54.46$^{a}$ | 53.86$^{ab}$ | | | L(*<0.05*) | |
| | YC-Zn | 52.27$^{ef}$ | 51.58 | 51.62 | 51.84 | 53.31 | 52.96 | | | NS | |
| | SEM[¶] | 0.56 | | | | | | | | | |
| **Rice straw** | | | | | | | | | | | |
| IVNDFD (%) | YC-Cr | 35.42 | 35.16 | 35.02 | 36.36 | 34.95 | 35.59 | 1.02 | NS | NS | <0.05 |
| | YC-Se | 34.22 | 35.18$^{ab}$ | 36.22$^{a}$ | 33.69$^{ab}$ | 32.66$^{b}$ | 33.39$^{ab}$ | | | L(*<0.05*) | |
| | YC-Zn | 35.31 | 35.16$^{ab}$ | 33.81$^{b}$ | 34.46$^{ab}$ | 37.73$^{a}$ | 35.39$^{ab}$ | | | Q(*<0.01*) | |
| | SEM[¶] | 0.59 | | | | | | | | | |
| IVDMD (%) | YC-Cr | 45.79$^{f}$ | 47.40 | 45.25 | 45.68 | 45.33 | 45.30 | 0.81 | <0.01 | NS | NS |
| | YC-Se | 47.47$^{e}$ | 47.41 | 48.31 | 48.01 | 46.98 | 46.68 | | | NS | |
| | YC-Zn | 46.40$^{ef}$ | 47.39$^{a}$ | 47.03$^{a}$ | 46.25$^{ab}$ | 46.43$^{ab}$ | 44.88$^{b}$ | | | *<0.05* | |
| | SEM[¶] | 0.36 | | | | | | | | | |

*[1] IVDMD, in vitro dry matter disappearance; IVNDFD, in vitro neutral detergent fiber disappearance; [2] YC-Cr=Zn-enriched yeast culture; YC-Se=Se-enriched yeast culture; YC-Zn=Zn-enriched yeast culture; [ab] Means within a row for doses that do not have a common superscript differ (P<0.05); [e,f] Means within a column for species that do not have a common superscript differ (P<0.05); [†] Mean=mean for individual species across doses including the dose of 0; [‡] SEM for yeast culture dose; [§] NS, not significant (P>0.05); L, linear effect of dose; Q, quadratic effect of dose; [¶] SEM for pooled mean of species including the dose of 0*

difference was observed when YC-Se supplementation dose increased (P>0.05). For *in vitro* $FRD_0$ of rice straw, the YC-Cr group was 35.06% and 21.13% higher than that of the YC-Se and YC- Zn (P<0.001). $FRD_0$ increased when the dose of YC-Cr supplementation level increased (linear, P<0.001). There was no significant difference in $FRD_0$ when YC-Se and YC-Zn dose increased (P>0.05). Besides, there was no interactive effects on $FRD_0$ for the three sources of yeast culture (P>0.05).

### *In Vitro NDFD and DMD*

The influence of three sources of yeast culture on IVNDFD and INDMD of maize stover and rice straw was shown in *Table 3*. IVDMD and IVNDFD were affected by three yeast culture supplementation for maize stover (P<0.05), IVNDFD and IVDMD for YC-Se were higher by 2.42% and 2.07% compared with YC-Cr treatment, while there was no significant difference between YC-Se and the other two yeast culture supplemented treatments (P>0.05). IVNDFD were not affected (P>0.05) by the supplementation levels of three yeast culture, while the IVDMD of the YC-Se group increased when the dose of YC-Se supplementation level increased (linear, P<0.05).There were no interactive effects (P>0.05) on IVDMD or IVNDFD for maize stover.

For rice straw, the average IVNDFD was not affected by the supplementation of three sources of yeast culture (P>0.05), while IVDMD of YC-Se was significantly higher than that of YC-Cr treatment (P<0.05). IVNDFD of rice straw decreased linearly with the increased YC-Se supplementation levels (P<0.05) as shown in *Table 2*. The largest IVNDFD of rice straw was observed when YC-Zn supplemented at 0.40%, and it increased quadratically with the increased supplementation levels (P<0.01). Compared to YC-Cr treatment, IVDMD of rice straw increased by 1.68% when added YC-Se, but the lower IVDMD for the YC-Zn group was observed at the levels of 0.55%. No significant difference was observed in IVDMD by the supplementation levels of YC-Se and YC-Cr (P>0.05). There were interactive effects (P<0.05) on IVNDFD for rice straw.

### *In Vitro $NH_3$-N Concentration and pH*

Effects of different yeast culture supplementation levels on *in vitro* $NH_3$-N concentration and pH were shown in *Table 4*. For maize stover, *in vitro* $NH_3$-N concentration was affected significantly by the supplementation of three yeast culture (P<0.05), the $NH_3$-N concentration of YC-Cr and YC-Zn were 35.14% and 32.07% higher than that of YC-Se (P<0.0001). As the *Table 3* described, the highest $NH_3$-N concentration of maize stover which obtained at the YC-Cr supplementation level of 0.10%, and it was 24.85% higher than the lowest concentration which obtained at the supplementation level of 0.55% (P<0.05). The $NH_3$-N concentration of control group was significantly higher than that of the other four groups after adding YC-Se, and presented cubic effect with the supplementation levels increased (P<0.01). The lowest (P<0.05) $NH_3$-N concentration for YC-Zn group was obtained at the levels

***Table 4.*** *Effects of three sources of yeast cultures on in vitro pH and $NH_3$-N concentration of maize stover and rice straw*

| Items[1] | Species[2] | Dose (%) | | | | | | SEM[‡] | Significance (*P*)[§] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean[†] | 0 | 0.10 | 0.25 | 0.40 | 0.55 | | Species | Dose | Species × Dose |
| **Maize stover** | | | | | | | | | | | |
| $NH_3$-N (mg/dL) | YC-Cr | 7.46$^{e}$ | 7.78$^{ab}$ | 8.14$^{a}$ | 7.58$^{ab}$ | 7.26$^{ab}$ | 6.52$^{b}$ | 0.36 | <0.0001 | *<0.05* | <0.0001 |
| | YC-Se | 5.52$^{f}$ | 7.78$^{a}$ | 4.12$^{b}$ | 4.96$^{b}$ | 5.49$^{b}$ | 5.24$^{b}$ | | | C(*<0.01*) | |
| | YC-Zn | 7.29$^{e}$ | 7.78$^{a}$ | 7.30$^{ab}$ | 7.21$^{ab}$ | 7.64$^{a}$ | 6.50$^{b}$ | | | *<0.05* | |
| | SEM[¶] | 0.16 | | | | | | | | | |
| pH | YC-Cr | 6.38$^{f}$ | 6.38 | 6.38 | 6.39 | 6.38 | 6.38 | 0.007 | <0.0001 | NS | <0.0001 |
| | YC-Se | 6.43$^{e}$ | 6.38$^{b}$ | 6.44$^{a}$ | 6.43$^{a}$ | 6.45$^{a}$ | 6.45$^{a}$ | | | C(*<0.05*) | |
| | YC-Zn | 6.38$^{f}$ | 6.38$^{ab}$ | 6.39$^{a}$ | 6.38$^{ab}$ | 6.37$^{ab}$ | 6.37$^{b}$ | | | L(*<0.05*) | |
| | SEM[¶] | 0.003 | | | | | | | | | |
| **Rice straw** | | | | | | | | | | | |
| $NH_3$-N (mg/dL) | YC-Cr | 5.06$^{ef}$ | 5.64$^{a}$ | 5.21$^{ab}$ | 4.78$^{ab}$ | 5.17$^{ab}$ | 4.53$^{b}$ | 0.32 | <0.0001 | *<0.05* | <0.05 |
| | YC-Se | 4.70$^{f}$ | 5.64$^{a}$ | 5.22$^{ac}$ | 4.62$^{abc}$ | 3.84$^{b}$ | 4.18$^{bc}$ | | | L(*<0.0001*) | |
| | YC-Zn | 5.57$^{e}$ | 5.64 | 5.25 | 5.67 | 5.29 | 6.01 | | | NS | |
| | SEM[¶] | 0.14 | | | | | | | | | |
| pH | YC-Cr | 6.36$^{f}$ | 6.35$^{b}$ | 6.36$^{ab}$ | 6.36$^{ab}$ | 6.37$^{a}$ | 6.37$^{a}$ | 0.004 | <0.0001 | L(*<0.0001*) | <0.0001 |
| | YC-Se | 6.48$^{e}$ | 6.35$^{b}$ | 6.52$^{a}$ | 6.52$^{a}$ | 6.52$^{a}$ | 6.52$^{a}$ | | | C(*<0.0001*) | |
| | YC-Zn | 6.36$^{f}$ | 6.35$^{b}$ | 6.35$^{b}$ | 6.37$^{a}$ | 6.36$^{ab}$ | 6.38$^{a}$ | | | C(*<0.01*) | |
| | SEM[¶] | 0.002 | | | | | | | | | |

*[1] $NH_3$-N=ammonia nitrogen; [2] YC-Cr=Zn-enriched yeast culture; YC-Se=Se-enriched yeast culture; YC-Zn=Zn-enriched yeast culture; [ac] Means within a row for doses that do not have a common superscript differ (P<0.05); [e,f] Means within a column for species that do not have a common superscript differ (P<0.05); [†] Mean = mean for individual species across doses including the dose of 0; [‡] SEM for yeast culture dose; [§] NS, not significant (P>0.05); L, linear effect of dose; Q, quadratic effect of dose; C, cubic effect of dose; [¶] SEM for pooled mean of species including the dose of 0*

of 0.55%. The pH value of *in vitro* fermentation fluid of maize stover was also affected by adding yeast culture. It was significantly higher for YC-Se than that of the other two kinds of yeast culture (P<0.0001), and the pH value of the control group was significantly lower than that of the another four groups for YC-Se supplemented treatments (P<0.05), the pH of YC-Zn group at the supplementation level of 0.25% was higher (P<0.01) than that of 0.55%, besides, there were cubic effect (P<0.05) and linear effect (P<0.05) with the supplementation dose increased, respectively. There were interactive effects (P<0.0001) on $NH_3$-N concentration and pH value with the increased addition doses of yeast culture for maize stover.

For rice straw, $NH_3$-N concentration in *in vitro* fermentation fluid was not affected when supplemented with yeast culture. However, The $NH_3$-N concentration of rice straw for YC-Se was affected when the supplementation dose changed, the highest $NH_3$-N concentration was observed at the control group and presented linear decreasing effects with the increased supplementation level (P<0.0001), the lowest (P<0.05) $NH_3$-N concentration was obtained at the supplementation level of 0.55%. pH in *in vitro* fermentation fluid of rice straw was affected when supplemented with yeast culture. The pH value for YC-Se was significant higher than that of another two sources of yeast culture (P<0.0001). Besides, different supplementation levels also significantly influenced the pH value in *in vitro* fermentation of rice straw. From the *Table 3*, we known that YC-Cr and YC-Se presented linear and cubic increased effects, respectively (P<0.0001). For YC-Zn, it also presented cubic increased effect with increased levels (P<0.01). Additionally, there was interactive effect on $NH_3$-N concentration and pH with supplementation levels changed for rice straw (P<0.05).

### *In Vitro VFA Concentration*

Effects of YC-Cr, YC-Se and YC-Zn supplementation on *in vitro* VFA concentration in fermentation fluid of maize stover and rice straw were shown in *Table 5a* and *5b*, respectively. For maize stover, the average acetate, propionate and TVFA concentration were not affected by three sources of yeast culture supplementation (P>0.05). It presented linear increasing effects on acetate, propionate, butyrate and TVFA concentration with increased supplementation dose for YC-Cr (P<0.01), while it presented a cubic effect on A: P (P<0.01). For YC-Se group, the largest acetate, propionate, TVFA concentration and A: P were observed at the supplementation dose of 0.55%, and presented linear increasing effects on acetate and TVFA concentration (P<0.05); For YC-Zn treatment. The largest acetate, TVFA concentration and were observed at the supplementation dose of 0.1%.While the largest propionate and butyrate concentration were observed at the supplementation dose of 0.55%. All of them presented cubic effect (P<0.01) with dose increased and without interactive effects (P<0.01) except A: P which presented quadratic effect (P<0.001).

For rice straw, effects of YC-Cr, YC-Se and YC-Zn on VFA

**Table 5a.** *Effects of three sources of yeast cultures on VFA concentration of in vitro incubation fluid of maize stover*

| Items[1] | Species[2] | Dose (%) | | | | | | SEM[‡] | Significance (*P*)[§] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean[†] | 0 | 0.10 | 0.25 | 0.40 | 0.55 | | Species | Dose | Species × Dose |
| Acetate (mmol/L) | YC-Cr | 21.05 | 17.66$^{b}$ | 21.87$^{ab}$ | 21.68$^{ab}$ | 22.83$^{a}$ | 21.18$^{ab}$ | 1.34 | NS | L(<0.01) | NS |
| | YC-Se | 21.73 | 17.67$^{b}$ | 21.53$^{b}$ | 23.47$^{ab}$ | 22.67$^{ab}$ | 23.70$^{a}$ | | | L(<0.05) | |
| | YC-Zn | 20.65 | 17.66$^{b}$ | 23.41$^{a}$ | 18.44$^{b}$ | 21.44$^{ab}$ | 22.27$^{a}$ | | | C(<0.01) | |
| | SEM[¶] | 0.59 | | | | | | | | | |
| Propionate (mmol/L) | YC-Cr | 6.81 | 5.37$^{b}$ | 7.15$^{a}$ | 7.05$^{a}$ | 7.49$^{a}$ | 6.97$^{a}$ | 0.43 | NS | L(<0.01) | NS |
| | YC-Se | 6.38 | 5.37$^{b}$ | 6.34$^{ab}$ | 6.86$^{a}$ | 6.47$^{ab}$ | 6.85$^{a}$ | | | <0.05 | |
| | YC-Zn | 6.48 | 5.37$^{b}$ | 7.08$^{ab}$ | 5.58$^{ab}$ | 6.99$^{ab}$ | 7.36$^{a}$ | | | C(<0.01) | |
| | SEM[¶] | 0.19 | | | | | | | | | |
| Butyrate (mmol/L) | YC-Cr | 2.02$^{e}$ | 1.56$^{b}$ | 2.09$^{a}$ | 2.10$^{a}$ | 2.20$^{a}$ | 2.13$^{a}$ | 0.13 | <0.05 | L(<0.01) | NS |
| | YC-Se | 1.79$^{f}$ | 1.56 | 1.53 | 2.08 | 1.81 | 1.98 | | | NS | |
| | YC-Zn | 1.93$^{ef}$ | 1.56$^{b}$ | 2.13$^{a}$ | 1.74$^{ab}$ | 2.02$^{ab}$ | 2.19$^{a}$ | | | C(<0.01) | |
| | SEM[¶] | 0.06 | | | | | | | | | |
| TVFA (mmol/L) | YC-Cr | 30.37 | 24.98$^{b}$ | 31.65$^{ab}$ | 31.35$^{ab}$ | 33.06$^{a}$ | 30.80$^{ab}$ | 1.93 | NS | L(<0.01) | NS |
| | YC-Se | 30.35 | 24.98$^{b}$ | 29.76$^{ab}$ | 32.81$^{a}$ | 30.93$^{ab}$ | 33.27$^{a}$ | | | L(<0.05) | |
| | YC-Zn | 29.53 | 24.98$^{c}$ | 33.14$^{a}$ | 26.17$^{bc}$ | 30.99$^{ab}$ | 32.34$^{a}$ | | | C(<0.01) | |
| | SEM[¶] | 0.86 | | | | | | | | | |
| A:P | YC-Cr | 3.14$^{g}$ | 3.33$^{a}$ | 3.09$^{b}$ | 3.09$^{b}$ | 3.09$^{b}$ | 3.10$^{b}$ | 0.05 | <0.0001 | C(<0.01) | <0.0001 |
| | YC-Se | 3.46$^{e}$ | 3.33 | 3.47 | 3.45 | 3.49 | 3.53 | | | NS | |
| | YC-Zn | 3.24$^{f}$ | 3.33$^{a}$ | 3.35$^{a}$ | 3.34$^{a}$ | 3.14$^{b}$ | 3.05$^{b}$ | | | Q(<0.001) | |
| | SEM[¶] | 0.02 | | | | | | | | | |

*[1] TVFA, total volatile fatty acids; A:P, ratio of acetate to propionate; [2] YC-Cr=Zn-enriched yeast culture; YC-Se=Se-enriched yeast culture; YC-Zn=Zn-enriched yeast culture; [ab] Means within a row for doses that do not have a common superscript differ (P<0.05); [e,f] Means within a column for species that do not have a common superscript differ (P<0.05); [†] Mean=mean for individual species across doses including the dose of 0; [‡] SEM for yeast culture dose; [§] NS, not significant (P>0.05); L, linear effect of dose; Q, quadratic effect of dose; C, cubic effect of dose; [¶] SEM for pooled mean of species including the dose of 0*

**Table 5b.** *Effects of three sources of yeast culture on VFA concentration of in vitro incubation fluid of rice straw*

| Items[1] | Species[2] | Dose (%) | | | | | | SEM[‡] | Significance (*P*)[§] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean[†] | 0 | 0.10 | 0.25 | 0.40 | 0.55 | | Species | Dose | Species × Dose |
| Acetate, (mmol/L) | YC-Cr | 19.72$^{ef}$ | 21.15$^{a}$ | 18.14$^{b}$ | 19.56$^{ab}$ | 18.48$^{b}$ | 21.29$^{a}$ | 0.98 | <0.001 | <0.05 | NS |
| | YC-Se | 18.31$^{f}$ | 21.14$^{a}$ | 20.47$^{a}$ | 16.45$^{b}$ | 16.31$^{b}$ | 17.17$^{b}$ | | | C(<0.05) | |
| | YC-Zn | 20.97$^{e}$ | 21.15 | 21.30 | 22.25 | 21.09 | 19.08 | | | NS | |
| | SEM[¶] | 0.44 | | | | | | | | | |
| Propionate (mmol/L) | YC-Cr | 5.80$^{f}$ | 6.87$^{a}$ | 5.26$^{b}$ | 5.58$^{b}$ | 5.18$^{b}$ | 6.11$^{ab}$ | 0.30 | <0.001 | C(<0.05) | <0.05 |
| | YC-Se | 5.38$^{g}$ | 6.87$^{a}$ | 5.78$^{b}$ | 4.79$^{c}$ | 4.64$^{c}$ | 4.85$^{c}$ | | | Q(<0.05) | |
| | YC-Zn | 6.19$^{e}$ | 6.87 | 6.34 | 6.33 | 5.95 | 5.43 | | | NS | |
| | SEM[¶] | 0.14 | | | | | | | | | |
| Butyrate (mmol/L) | YC-Cr | 1.69$^{ef}$ | 1.72 | 1.64 | 1.67 | 1.58 | 1.84 | 0.08 | <0.05 | NS | <0.001 |
| | YC-Se | 1.29$^{f}$ | 1.72$^{a}$ | 1.41$^{b}$ | 1.12$^{c}$ | 1.13$^{b}$ | 1.10$^{c}$ | | | L(<0.01) | |
| | YC-Zn | 1.77$^{e}$ | 1.72 | 1.79 | 1.85 | 1.75 | 1.73 | | | NS | |
| | SEM[¶] | 0.04 | | | | | | | | | |
| TVFA (mmol/L) | YC-Cr | 27.91$^{e}$ | 30.17$^{a}$ | 26.82$^{ab}$ | 27.22$^{ab}$ | 25.63$^{b}$ | 29.69$^{ab}$ | 1.33 | <0.0001 | L(<0.01) | <0.01 |
| | YC-Se | 25.30$^{f}$ | 30.17$^{a}$ | 27.96$^{a}$ | 22.63$^{b}$ | 22.36$^{b}$ | 23.41$^{b}$ | | | L(<0.01) | |
| | YC-Zn | 29.35$^{e}$ | 30.17 | 29.84 | 30.87 | 29.22 | 26.65 | | | NS | |
| | SEM[¶] | 0.60 | | | | | | | | | |
| A:P | YC-Cr | 3.59 | 3.16$^{b}$ | 4.03$^{a}$ | 3.58$^{ab}$ | 3.65$^{ab}$ | 3.58$^{ab}$ | 0.14 | NS | <0.05 | NS |
| | YC-Se | 3.54 | 3.14$^{b}$ | 3.71$^{a}$ | 3.55$^{a}$ | 3.64$^{a}$ | 3.64$^{a}$ | | | Q(<0.01) | |
| | YC-Zn | 3.49 | 3.14$^{c}$ | 3.39$^{b}$ | 3.60$^{a}$ | 3.65$^{a}$ | 3.61$^{a}$ | | | Q(<0.01) | |
| | SEM[¶] | 0.06 | | | | | | | | | |

*[1] TVFA, total volatile fatty acids; A:P, ratio of acetate to propionate; [2] YC-Cr=Zn-enriched yeast culture; YC-Se=Se-enriched yeast culture; YC-Zn=Zn-enriched yeast culture; [ac] Means within a row for doses that do not have a common superscript differ (P<0.05); [e,f] Means within a column for species that do not have a common superscript differ (P<0.05); [†] Mean=mean for individual species across doses including the dose of 0; [‡] SEM for yeast culture dose; [§] NS, not significant (P>0.05); L, linear effect of dose; Q, quadratic effect of dose; C, cubic effect of dose; [¶] SEM for pooled mean of species including the dose*

concentration in *in vitro* fermentation fluid were shown in *Table 4*. The YC-Zn treatment obtained the higher acetate, propionate, butyrate, and TVFA concentration and lower A: P compared to another two treatments. For the YC-Cr treatment, the control group obtained the larger propionate (cubic, P<0.05), TVFA concentration (linear, P<0.01) and lower A: P values (P<0.05). For the YC-Se treatment, the larger acetate (cubic, P<0.05), propionate (quadratic, P<0.05), butyrate (linear, P<0.01), TVFA concentration (linear, P<0.0001) and lower A: P (quadratic, P<0.01) value were observed at the supplementation dose of 0% with dose increased. No significant difference in acetate, propionate, butyrate and TVFA concentration were observed when YC-Zn supplementation dose improved except A: P which the lower value was observed at the control group and presented quadratic effect (P<0.01). Besides, there were interactive effects on propionate, butyrate and TVFA for three sources of yeast culture.

## DISCUSSION

Unlike the yeast cultures in which the influence on the rumen fermentation have been relatively intensively studied, available reports on the effects of Cr-, Se- and Zn-enriched yeast cultures on gas production parameters of *in vitro* ruminal fermentation are rather insufficient. In the current study, supplementation of three kinds of yeast cultures elevated the theoretical maximum of gas production ($V_f$) for maize stover, meanwhile, supplementation of YC-Se increased the $V_f$ of rice straw. This finding was in accordance with Tang et al.[1], which reported that supplementation of yeast culture increased the theoretical maximum of gas production, rate of gas production and IVDMD and decreased the lag time for each type of straw (rice straw, wheat straw, maize stover and maize stover silage). However, Tang and Wang [23] reported that there was no significant influence of yeast culture supplementation on *in vitro* gas production of alfalfa, leymuschinensis, this was not in agreement with the results in the current study, the disparity was probably ascribed to the difference in yeast culture and fermentation substrates. Additionally, among the three sources of yeast culture, supplementation of YC-Se was more efficient in improving *in vitro* $V_f$ of maize stover and rice straw compared to the other two yeast cultures. This findings possibly ascribed to that YC-Se element increased the activity of rumen microorganism in *in vitro* fermentation fluid. Wang et al.[24] reported that Se-yeast had a positive influence on rumen fermentation through improving the activity of rumen microorganism. Indexes of $FRD_0$ and $T_{0.5}$ usually reflect the rate of degradation at early incubation stages of '<12 h' and the incubation time of reaching half of the maximum gas production, respectively. Generally speaking, the faster $FRD_0$ is, the shorter $T_{0.5}$ becomes [25]. In *in vitro* fermentation of maize stover and rice straw, the addition of YC-Cr increased $FRD_0$ but decreased $T_{0.5}$ at the supplementation dose of 0.25% and 0.40%, suggesting that the rate of degradation would be accelerated at the early stage of *in vitro* fermentation. Besides, the addition of YC-Cr increased $FRD_0$ and decreased $T_{0.5}$ more efficiently than that of another two sources of yeast cultures. Wang et al.[20] reported that supplementation of two active yeast (Angel yeast & Lesaffre yeast) did not exert a significant influence on IVNDFD, parameters of LE model which including $V_f$, $FRD_0$ and $T_{0.5}$, but it increased the IVDMD of maize stover and rice straw, the difference in results between Wang et al.[20] and the current study may ascribe to the difference in yeast culture supplementation. Previous research indicated that *S. cerevisiae* culture filtrate stimulated the initial rate of cellulose degradation [26]. This may explain the increase in the rate of gas production by the addition of YC-Cr in the current study, the addition of YC-Cr might increase the number of total and cellulolytic bacteria in fermentation liquid.

*In vitro* DMD and NDFD disappearance were important indexes in the using of forage during the process of rumen fermentation. The findings in the current study indicated that supplemented with YC-Se got better effects on IVDMD and IVNDFD for maize stover and IVDMD for rice straw than that of YC-Cr and YC-Zn. Wang et al.[24] reported selenium yeast supplementation effectively increased the number of cellulose decomposition microbes or promoted the vitality of the cellulose decomposition microbes so that the degradation rate increased. These could be explained by that supplemented with YC-Se increased the number of total cellulolytic bacteria in *in vitro* fermentation fluid compared to that of the addition of the other two yeast cultures. However, except for YC-Zn, there was no effect on *in vitro* DMD and NDFD compared to control group with supplementation dose changed. Many similar results have been published; Tripathi and Karim [27] have reported that yeast culture supplementation did not influence intake and digestibility of organic matter, neutrtal detergent fiber (NDF) and acid detergent fiber (ADF). Fokkink et al.[28] have found that there was no effect on calf performance when Se was supplied in the form of YC-Se. Titi et al.[29] also found that addition of yeast culture had no effect on apparent digestibility of DM, CP and NDF. However, opposite finding to the results of the current results was also reported. Miranda et al.[30] had reported that *Saccharomyces cereuisiae* cultures increased *in situ* alfalfa NDF digestion at 48 h. This difference might be resulted from the different experiment method.

Since pH value is an important index reflecting the internal homeostasis of rumen environment, therefore maintaining a relatively stable ruminal pH is vital to assuring efficient rumen fermentation. Ruminants usually possess highly developed systems to maintain ruminal pH within a physiological range of about 5.5-7.0 [31]. In the current study, despite the varying drops in response to the addition of YC-Se, YC-Cr and YC-Zn, the pH value across all treatments ranged from 6.35 to 6.52, providing a suitable

circumstance for fermentation, growth of microorganisms and fiber degradation in the rumen [32].

Hristov et al.[33] suggested that deficiency of $NH_3$-N restricts the microbial protein synthesis, while the over-high $NH_3$-N concentration also inhibits the microbial utilization of $NH_3$-N. Satter and Slyter [34] reported that lowest $NH_3$-N concentration of rumen fluid should not be less than 5 mg/dL to maintain the higher growth rate of bacteria. In this study, concentration of $NH_3$-N in response to the addition of YC-Se was lower than that of YC-Cr and YC-Zn and the lowest concentration of $NH_3$-N was observed at the dose of 0.10% for maize stover and 0.40% for rice straw by adding YC-Se respectively, both of the lowest concentration were lower than 5 mg/dL. The reasons for these results may ascribe to the improvement in synthesis of microbial protein. Lu et al.[35] suggested that supplemented with Se-enriched probiotics could increase synthesis of microbe protein by rumen bacteria through using $NH_3$-N in fermentation fluid. Tripathi and Karim [27] have reported yeast culture supplementation improved microbial CP synthesis. Kamalamma et al.[36] also found that Yea-sacc$^{1026}$ appeared to enhance incorporation of ammonia N into microbial cells. But many published results shown that lack of effect on ammonia concentration in the rumen using *Saccaromyces cerevisiae* yeast culture [37-39]. Different kinds of yeast culture and experiment method might result in this difference.

Ruminal volatile fatty acids (VFAs) are major energy sources for ruminants, rumen VFAs could provide 50-80% of the energy needed by ruminants [40,41], their content and composition are important physiological indexes that reflect rumen digestion and metabolism. In the current study, adding three kinds of yeast cultures to *in vitro* fermentation systems made changes to varying degrees in VFA concentration for maize stover and rice straw respectively, especially VFA concentration increased for maize stover with the enhancement of supplementation dose, while there was a decreased trend in that of rice straw. The changes in complex microflora were major reason for the difference in VFA concentration, *in vitro* fermentation was changed in response to the changes in microflora when yeast culture added. Wang et al.[13] reported that the VFA concentration was significantly related to the numbers of *Selenomonas ruminantium* and *Megasphaera elsdenii* organisms in the rumen. Pinloche et al.[42] found that diet supplementation of probiotic yeast changed the main fibrolytic group *(Fibrobacter* and *Ruminococcus)* and lactate utilizing bacteria *(Megasphaera* and *Selenomonas)*. The findings in TVFA concentration of maize stover increased due to three kinds of yeast cultures supplementation, which was similar to Guedes et al.[37]. Many published results shown that TVFA concentration was not affected in response to SC (*Saccaromyces cerevisiae*) yeast supplementation [38,43].

In this study, besides the stimulating effect of three kinds of yeast cultures on TVFA concentration, supplementation with them also changed the molar proportion of VFA in *in vitro* fermentation fluid. Different acetate: propionate ratio was obtained in response to different yeast culture and supplementation dose. Mwenya et al.[44] found an increase in acetate: propionate ratio when fistulated non-lactating cows were supplemented with *Trichosporum sericeum* yeast culture (forage to concentrate ratio, 70:30), while other researchers found a lower ratio due to SC (*Saccaromyces cerevisiae*) yeast supplementation [4,45].

In summary, YC-Se is preferred compared to YC-Cr and YC-Zn and its optimal dose should be 0.25% and 0.1% for maize stover and rice straw, respectively, as YC-Se improved the *in vitro* fermentation characteristics of maize stover and rice straw, *in vitro* DM and NDF disappearance, and enhanced most of VFA concentration.

## FUTURE RECOMMENDATION

The present *in vitro* results should be tested further using *in vivo* experiments to explore the effects of different yeast culture on milk production in dairy cows in future.

## Conflict of Interest

There was no Conflict of interest.

## Acknowledgements

The authors wish to acknowledge the financial support received from the National Natural Science Foundation of China (No.31001024, No. 31320103917), Ministry of Science and Technology of the People's Republic of China (No. 2012BAD14B17), Chinese Academy of Sciences (No. KFJ-EW-STS-071) and Hunan Provincial Science and Technology Department (2013TF3006).

## REFERENCES

**1. Tang SX, Tayo GO, Tan ZL, Sun ZH, Shen LX, Zhou CS, WJ Xiao, Ren GP, Han XF, Shen SB:** Effects of yeast culture and fibrolytic enzyme supplementation on *in vitro* fermentation characteristics of low-quality cereal straws. *J Anim Sci,* 86, 1164-1172, 2008. DOI: 10.2527/jas.2007-0438

**2. Lesmeister KE, Heinrichs AJ, Gabler MT:** Effects of supplemental yeast *(Saccharomyces cerevisiae)* culture on rumen development, growth characteristics, and blood parameters in neonatal dairy calves. *J Dairy Sci,* 87, 1832-1839, 2004. DOI: 10.3168/jds.S0022-0302(04)73340-8

**3. Plata PF, Mendoza GDM, Bárcena-Gama JR, González MS:** Effect of a yeast culture *(Saccharomyces cerevisiae)* on neutral detergent fiber digestion in steers fed oat straw based diets. *Anim Feed Sci Technol,* 49, 203-210, 1994. DOI: 10.1016/0377-8401(94)90046-9

**4. Williams PE, Tait CAG, Innes GM, Newbold CJ:** Effects of the inclusion of yeast culture *(Saccharomyces cerevisiae* plus growth medium) in the diet of cows on milk yield and forage degradation and fermentation patterns in the rumen of sheep and steers. *J Anim Sci,* 69, 3016-3026, 1991. DOI: 10.2527/1991.6973016x

**5. Martin SA, Nisbet DJ, Dean RG:** Influence of a commercial yeast supplement on the *in vitro* ruminal fermentation. *Nutr Rep Int,* 40, 395-403,1989.

**6. Callaway ES, Martin SA:** Effects of *Saccharomyces cerevisiae* culture on ruminal bacteria that utilize lactate and digest cellulose. *J Dairy Sci,* 80, 2035-2044, 1997. DOI: 10.3168/jds.S0022-0302(97)76148-4

**7. Mazo VK, Gmoshinski IV, Zorinand SN:** New food sources of essential trace elements produced by biotechnology facilities. *Biotechnol J,* 2, 1297-1305, 2007. DOI: 10.1002/biot.200700015

**8. Ortman K, Pehrson B:** Selenite and selenium yeast as feed supplements for dairy cows. *J Vet Med,* 4, 373-380, 2007.

**9. Zhang SQ, Zhang Y, Peng N, Zhang HB, Yao J, Li ZH, Liu LG:** Pharmacokinetics and biodistribution of zinc-enriched yeast in rats. *Sci Word J,* 2014, 217142, 2014. DOI: 10.1155/2014/217142

**10. Anderson RA:** Chromium, glucose intolerance and diabetes. *J Am Coll Nutr,* 17, 548-555, 1998. DOI: 10.1080/07315724.1998.10718802

**11. Mertz W:** Chromium in human nutrition: A review. *J Nutr,* 123, 626-633, 1993. DOI: 10.1093/jn/123.4.626

**12. Yue WB, Zhang CX, Shi LG, Ren YS, Jiang YS, Kleemann DO:** Effect of supplemental selenomethionine on growth performance and serum antioxidant status in Taihang black goats. *Asian-Austral J Anim Sci,* 22, 365-370, 2009. DOI: 10.5713/ajas.2009.80474

**13. Shi L, Zhang CX, Yue WB, Shi LG, Zhu XM, Lei FL:** Short-term effect of dietary selenium-enriched yeast on semen parameters, antioxidant status and Se concentration in goat seminal plasma. *Anim Feed Sci Technol,* 157, 104-108, 2010. DOI: 10.1016/j.anifeedsci.2010.02.006

**14. Wang XX, Li XB, Zhao CX, Hu P, Chen H, Liu ZX, Liu GW, Zhe W:** Correlation between composition of the bacterial community and concentration of volatile fatty acids in the rumen during the transition period and ketosis in dairy cows. *Appl Environ Microb,* 78, 2386-2392, 2012. DOI: 10.1128/AEM.07545-11

**15. Zhang CH, Jiang JY, Ren HY, Zhu FH, Li WL:** Effects of chromium yeast on immune function and carcass quality in heat stressed broilers. *Acta Ecol Anim Domast,* 30, 56-61, 2009.

**16. Cope CM, Mackenzie AM, Wilde D, Sinclair LA**: Effects of level and form of dietary zinc on dairy cow performance and health. *J Dairy Sci,* 92, 2128-2135, 2008. DOI: 10.3168/jds.2008-1232

**17. Tang SX, Tan ZL, Zhou CS, Jiang H, Jiang Y, Sheng L:** A comparison of *in vitro* fermentation characteristics of different botanical fractions of mature maize stover. *J Anim Feed Sci,* 15, 505-515, 2006. DOI: 10.22358/jafs/66920/2006

**18. AOAC:** Official Methods of Analysis of AOAC International. 16th edn. Association of Official Analytical Chemists, MD, Washington, DC, USA,1999.

**19. Van Soest PJ, Robertson JB, Lewis BA:** Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci,* 74, 3583-3597, 1991. DOI: 10.3168/jds.S0022-0302(91)78551-2

**20. Wang M, Sun XZ, Janssen PH, Tang SX, Tan ZL:** Responses of methane production and fermentation pathways to the increased dissolved hydrogen concentration generated by eight substrates in *in vitro* ruminal cultures. *Anim Feed Sci Technol,* 194, 1-11, 2014. DOI: 10.1016/j.anifeedsci.2014.04.012

**21. Wang Z, He ZX, Beauchemin KA, Tang SX, Zhou CS, Han XF, Wang M, Kang JH, Odongo NE, Tan ZL:** Comparison of two live *Bacillus* species as feed additives for improving *in vitro* fermentation of cereal straws. *Anim Sci J*, 87, 27-36, 2016. DOI: 10.1111/asj.12346

**22. Wang M, Tang SX, Tan ZL:** Modeling *in vitro* gas production kinetics: Derivation of Logistic-Exponential (LE) equations and comparison of models. *Anim Feed Sci Technol*, 165, 137-150, 2011. DOI: 10.1016/j.anifeedsci.2010.09.016

**23. Tang HC, Wang T:** The effects of yeast culture on *in vitro* fermentation of rumen microorganisms and the metabolism of goat rumen[D]. Nanjing Agricultural University, Nanjing, p: 32-39, 2006.

**24. Wang C, Liu Q, Huang YX, Miao CH, Gao DH**: Effects of dietary Se-yeast supplementation on rumen fermentation and purine derivatives in Simmental steers. *Chin J Anim Nutr*, 19, 725-730, 2007.

**25. Wang M, Sun XZ, Tang SX, Tan ZL, Pecheco D:** Deriving fractional rate of degradation of logistic-exponential (LE) model to evaluate early *in vitro* fermentation. *Animal*, 7, 920-929, 2013. DOI: 10.1017/S1751731112002443

**26. Sullivan HM, Martin SA:** Effects of a Saccharomyces cerevisiae culture on *in vitro* mixed ruminal microorganism fermentation. *J Dairy Sci*, 82, 2011-2016, 1999. DOI: 10.3168/jds.S0022-0302(99)75438-X

**27. Tripathi MK, Karim SA:** Effect of individual and mixed live yeast culture feeding on growth performance, nutrient utilization and microbial crude protein synthesis in lambs. *Anim Feed Sci Technol*, 155, 163-171, 2010. DOI: 10.1016/j.anifeedsci.2009.11.007

**28. Fokkink WB, Hill TM, Bateman G, Aldrich JM, Schlotterbeck RL:** Selenium yeast for dairy calf feeds. *Anim Feed Sci Technol*, 153, 228-235, 2009.

**29. Titi HH, Dmour RO, Abdullah AY:** Growth performance and carcass characteristics of Awassi lambs and Shami goat kids fed yeast culture in their finishing diet. *Anim Feed Sci Technol*, 142, 33-43, 2008. DOI: 10.1016/J.ANIFEEDSCI.2007.06.034

**30. Miranda RLA, Mendoza MGD, Barcena-Gama JR, Gonzalez MSS, Ferrara R, Ortega CME, Cobos PMA:** Effect of *Saccharomyces cerevisiae* or *Aspergillus oryzae* cultures and NDF level on parameters of ruminal fermentation. *Anim Feed Sci Technol,* 63, 289-296, 1996. DOI: 10.1016/S0377-8401(96)01008-5

**31. Krause KM, Oetzel GR:** Understanding and preventing subacute ruminal acidosis in dairy herds: A review. *Anim Feed Sci Technol,* 126, 215-236, 2006. DOI: 10.1016/J.ANIFEEDSCI.2005.08.004

**32. Stewart CS, Flint HJ, Bryant MP:** The rumen bacteria. **In,** Hobson PN, Stewart CS (Eds): The Rumen Microbial Ecosystem, 10-72. Chapman and Hall, London, 1997.

**33. Hristov AN, Ropp JK, Hunt CW:** Effect of barley and its amylopectin content on ruminal fermentation and bacterial utilization of ammonia-N *in vitro*. *Anim Feed Sci Technol,* 99, 25-36, 2002. DOI: 10.1016/S0377-8401(02)00076-7

**34. Satter LD, Slyter LL:** Effect of ammonia concentration of rumen microbial protein production *in vitro*. *Br J Nutr,* 32, 199-208, 1974. DOI: 10.1079/BJN19740073

**35. Lu M, Ran LW, Ye GG, Wu XM, Huang KH:** Effects of dietary addition of Se-enriched probiotics on blood parameters and antioxidant capacity of goats. *Anim Husbandry Vet Med,* 45, 94-97, 2013.

**36. Kamalamma UK, Krishnappa P:** Effect of feeding yeast culture (Yea-sacc[1026]) on rumen fermentation *in vitro* and production performance in crossbred dairy cows. *Anim Feed Sci Technol,* 57, 247-256, 1996. DOI: 10.1016/0377-8401(95)00829-2

**37. Guedes CM, Goncalves D, Rodrigues MAM, Dias-da-Silva A:** Effects of a *Saccharomyces cerevisiae* yeast on ruminal fermentation and fibre degradation of maize silages in cows. *Anim Feed Sci Technol,* 145, 27-40, 2008. DOI: 10.1016/j.anifeedsci.2007.06.037

**38. Doreau M, Jouany JP:** Effect of a *Saccharomyces cerevisiae* culture on nutrient digestion in lactating dairy cows. *J Dairy Sci,* 81, 3214-3221, 1998. DOI: 10.3168/jds.S0022-0302(98)75885-0

**39. Newbold CJ, Wallace RJ, Chen XB, McIntosh FM:** Different strains of *Saccharomyces cerevisiae* differ in their effects on ruminal bacterial numbers *in vitro* and in sheep. *J Anim Sci,* 73, 1811-1818, 1995. DOI: 10.2527/1995.7361811x

**40. Bergman EN, Reid RS, Murray MG, Brockway JM, Whitelaw FG:** Interconversions and production of volatile fatty acids in sheep rumen. *Biochem J,* 97, 53-58, 1965. DOI: 10.1042/bj0970053

**41. Sutton JD:** Digestion and absorption of energy substrates in the lactating cow. *J Dairy Sci,* 68, 3376-3393, 1985. DOI: 10.3168/jds.S0022-0302(85)81251-0

**42. Pinloche E, McEwan N, Marden JP, Bayourthe C, Auclai E, Newbold CJ:** The effects of a probiotic yeast on the bacterial diversity and population structure in the rumen of cattle. *Plos One,* 8, 1-10, 2013. DOI: 10.1371/journal.pone.0067824

**43. Enjalbert F, Garrett JE, Moncoulon R, Bayourthe C, Chicoteau P:** Effects of yeast culture (*Saccharomyces cerevisiae*) on ruminal digestion in non-lactating cows. *Anim Feed Sci Technol,* 76, 195-206, 1999. DOI: 10.1016/S0377-8401(98)00230-2

**44. Mwenya B, Santoso B, Sar C, Pen B, Morikawa R, Takaura K, Umetsu K, Kimura K, Takahashi J:** Effects of yeast culture and galacto-oligosaccharides on ruminal fermentation in Holstein cows. *J Dairy Sci,* 88, 1404-1412, 2005. DOI: 10.3168/jds.S0022-0302(05)72808-3

**45. Erasmus LJ, Botha PM, Kistner A:** Effects of yeast culture supplement on production, rumen fermentation and duodenal nitrogen flow in dairy cows. *J Dairy Sci,* 75, 3056-3065, 1992. DOI: 10.3168/jds.S0022-0302(92)78069-2

Kafkas Univ Vet Fak Derg
24 (5): 761-767, 2018
DOI: 10.9775/kvfd.2018.19997

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Investigation of Rotavirus, Adenovirus and Astrovirus in Mussels and Shrimps Using Multiplex Real-time PCR

Farouk GHALYOUN [1,a] Ayla ÜNVER ALÇAY [2,b]

[1] Istanbul Aydin University, Institute of Natural and Applied Sciences, Department of Food Safety, TR-34513 Esenyurt, Istanbul - TURKEY
[2] Istanbul Aydin University, ABMYO, Food Technology Program, Florya Main Campus, TR-34295 Sefakoy/Kucukcekmece, Istanbul - TURKEY
[a] ORCID: 0000-0002-1304-4657; [b] ORCID: 0000-0003-3254-155X

**Article Code: KVFD-2018-19997 Received: 22.04.2018 Accepted: 23.07.2018 Published Online: 23.07.2018**

**How to Cite This Article**

**Ghalyoun F, Ünver Alçay A:** Investigation of Rotavirus, Adenovirus and Astrovirus in mussels and shrimps using multiplex real-time PCR. *Kafkas Univ Vet Fak Derg,* 24 (5): 761-767, 2018. DOI: 10.9775/kvfd.2018.19997

## Abstract

Viruses are one of the most common pathogens transmitted via food. Based on epidemiological evidence, human enteric viruses are considered to be as the most commonly transmitted etiological agents by bivalve shellfish. In Istanbul, the most widely consumed sea products after fish are mussels and shrimps. In this study, the presence of rotavirus, adenovirus and astrovirus were investigated in mussel and shrimp samples consumed by hunting on the shores of Istanbul. For this purpose, a total of 28 groups of shrimp and 52 groups of mussel were collected from different places in Istanbul. Each group was analyzed separately as a sample. Viruses were extracted from the digestive tissue by direct elusion method in a glycine/NaCl, pH 9.5 buffer followed by PEG-6000 precipitation. Multiplex Real-time RT-PCR technique was used to analyze the shellfish samples. Astrovirus was found in 63.46% of the total 52 analyzed mussel samples, whereas adenovirus was found in 46.15%. None of the mussel samples was positive for rotavirus. All tested shrimp samples were negative for all three researched viruses. This is the first report on the prevalence of rotavirus, adenovirus and astrovirus in mussels and shrimps in Turkey. The results show that mussels sold in Istanbul are highly contaminated with adenovirus and astrovirus and this could pose a great threat to public health.

***Keywords:*** *Mussel, Shrimp, Rotavirus, Adenovirus, Astrovirus, RT-PCR*

# Midye ve Karideslerde Rotavirus, Adenovirus ve Astrovirus Varlığının Multiplex Real-time PCR Kullanılarak Araştırılması

## Öz

Viruslar gıda vasıtasıyla en yaygın bulaşan patojenlerden biridir. Epidemiyolojik bulgulara göre insana ait enterik viruslar kabuklu deniz ürünleri vasıtasıyla en çok aktarılan etiyolojik ajan olarak tanımlanmaktadır. İstanbul'da balıktan sonra en çok tüketilen deniz ürünleri midye ve karidestir. Bu çalışmada, İstanbul kıyılarında avlanarak tüketilen midye ve karides numunelerinde rotavirus, adenovirus ve astrovirus varlığı araştırılmıştır. Bu amaçla toplam 28 karides ve 52 midye grubu İstanbul'un farklı yerlerinden toplanmıştır. Her bir grup ayrı bir numune olarak analize alınmıştır. Viruslar glisin/NaCl, pH 9.5 tamponu kullanarak direkt elüsyon metodu ile doğrudan sindirim dokusundan ayrıştırılmış ve PEG-6000 ile konsantre edilmiştir. Alınan midye ve karides numuneleri Multiplex Real-time PCR yöntemi ile analiz edilmiştir. Toplam çalışılan 52 midye numunesinin %46.15'inde adenovirus ve %63.46'sında astrovirus saptanmıştır. Rotavirus hiçbir midye numunesinde tespit edilmemiştir. Karides numunelerinin hiçbirinde araştırılan virusların hiçbiri saptanmamıştır. Bu, Türkiye'deki midyelerde ve karideslerde rotavirus, adenovirus ve astrovirus prevalansı hakkındaki ilk rapordur. Sonuçlar, İstanbul'da satılan midyelerin adenovirus ve astrovirus ile kontamine olduğunu ve bu durumun halk sağlığı için büyük bir tehdit oluşturabileceğini göstermektedir.

***Anahtar sözcükler:*** *Midye, Karides, Rotavirus, Adenovirus, Astrovirus, RT-PCR*

## INTRODUCTION

Viral contamination of food and water represents a significant threat to human health [1]. Viruses are now recognized as a major cause of food-borne diseases and in recent years the incidence of such diseases has been increasing worldwide. Over 100 virus species which cause a wide variety of illnesses in humans may be present in sewage

**İletişim (Correspondence)**
+90 534 5136567
faroukghalyoun@stu.aydin.edu.tr

contaminated waters [2]. Water quality and poor hygiene practices are the key factors having the highest influence on fresh produce contamination [3].

Diarrhea is the second leading cause of death in children under five years old worldwide and is held responsible for around 525000 child deaths annually [4]. Different enteric viruses like group A rotaviruses, adenoviruses, astroviruses and caliciviruses may infect children in the early childhood phase and cause gastroenteritis [5]. Moreover, several studies have described human adenoviruses (HAdV) as the third causative agent of acute gastroenteritis in infants and young children right after rotaviruses and noroviruses [6].

Rotaviruses are found in waste water and can also be concentrated by shellfish [7,8]. Human astrovirus is a significant cause of acute diarrhea among children. Fresh products such as shellfish, infected water, lettuce, green onions and other green vegetables, soft berries such as strawberries and raspberries are among the susceptible foods that can be contaminated with human astrovirus in the pre-harvest stage [9]. Adenoviruses are associated with a variety of clinical diseases involving nearly every organ of the human body. Adenoviruses can be transmitted through the fecal-oral route and infected food and water intake [10]. In a study on the prevalence of multiple viral agents in patients with upper respiratory tract infection, adenovirus was found in 11 samples (6.4%) of the total 171 patients tested [11]. Adenoviruses can also be transmitted during food processing. In a study on the presence of adenovirus genomes, it was reported that 5.8% of 291 swabs contained the adenovirus genome, particularly in restaurants and canteen kitchens. These findings indicate that kitchen surfaces can lead to viral contamination and that food workers should be educated on virus transmission [12]. Enteric HAdV has also been reported to be very common in aquatic environments [13-15].

In spite of the fact that foodborne diseases are a notable problem, testing of foods for viral contamination is done infrequently. The most used methods for clinical diagnosis are electron microscopy, passive particle agglutination tests or enzyme-linked immunosorbent assays (ELISA). Nonetheless, these methods do not possess a sensitivity high enough in order to be used to recognize viral particles usually present in low numbers in environmental samples. Therefore, methods based on the amplification of viral nucleic acids by polymerase chain reaction (PCR) have been applied to an increasing extent for the detection of viruses in water and food samples [16].

Virus detection in shellfish has never been a trouble-free process. It is to be expected that viruses are present in very low numbers in shellfish tissue, which nevertheless are still enough to cause an infection. Special methodologies should be used in order to achieve a highly efficient virus recovery from shellfish tissues. Additionally, when viruses are extracted from shellfish tissues, the resulting extracts are highly cytotoxic because inhibitory substances are concentrated and recovered along with the viruses. Shellfish extracts will often cause inhibition to the molecular assay, especially if PCR based methodologies are to be used. Consequently, the ultimate objective would be the development of new procedures for shellfish analysis which result in a low volume of none-cytotoxic or highly pure nucleic acid preparation with no inhibitory effects to the PCR. Molecular techniques, especially RT-PCR, serve as excellent tools for the detection of health-significant viruses in food and environmental samples [17].

In the Turkish Food Codex Microbiological Criteria Regulation, acceptable limits of *E. coli* and *Salmonella* for live bivalve molluscs; acceptable limits of Histamine, *Salmonella* and *L. monocytogenes* for processed bivalve molluscs and shellfish have been defined. There are no regulations for virus contamination in the Turkish Food Codex [18]. There is also no official system of monitoring of the virus for bivalve molluscs and shellfish; in addition, there are very few scientific studies on this subject in Turkey. Although most cases of foodborne illness caused by enteric viruses, there is a lack of knowledge about sources of enteric viruses and outbreak reports.

The research about the presence of norovirus in shellfish was carried out in Turkey [19]; but there is no study of adenovirus, rotavirus and astrovirus. For this reason, in this study, the prevalence of rotavirus, adenovirus and astrovirus in mussles and shrimps that are frequently consumed in Istanbul has been researched.

# MATERIAL and METHODS

## Materials

### *Sampling*

A total of 80 shellfish samples (52 Mediterranean black mussels *[Mytilus galloprovincialis]* and 28 deep sea pink shrimps *[Parapenaeus longirostris]*) were collected in April 2017 from three different locations in Istanbul before the end of the fishing season. The shellfish were collected live, fresh and unfrozen. Additionally, it was made sure that no chemical substances nor additives were added to the shellfish. Each sample contained either 400 g shrimp or 25 mussel pieces. Sampling quantity and location is shown in *Table 1*.

Immediately after collection, shellfish were placed in sterile blender bags (Stomacher® bags) and transported under cold conditions to the laboratory.

## Methods

Analyses were carried out at Istanbul Aydin University Food Processing Laboratory and Intertek Test Laboratories (Yenibosna/Bahcelievler/Istanbul).

**Table 1.** *Sampling quantity and location*

| Sample Type | Quantity | Groups Analyzed (n) | Sampling Location |
|---|---|---|---|
| Shrimp | 8 kg | 20 | Beylikduzu - Gurpinar Seafood market |
| Shrimp | 3.2 kg | 8 | Buyukcekmece - Mimarsinan Fishers port |
| Mussel | 500 pieces | 20 | Beylikduzu - Gurpinar Seafood market |
| Mussel | 350 pieces | 13 | Rumeli Kavagi - Fresh fish selling shops |
| Mussel | 500 pieces | 19 | Beyoglu - Restaurants |

### *Shellfish Processing for Virus Concentration*

After arrival at the laboratory, shellfish were washed with sterile distilled water and shucked with a sterile knife. It has been reported that most viruses are found in the highest concentrations in the stomach and digestive diverticula of shellfish [17]. Using these organs for virus analysis makes the isolation of viral nucleic acids easier and faster and also increases the sensitivity of the molecular test by increasing the number of shellfish analyzed [17,20]. Therefore, the digestive tissues were dissected for analysis under aseptic conditions and used for virus isolation. For each sample 18-22 g of shellfish digestive tissue were extracted. For each 18-22 g sample a total of 400 g of shrimp or 25 mussel pieces were processed. Viral particles were eluted by the methods described below.

### *Virus Elusion and Concentration*

Direct glycine elution and virus concentration was applied according to the method by Kingsley and Richards [21] with minor modifications. First, viral particles were eluted from shellfish tissue using glycine buffer (0.1 M glycine and 0.3 M NaCl, pH 9.5) (Glycine GR for analysis-Merck Millipore-Germany). Later, PEG-6000 (Polyethylene Glycol 6000-Merck Millipore-Germany) was used for the concentration of viruses.

For direct elusion, 18-22 g of shellfish digestive tissue sample was homogenized with 175 mL of glycine buffer (0.1 M glycine and 0.3 M NaCl, pH 9.5) in a blender for 3 min at maximum speed. Then, 40 mL of the homogenate were centrifuged at 7000 x g for 30 min at 4°C.

Virus particles present in the supernatant were concentrated using PEG-6000. A 16% PEG-6000 and 0.525 M NaCl solution was added to an equal volume of the supernatant and left for one hour on ice for precipitation. After precipitation, samples were centrifuged at 7000 x g for 10 min at 4°C. Resulting pellets were diluted in 400 µL of distilled nuclease free water and stored at -30°C to be used later for virus nucleic acid isolation.

### *Isolation of Viral DNA/RNA*

Viral nucleic acid extraction was performed using a commercial DNA/RNA kit (InnuPrep Virus DNA/RNA Kit-AnalytikJena-Germany) which is an extraction kit for isolating viral DNA and RNA at the same time and from the same sample. The isolation procedure was carried out based on the manufacturer's instructions. For isolation 400 µL of sample solutions containing the concentrated viruses prepared in the previous step were used. Standard precautions were applied during handling of samples in order to reduce the probability of contamination. The isolation procedure combines lysis of starting material with subsequent binding of viral nucleic acids onto the surface of a spin filter membrane. After several washing steps the viral nucleic acids are eluted from the membrane using RNase-free water. The extracted viral nucleic acids are suitable for use with applications like PCR, real-time PCR or any kind of enzymatic reaction.

Before the molecular analysis, the resulting nucleic acid quantity was measured for each sample using a spectrophotometer (Spectrophotometer for Life Science, Shimadzu Corporation Analytical & Measuring Instruments Division, Japan). In order to prevent PCR inhibition and to acquire accurate results, a dilution step was applied to samples with a DNA/RNA concentration higher than 40 ng/µL.

### *Multiplex Real-time PCR*

Real-time PCR assay was performed using the PowerChek Adeno/Astro/Rota Real-time PCR Kit (Kogene Biotech-Korea) according to the manufacturer's instructions. The kit contains the primer/probe mix (Adenovirus specific primer and probe, Astrovirus specific primer and probe, Rotavirus specific primer and probe, IC specific primer and probe and DNA for IC), RT-PCR Reaction Buffer (containing dNTPs and MgCl2), RT Enzyme Mix (One-step RT-PCR Enzyme Mix), Exogenous Positive Control (EPC) and Adeno/Astro/Rota Virus positive control. The Internal Control (IC) allows the user to determine and control possible PCR inhibition. The IC reagents are built in the Primer/Probe Mix. Also, it needs not to be run separately. The IC is introduced into each amplification reaction and is co-amplified with target DNA from specimen. The Real-time RT-PCR test includes cDNA synthesis by reverse transcriptase and PCR amplification using *Taq.* DNA polymerase and specific primer and probe labeled with the fluorescent dye in a single tube. The kit is suitable for detection of the target gene region without requirement for any additional reagents between the reverse transcription and PCR amplification steps, which minimizes contamination risk for the samples.

The PCR test was performed in optical grade 96-well plates. The required PCR reaction mix was prepared based on the number of wells available in each PCR cycle. Each well was filled with 15 µL of PCR reaction mix and 5 µL of extracted sample (total 20 µL). For the positive control (C+), 5 µL of the positive control available in the kit was used instead of the sample and 5 µL of nuclease-free water was used for the negative control (NTC). RT-PCR runs were performed on a real-time thermocycler (Agilent Technologies-USA, Mx3005P). Reaction conditions were as follows: 30 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 seconds at 94°C and 60 seconds at 55°C as recommended by the manufacturer. According to the information given by the kits manufacturer company, test sensitivity is 100-1000 copies limit of detection (LOD) and specificity is 100% exclusivity for about 35 non-target strains.

After preparation of the reaction mix and addition of isolated viral nucleic acids to the reaction tubes, the target sequences of adenovirus, astrovirus and rotavirus specific genes and the Internal Control (IC) were detected through the FAM, VIC (HEX), ROX and Cy5 channels respectively.

## RESULTS

Of the total 52 tested mussel sample groups (1350 pieces), 24 groups (46.15%) contained adenovirus and 33 groups (63.46%) contained astrovirus while rotavirus was not detected in any of the tested mussel samples. Additionally, it was determined that 40.38% of analyzed mussels groups (21 groups) contained both astrovirus and adenovirus. Rotavirus, adenovirus and astrovirus were not detected in any of the analyzed shrimp samples.

The distribution of adenovirus (AdV) and astrovirus (AsV) in mussel samples based on sampling location is shown in *Table 2*.

The obtained Ct values during the PCR run were used to establish a standard curve. A different color was specified

***Table 2.*** *Adenovirus (AdV) and astrovirus (AsV) in mussel samples based on sampling location*

| **Mussel Samples** | | **AdV Positive** | | **AsV Positive** | | **AdV & AsV Positive** | |
|---|---|---|---|---|---|---|---|
| **Sampling Location** | **No. of Groups** | **N*** | **%** | **N** | **%** | **N** | **%** |
| Gurpinar Seafood market | 20 | 17 | 85 | 19 | 95 | 16 | 80 |
| Rumeli Kavagi | 13 | 5 | 38.46 | 12 | 92.31 | 4 | 30.77 |
| Beyoglu | 19 | 2 | 10.53 | 2 | 10.53 | 1 | 5.26 |
| Total | 52 | 24 | 46.15 | 33 | 63.46 | 21 | 40.38 |

*N: Number of positive samples*

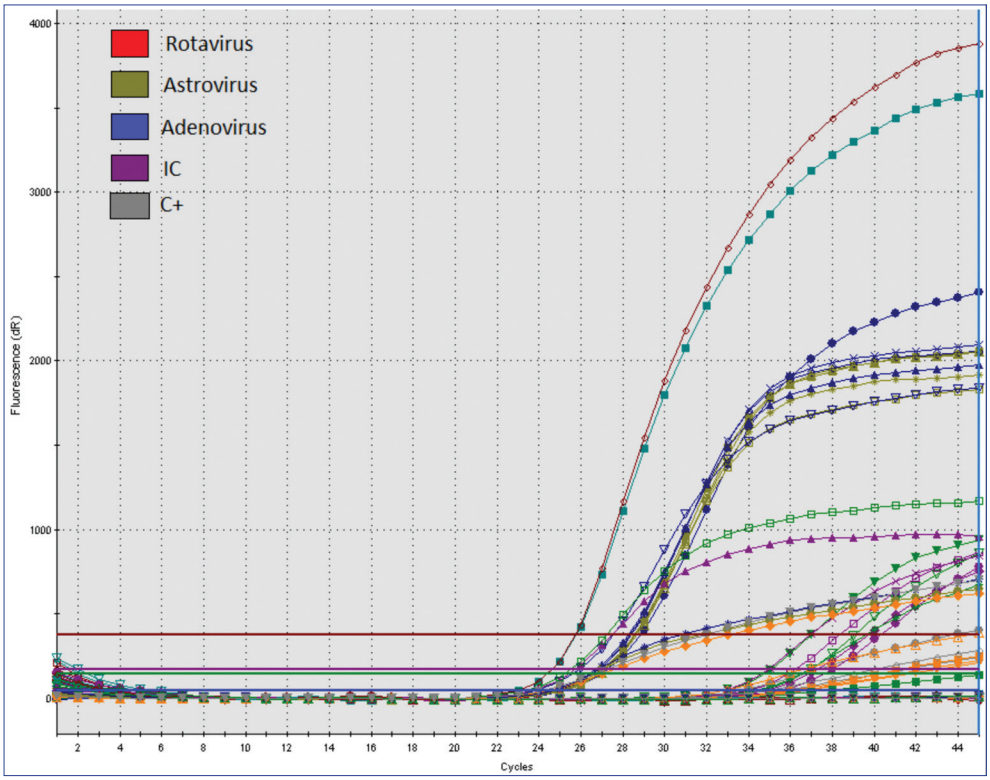



**Fig 1.** PCR amplification plots

for each of the researched viruses as well as for the internal control (IC) and positive control (C+). The resulting PCR amplification plots were depicted on a diagram as shown in *Fig.1*.

## DISCUSSION

As a result of eating raw or undercooked fish and other seafoods, it is possible for consumers to become infected with zoonotic diseases (parasitic, bacterial and viral). Extracting more than 100 types of enteric viruses in their feces, seafoods, especially shellfish, are considered to be the main contamination source of enteric viruses in humans. Fish, crabs, clams, mussels, shrimps and oysters living in sewage contaminated waters have been shown to carry bacteria and viruses of enteric origin. Based on experimental studies, enteric viruses were present in the structures of fish and mussels feeding on lobster, sand worms and other residuals. Especially oysters and mussels are of big importance because these types of seafoods can be consumed raw or half-cooked [22].

Enteric viral pathogens like adenovirus, astrovirus, norovirus and hepatitis E virus (HEV) are health significant viruses and may be associated with the consumption of contaminated water or shellfish. During their filter feeding process, shellfish tend to concentrate viruses and bacteria in their edible tissues and the concentrations on these microorganisms are expected to be much higher in shellfish than in the surrounding waters [2].

In the study by Le Guyader et al.[23], astrovirus was detected in 50% of the analyzed mussel samples. Elamri et al.[24] tested the prevalence of multiple enteric viruses in shellfish samples collected from different locations in Tunisia. Astrovirus was found in 61% of the total analyzed mussel and clam samples. In a study in south Italy by Fusco et al.[25], the prevalence of different enteric viruses in Mediterranean mussels *(Mytilus galloprovincialis)* has been researched. In the course of two years, 108 mussel groups (25 groups in 2014 and 83 groups in 2015) were collected and analyzed using Real-time RT-PCR. Among the researched viruses, astrovirus was found in 16% of the first mussel group collected in 2014 and in 32.53% of the second group collected in 2015. In present study, astrovirus was found in 63.46% of the total 52 tested mussel samples which is higher than the results of Le Guyader et al.[23], Elamri et al.[24] and Fusco et al.[25].

Adenovirus was found positive in 18.6% of the total 86 mussel samples collected from the Norwegian coast [26]. Formiga-Cruz et al.[27] tested the prevalence of enteric viruses in *(Mytilus edulis)* mussel samples in a wide range study conducted in many geographical regions throughout Europe. Adenovirus was found in 33% of 144 samples collected from Greece, in 33% of 54 samples collected from Sweden and in 36% of 104 samples collected from Spain. These ratios are relatively low when compared to the results of this study. In this study adenovirus was found in 46.15%.

For the 173 samples collected from England adenovirus was found positive in 46% which matches the results of this study. Muniain-Mujika et al.[28]. researched the prevalence of human pathogenic viruses in Mediterranean black mussels *(Mytilus galloprovincialis)* and clams *(Crassostrea gigas)*. While hepatitis A virus was found in 24% of the analyzed samples, adenovirus was found in 47%. This result is almost the same with the result of this study in which adenovirus was found in 46.15%.

The results of this study show that as high as 85% of mussel samples collected from Gurpinar seafood market in Beylikduzu were positive for adenovirus, 95% were positive for astrovirus and 80% contained both adenovirus and astrovirus at the same time. In addition, while 38.46% of mussel samples collected from Rumeli Kavagi contained adenovirus, as high as 92.31% contained astrovirus and 30.77% contained both adenovirus and astrovirus. This is an indicator of the high levels of contamination of mussels collected and sold in Beylikduzu and Rumeli Kavagi counties. On the other hand, only 10.53% of mussel samples collected from Beyoglu county contained adenovirus and 10.53% as well contained astrovirus which is relatively low compared to other sampling locations.

In present study, all of the tested shrimp samples were negative for all researched viruses. In a study on shrimps, the biological accumulation of adenovirus in pink shrimps collected from south Brazil was researched by Luz et al.[29]. One hundred pink shrimp samples *(Farfantepenaeus paulensis)* were collected between April 2012 and May 2013 and examined using Real-time qPCR for the prevalence of multiple types of adenovirus. 35% of the total analyzed samples contained various types of adenovirus (Avian adenovirus %17, Bovine adenovirus %13, Canine adenovirus 7% and Porcine adenovirus %2) [29]. However, and in a similar way to this study, none of the tested shrimp samples contained human adenovirus. In a different case, 20 shrimp samples *(Penaeus monodon)* were collected from the southwestern shores of India and analyzed using RT-PCR method for the prevalence of enterovirus, adenovirus, norovirus and hepatitis A virus. While only 15% of the tested samples contained enterovirus, none of them contained neither adenovirus nor any of the other researched viruses [30].

Differently than mussels, shrimps are usually collected from deep places in the sea. Mussels normally live close to rocky seashores and are collected from such locations. Wastewater, which is the main contamination source of enteric viruses, is discharged close to seashores. Therefore, it is to be expected that mussels will be more contaminated than shrimps. In the highly populated city of Istanbul, the amount of sewage discharge is expected to be extremely

high. Because Istanbul is one of the most crowded cities in the world, high contamination of seashores can be the reason for the high contamination ratios of mussels that were found in present study.

Foodborne viruses continuously show high resistance to environmental conditions and are always posing a threat to our health. Such viruses are transmitted mainly through food and the fecal-oral route and can cause different diseases such as gastroenteritis and diarrhea. The prevalence of enteric viruses has been detected in seafoods in different studies around the world. On the other hand, there are few studies related to enteric viruses in mussels and shrimps in Turkey. Erol et al.[31] investigated hepatitis A virus (HAV) and norovirus (NoV) contamination in mussels collected from 8 stations in the gulf of Izmir between August 2009 and September 2010. In this study, it was determined that mussels in the Gulf of Izmir in Turkey were heavily contaminated with HAV and NoV; 9 of the 30 digestive tissue samples (30%) were positive for NoV and 8 samples (26.7%) were positive for HAV by direct PCR, RT-nested PCR and RT-booster PCR [31]. These rates are higher than the contamination rate detected in the study by Terzi et al.[32]. In the study by Terzi et al.[32], RT-PCR was used to analyze 60 mussel samples collected from the middle Black Sea region in Turkey for the prevalence of HAV which was detected only in two samples [32]. A previous study conducted by Yilmaz et al.[19] in Turkey researched NoV (Genogroup I and II) positivity by RT-PCR in mussel samples. For this study, a total of 320 mussels were collected from fish distributors and samples were pooled. NoV Genogroup II was detected in 5 (4.5%) of 110 mussel pools collected from the Bosphorus, Istanbul, Turkey [19]. There is no research on other enteric viruses in mussels and shrimps in Turkey. Since there is limited scientific data on the prevalence of enteric viruses in shellfish in Turkey, further research is needed.

*Mytilus galloprovincialis* (Mediterranean black mussel) is widely available in Turkey and mostly consumed as stuffed and fried mussels. Stuffed mussels are sold as ready-to-eat foods by most street sellers in Istanbul. It is known that hygienic and storage conditions are not sufficiently taken into account during their production and/or sale and that microbiological qualities are generally low. Therefore, consuming raw, undercooked and cross contaminated mussels can result in illness. In this sense, taking necessary measures during obtaining, processing and selling of raw black mussels is very important in terms of public health and food safety.

In the present study, adenovirus and astrovirus were detected in high ratios in mussels. This is an indicator of the high level of contamination of mussels sold in Istanbul with these viruses and possibly other enteric viruses. This study and a few other studies aforementioned in Istanbul show that increasing the awareness of food workers and restaurants about the correct handling and cooking of shellfish is of great importance from the perspective of food safety.

## REFERENCES

**1. Goyal SM:** Methods of virus detection in foods. **In,** Goyal SM (Ed): Viruses in Foods. 101-119, Springer-Verlag US, 2006. DOI: 10.1007/0-387-29251-9

**2. Bosch A:** Human enteric viruses in the water environment: A mini-review. *Int Microbiol,* 1 (3): 191–196, 1998.

**3. Greening GE:** Human and animal viruses in food (Including taxonomy of enteric viruses). **In,** Goyal SM (Ed): Viruses in Foods. 5-42, Springer-Verlag US, 2006. DOI: 10.1007/0-387-29251-9

**4. WHO (World Health Organization):** Diarrhoeal disease–Fact Sheet, WHO Media Centre. Available from: http://www.who.int/mediacentre/factsheets/fs330/en/ *Accessed:* July 31, 2017.

**5. Richards GP:** Shellfish-associated viral disease outbreaks. **In,** Goyal SM (Ed): Viruses in Foods. 223-238, Springer-Verlag US, 2006. DOI: 10.1007/0-387-29251-9

**6. Portes SAR, Lanzarini NM, Vieira CB, Fernandes J, Silva ADS, de Oliveira RC, da Costa EV, de Lemos ERS, Fumian TM:** Viruses in mass gatherings: Viral waterborne and foodborne diseases. *Virus Rev Res J Brazil Soc Virol,* 21 (2): 32-40, 2016.

**7. Cook N, Bridger J, Kendall K, Gomara MI, El-Attar L, Gray J:** The zoonotic potential of rotavirus. *J Infect*, 48 (4): 289-302, 2004. DOI: 10.1016/j.jinf.2004.01.018

**8. Lees D:** Viruses and bivalve shellfish. *Int J Food Microbiol*, 59 (1-2): 81-116, 2000. DOI: 10.1016/S0168-1605(00)00248-8

**9. Pinto RM, Bosch A:** Rethinking virus detection in food. **In,** Koopmans M, Cliver DO, Bosch A (Eds): Foodborne Viruses: Progress and Challenges. 171-188, ASM Press, Washington, DC, USA, 2008.

**10. Boone SA, Gerba CP:** Significance of fomites in the spread of respiratory and enteric viral disease. *Appl Environ Microbiol*, 73 (6): 1687-1696, 2007. DOI: 10.1128/AEM.02051-06

**11. Celebi Akarsu D, Altoparlak U:** The investigation of some viral agents in the patients with upper respiratory tract in Erzurum region. *Kafkas Univ Vet Fak Derg*, 15 (6): 951-955, 2009. DOI: 10.9775/kvfd.2009.785

**12. Maunula L, Ronnqvist M, Aberg R, Lunden J, Nevas M:** The presence of norovirus and adenovirus on environmental surfaces in relation to the hygienic level in food service operations associated with a suspected gastroenteritis outbreak. *Food Environ Virol*, 9 (3): 334-341, 2017. DOI: 10.1007/s12560-017-9291-7

**13. Miagostovich MP, Ferreira FFM, Guimaraes FR, Fumian TM, Diniz-Mendes L, Luz SLB, Silva LA, Leite JPG:** Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream waters of Manaus, central Amazonia. *Appl Environ Microbiol*, 74 (2): 375-382, 2008. DOI: 10.1128/AEM.00944-07

**14. Barrella KM, Garrafa P, Monezi TA, Harsi CM, Salvi C, Violante PABC, Mehnert DU:** Longitudinal study on occurrence of adenoviruses and hepatitis A virus in raw domestic sewage in the city of Limeira, Sao Paulo. *Braz J Microbiol*, 40 (1): 102-107, 2009. DOI: 10.1590/S1517-838220090001000017

**15. Prado T, Miagostovich MP:** Environmental virology and sanitation in Brazil: A narrative review. *Cad Saude Publica*, 30 (7): 1367-1378, 2014. DOI: 10.1590/0102-311X00109213

**16. Vasickova P, Dvorska L, Lorenkova A, Pavlik I:** Viruses as a cause of foodborne diseases: A review of the literature. *Vet Med*, 50 (3): 89-104, 2005.

**17. Bosch A, Pinto RM, Le Guyader FS:** Viral contaminants of molluscan shellfish: Detection and characterization. **In,** Shumway SE (Ed): Shellfish Safety and Quality. 608, Woodhead Publishing Limited, 2009.

**18. Turkish Food Codex Regulation on Microbiological Criteria:** 29 December 2011-28157.

**19. Yilmaz H, Bostan K, Turan N, Muratoglu K, Yilmaz A, Ozkul AA, Kocazeybek B, Helps C:** Real-time PCR detection of norovirus in mussels

collected from the Bosphorus in Istanbul, Turkey. *Food Environ Virol*, 2 (2): 64-68, 2010. DOI: 10.1007/s12560-010-9028-3

**20. Atmar RL, Neill FH, Woodley CM, Manger R, Fout GS, Burkhardt W, Leja L, McGovern ER, Le Guyader F, Metcalf TG, Estes MK:** Collaborative evaluation of a method for the detection of Norwalk virus in shellfish tissues by PCR. *Appl Environ Microbiol*, 62 (1): 254-258, 1996.

**21. Kingsley DH, Richards GP:** Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and norwalk-like viruses in shellfish. *Appl Environ Microbiol*, 67 (9): 4152-4157, 2001. DOI: 10.1128/AEM.67.9.4152-4157.2001

**22. Arslan A, Dincoglu A, Guven A:** The zoonoses produced by the sea foods consumption in humans. *Kafkas Univ Vet Fak Derg,* 1, 103-107, 1995.

**23. Le Guyader F, Haugarreau L, Miossecm L, Dubois E, Pommepuy M:** Three-year study to assess human enteric viruses in shellfish. *Appl Environ Microbiol*, 66 (8): 3241-3248, 2000. DOI: 10.1128/AEM.66.8.3241-3248.2000

**24. Elamri DE, Aouni M, Parnaudeau S, Le Guyader FS:** Detection of human enteric viruses in shellfish collected in Tunisia. *Lett Appl Microbiol*, 43 (4): 399-404, 2006. DOI: 10.1111/j.1472-765X.2006.01978.x

**25. Fusco G, Bartolo ID, Cioffi B, Ianiro G, Palermo P, Monini M, Amoroso MG:** Prevalence of foodborne viruses in mussels in southern Italy. *Food Environ Virol*, 9 (2): 187-194, 2017. DOI: 10.1007/s12560-016-9277-x

**26. Myrmel M, Berg EMM, Rimstad E, Grinde B:** Detection of enteric viruses in shellfish from the Norwegian coast. *Appl Environ Microbiol*, 70 (5): 2678-2684, 2004. DOI: 10.1128/AEM.70.5.2678-2684.2004

**27. Formiga-Cruz M, Tofino-Quesada G, Bofill-Mas S, Lees DN, Henshilwood K, Allard AK, Conden-Hansson AC, Hernroth BE, Vantarakis A, Tsibouxi A, Papapetropoulou M, Furones MD, Girones R:** Distribution of human virus contamination in shellfish from different growing areas in Greece, Spain, Sweden, and the United Kingdom. *Appl Environ Microbiol*, 68 (12): 5990-5998, 2002. DOI: 10.1128/AEM.68.12.5990-5998.2002

**28. Muniain-Mujika I, Calvo M, Lucena F, Girones R:** Comparative analysis of viral pathogens and potential indicators in shellfish. *Int J Food Microbiol*, 83 (1): 75-85, 2003. DOI: 10.1016/S0168-1605(02)00324-0

**29. Luz RB, Staggemeier R, Fabres RB, Soliman MC, Souza FG, Gonçalves R, Fausto IV, Rigotto C, Heinzelmann LS, Henzel A, Fleck JD, Spilki FR:** Bioaccumulation of animal adenoviruses in the pink shrimp. *Braz J Microbiol*, 46 (3): 715-723, 2015. DOI: 10.1590/S1517-838246320140323

**30. Umesha KR, Bhavani NC, Venugopal MN, Karunasagar I, Krohne G, Karunasagar I:** Prevalence of human pathogenic enteric viruses in bivalve molluscan shellfish and cultured shrimp in south west coast of India. *Int J Food Microbiol*, 122 (3): 279-286, 2008. DOI: 10.1016/j.ijfoodmicro.2007.12.024

**31. Erol N, Delibas SB, Ozkoc S, Erguden C, Aksoy U:** Investigation of parasitic and viral pathogens in *mussels (Mytilus galloprovincialis)* in the Gulf of Izmir, Turkey. *Saudi Med J*, 37 (6): 703-706, 2016. DOI: 10.15537/smj.2016.6.15034

**32. Terzi G, Albayrak H, Yazici Z, Gumusova SO, Celik TH:** First identification of hepatitis A virus RNA in mussels *(Mytilus spp.)* in Turkey. *J Anim Vet Adv*, 9 (11): 1584-1587, 2010. DOI: 10.3923/javaa.2010.1584.1587

Kafkas Univ Vet Fak Derg
24 (5): 769-774, 2018
DOI: 10.9775/kvfd.2018.20031

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

# Bimanual Capsulorhexis Using a New Hand Tool: An Experimental Study in Sheep Eye

Koray KARADAYI [1] Çağrı GÜLTEKİN [2,a] Ersin AYTAÇ [3,b]

[1] This work was supported by the Near East University [grant numbers: CE028-2016]

[1] Near East University School of Medicine, Department of Ophthalmology, 99138 Lefkoşa, TRNC
[2] Department of Surgery, Faculty of Veterinary Medicine, Near East University, 99138 Lefkoşa, TRNC
[3] Near East University Innovation and Information Technologies Center, NEURobotics Lab, 99138 Lefkoşa, TRNC
[a] ORCID:0000-0001-8586-1472; [b] ORCID: 0000-0001-6401-2949

**Article Code: KVFD-2018-20031 Received: 27.04.2018 Accepted: 29.07.2018 Published Online: 29.09.2018**

**How to Cite This Article**

**Karadayi K, Gültekin Ç, Aytaç E:** Bimanual capsulorhexis using a new hand tool: An experimental study in sheep Eye. *Kafkas Univ Vet Fak Derg,* 24 (5): 769-774, 2018. DOI: 10.9775/kvfd.2018.20031

## Abstract

The purpose of this study was to design a cheap and easy-to-use hand tool that allows a better-centered capsulorhexis of consistent shape and size for inexperienced and experienced surgeons in cataract surgery. Forty-five eyes of dead adult sheep from a local slaughterhouse were grouped as; group 1, the continuous curvilinear capsulorhexis (CCC) group (n: 15); group 2, the cerclage wire group (n: 15); and group 3, the polylactic acid (PLA) hand tool group (n: 15). In group 1, one corneal incision was made. In groups 2 and 3, two incisions were made at 3:00 and 11:00 o'clock directions. Methylene blue was used for capsular staining. After injecting viscoelastic into the anterior chamber and the capsule was punctured, the devices were inserted at 3:00 o'clock. All capulorhexes were performed using the Utrata forceps following the internal contour of the devices. As a result of the study, capsulorhexes were successfully performed in 9 of 15 eyes in group 1; 2 of 15 eyes in group 2; and 12 of 15 eyes in group 3. Statistically, the correlation between the distributions of groups 1 and 2 ($\chi^2$=7.033, P=0.008); and for groups 1 and 3 ($\chi^2$=6.171, P=0.002) were found to be significant. This study showed that CCC was performed using the newly designed PLA hand tool more successfully than the classical method by the beginning surgeon.

***Keywords:*** *Polylactic acid, Capsulorhexis, Continuous curvilinear capsulorhexis, Cataract surgery*

# Yeni Bir El Aleti Kullanarak Bimanuel Kapsüloreksis: Koyun Gözünde Deneysel Çalışma

## Öz

Bu çalışma, katarakt cerrahisinde deneyimsiz ve deneyimli cerrahlar için kullanımı kolay, ucuz ve uygun şekil ve boyutta sentralize bir kapsüloreksis yapılabilmesine olanak tanıyan bir el aleti tasarlamak amacıyla yapıldı. Yerel bir mezbahaneden toplanan 45 ölü koyun gözü; grup 1, sürekli eğrisel kapsüloreksis (CCC) grubu (n: 15); grup 2, serklaj tel grubu (n: 15); ve grup 3, polilaktik asit (PLA) aleti grubu (n: 15) olarak ayrıldı. Grup 1'de bir korneal ensizyon yapıldı. Grup 2 ve 3'te, saat 3:00 ve 11:00 yönlerinde iki ensizyon yapıldı. Kapsül, metilen mavisi ile boyandı. Ön kamaraya viskoelastik enjekte edildikten sonra kapsül delindi, cihazlar saat 3: 00 yönünden yerleştirildi. Tüm kapsüloreksisler, cihazların iç kontürünü izleyen Utrata forsepsi kullanılarak gerçekleştirildi. Çalışma sonucunda, grup 1'deki 15 gözün 9'u, grup 2'deki 15 gözün 2'si; ve grup 3'teki 15 gözün 12'sinde kapsüloreksis başarıyla gerçekleştirildi. İstatistiksel olarak, grup 1 ve 2'nin ($\chi^2$=7.033, P=0.008); ve grup 1 ve 3'ün ($\chi^2$=6.171, P=0.002) dağılımları arasındaki korelasyon anlamlı bulundu. Bu çalışma, deneyimsiz cerrah tarafından yeni tasarlanmış PLA cihaz kullanılarak yapılan CCC'nin klasik yönteme göre daha başarılı olduğunu göstermiştir.

***Anahtar sözcükler:*** *Polilaktik asit, Kapsüloreksis, Sürekli eğrisel kapsüloreksis, Katarakt cerrahisi*

## INTRODUCTION

Cataract surgery is the most commonly performed eye surgery in most countries. The surgery basically consists of the stages of corneal incision, capsulorhexis, phaco-fragmentation/aspiration and intraocular lens (IOL) insertion. One study demonstrates that capsulorhexis is the most difficult step in cataract surgery and depends on the surgeon's experience [1]. Gimbel and Neuhann's continuous curvilinear capsulorhexis (CCC) technique made it possible for capsulorhexis to be of appropriate shape and diameter. Although the shape and diameter of CCC is not always

**İletişim (Correspondence)**
+90 533 31042 81
cagri.gultekin@neu.edu.tr

consistent, with this method, the fixation and centration of the IOL is highly assured [2]. To create a CCC of more consistent shape and diameter, various techniques and devices have been used. Because of the increasing use of premium multifocal IOLs, a standard diameter and shape of rhexis as well as a smooth capsule edge is now of utmost importance. It is known that the rhexis edge created using the highly priced Femtosecond Laser Assisted Cataract Surgery System (FLACS) is not as smooth and strong as that which created by the classical manual capsulorhexis [3]. Studies are conducted on animal eyes or surgical simulators to enhance surgical training and practice, while new instruments are continuously developed in order to facilitate capsulorhexis [4-11]. However, many of the instruments developed so far have not been applied to clinical-use with sufficient success due to either their high cost or lack of practicality [12-15].

As a new manufacturing technology, three-dimensional printers are used in producing custom shaped prostheses, implants, and various surgical instruments [16-19]. One of the most durable and bioadaptable thermoplastic materials used in the production of surgical instruments is the polylactic acid (PLA) polymer [15]. PLA, which is an FDA-approved biocompatible, biodegradable, and environment friendly polymer, is a lactic acid- based aliphatic polyester. Its filaments are dissolved by means of bacteria or through a hydrolysis process [20]. These manufactured PLA devices can be readily sterilized with plasma sterilization technology [21]. Furthermore, their production cost is lower than that of other similar surgical instruments [18].

We aimed in this study to develop a new cost-efficient and easy-to-use instrument to facilitate capsulorhexis especially for the beginning cataract surgeon, also to insure a better-centered capsulorhexis of consistent shape and size for the experienced surgeon wishing to implant a premium IOL.

## MATERIAL and METHODS

Forty five eyes of dead adult sheep were obtained from a local slaughterhouse. In the study, we compared the bending cerclage wire and PLA tool, which are the two low costly materials with the CCC method. Routine CCC was performed in group 1 (n: 15) using a bent cystotome and a Utrata forceps *(Fig. 1)*. In group 2 (n:15), capsulorhexis was performed bimanually with the aid of a cerclage wire improvised by bending a cerclage wire of 70 mm length and 0.8 mm width (20 gauge, Tektel surgical steel wire, Doğsan Inc., Turkey) into a semicircle of 7 mm-diameter *(Fig. 2)*.

The PLA instrument used in group 3 (n: 15) was designed in Solidworks 2016 (Dassault Systems Solidworks Corp.) and produced in Ultimaker Extended 2 Plus (Ultimaker B.V., Netherlands), a 3D printer was used. PLA filaments used in production were in the form of standard PLA of (Ultimaker PLA - Ultimaker B.V.) 2.85 mm in diameter. Ultimaker 3D Printer is using FDM (Fused Deposition Modeling) technique. FDM is an additive manufacturing technology working as follows; the polymer is pulled from the spool via a stepper motor and introduced in the hot end which is 215°C where the polymer reaches superfluidity. The polymer was produced through the method of stacking up over printing tray in which it was poured from a height of 0.1 mm using stepper motors working in a precision of 0.1 mm in the X-Y-Z coordinate axis system. Also through the printing process, the print bed is kept at 60°C which is the critical temperature for the PLA to start its crystallization.

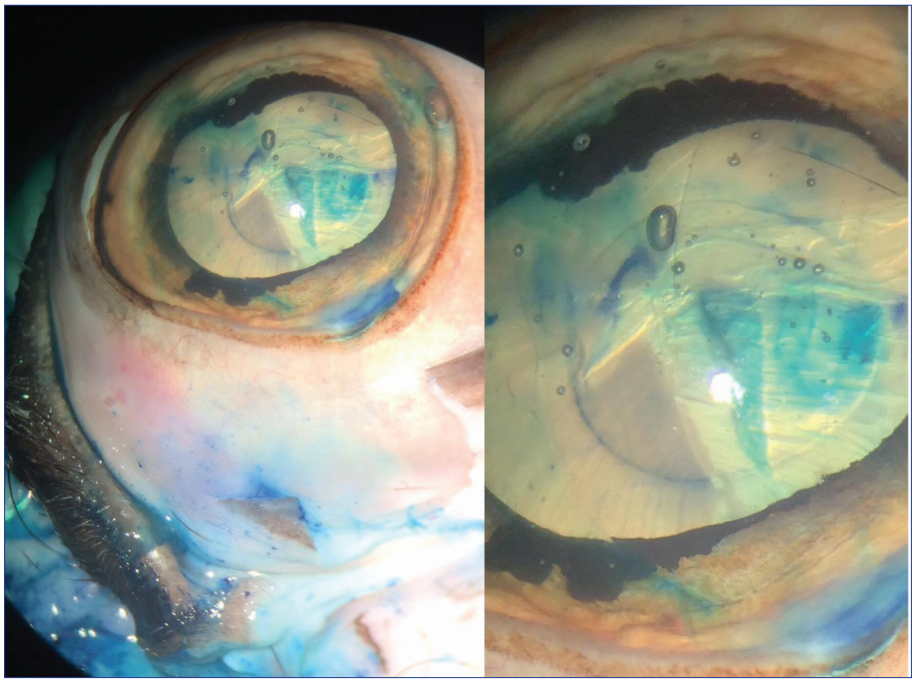


**Fig 1.** CCC was successfully performed using a cystotome and a Utrata forceps in Group 1

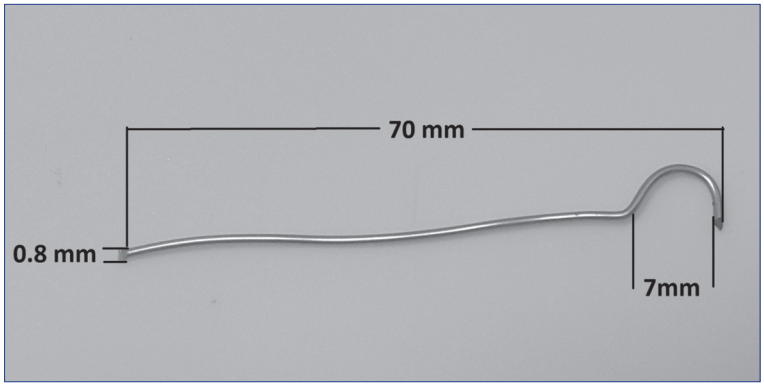



**Fig 2.** The cerclage wire device

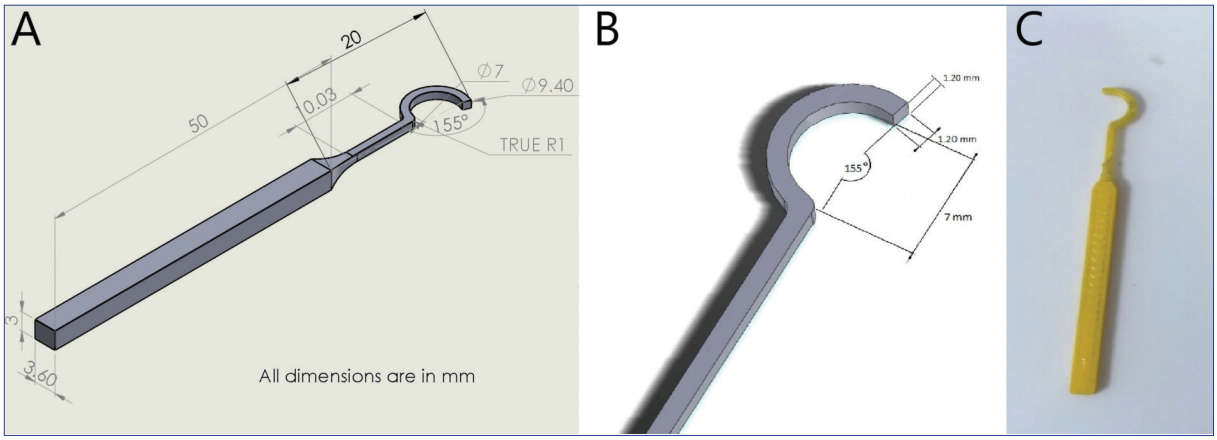



**Fig 3.** A) Technical Solidworks manufacturing drawing of invented PLA hand tool, B) Detailed technical drawing of the tip, C) Actual 3D printed invented PLA hand tool

After the instrument is designed in Solidworks, designed part tested by Solidworks simulation tool. The simulation process was to hold the ring part on its place and applying force to the handle bar to observe the stress in the transition part. The applied force ranged from 1Newton (N) to 10N and the von Mises stress on the transition part changed from 1.244e+0.8 to 1.244e+0.9 in which the part is still in the range of elastic deformation. Elastic deformation is the type of deformation where the object turns back to its original shape. In capsulorhexis technique; to tear the anterior capsule with constant shape and size the applied force will never exceed 1.01kilogram force (kgf), since the invented tool is a disposable item, therefor there were no need for fatigue analysis. After the simulation the design is exported as STL (STereo Lithography) format. An STL file describes a raw, unstructured triangulated surface by the unit normal and vertices of the triangles using a three dimensional Cartesian coordinate system. The STL is then introduced to a slicing software called Cura. Cura is a 3D printing slicing application. Cura exports the file in G-Code format, which is one of the variants of the most widely used numerical control (NC) programming language. The Ultimaker 3D printer accepts G-Code to manufacture the device as designed. The length of the instrument produced is 70.45 mm. The width of its stem part is 3.6 mm and its height 3 mm, forming a circle of 205° at the end of the instrument. The inner diameter of the circle is 7 mm and the outer 9.4 mm. The tip at the end is 1.2 mm-thick and square-shaped providing a smooth bottom contact surface with square edges as opposed to the circular shape of cerclage wire *(Fig. 3)*.

All eyes collected from the local slaughter house were preserved in 7% NaCl solution in cold chain and studied within 12 h. The eyes had been removed with the eyelids. In all groups the eyes were exposed open by medial canthotomy, then fixed onto a polystyrene foam with 2 needles. Anterior chamber was entered at 11 o'clock position using a 3.2 mm slit angled corneal knife. Anterior capsule was stained with methylene blue after air was injected into the anterior chamber. Following irrigation of the anterior chamber, a viscoelastic substance (Viscoat, Alcon Lab. Inc., Texas, USA) was injected to fill the anterior chamber as usual. The lens capsule was punctured with a cystotome. In group 1, capsulorhexis was performed monomanually as usual using a Utrata forceps inserted through the incision at 11 o'clock.

In groups 2 and 3, the instruments to be tested were inserted into the anterior chamber through the second incision at 3 o'clock as to supply a crescent guide inferiorly in the direction of 3-6-9 o'clock hours *(Fig. 4A)*. After laying the improvised guide onto the capsule, a capsulorhexis of

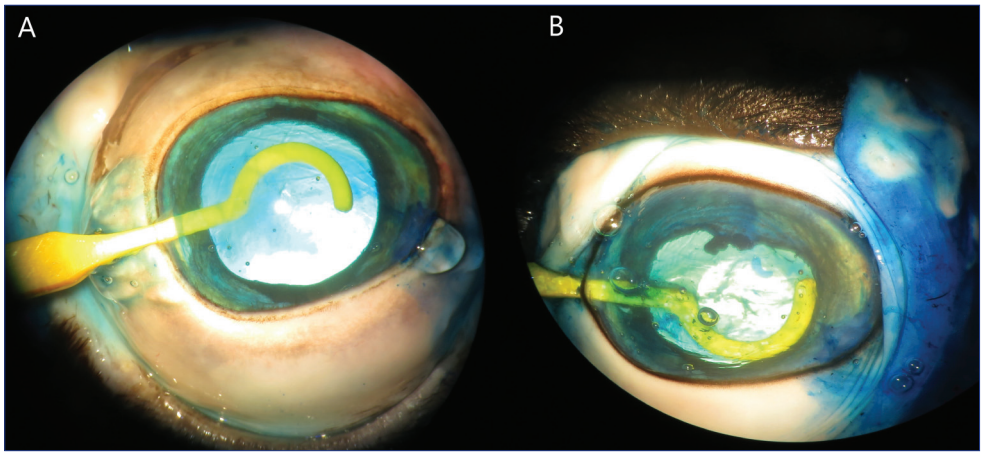



**Fig 4.** A) In group 3, the instruments to be tested were inserted into the anterior chamber through the incision at 3 o'clock and to supply a crescent guide inferiorly in the direction of 3-6-9 o'clock hours, B) The PLA hand tool turned around 180 degrees on its long axis into the anterior chamber, and reinserted over the anterior capsule in the direction of 9-12-3 o'clock hours

80-200 degrees was fashioned using a Utrata forceps inserted at the 11 o'clock entrance after the puncturing the capsule with the cystotome. At that stage, the improvised instrument (the cerclage wire or the PLA guide) was taken out of the anterior chamber, turned around 180 degrees on its long axis and reinserted into the anterior chamber, thus laying superiorly over the anterior capsule in the direction of 9-12-3 o'clock hours *(Fig. 4B)*. Then the capsular flap was regrasped with the Utrata forceps to complete the other (160-180 degrees) half of the rhexis, following the contour of the internal edge of the instrument just as the inferior half.

Successful CCC was defined as a smooth continuous curvilinear circle of 6 to 7 mm of diameter as the acronym CCC implies. In all 3 groups, the rate of success was recorded either as successful or unsuccessful. Statistically, the data obtained was evaluated with the Chi-square test.

All surgeries were performed by the same veterinary surgeon that had no prior experience in capsulorhexis. As the beginning surgeon, he started with the first group of 15 eyes and then proceeded with the second group and the third, consecutively.

## RESULTS

In the first group, successful CCC was obtained in 9 of 15 eyes. In the unsuccessful 6 eyes, either the capsule was torn or the rhexis was not of a full-circle shape. In group 2, a full circle of CCC was created in only 2 of 15 eyes. The cause of unsuccessful rhexis in this group was tearing of the rhexis edge off course close to the distal end of the wire.

In group 3, a successful CCC of 7 mm diameter was created in 12 of 15 eyes. Of note, in this group the unsuccessful cases were the first 3 cases. In all 3 cases, the rhexis edge was observed to tear away at the same point consistently close to the middle part of the guide all in the same manner. On magnified close inspection, we observed a flaw at this region- a very small protuberance at the base of the inferior surface (which was supposed to be immaculate) of the guide. After this protuberance was polished away, the remaining 12 cases were all successful.

**Table 1.** *The distribution of results for group 1 and group 2*

| Groups | | Results | | Total |
|---|---|---|---|---|
| | | Unsuccessful | Successful | |
| CCC group | n | 6 | 9 | 15 |
| | % Line | 40.0% | 60.0% | 100.0% |
| | % Total | 20.0% | 30.0% | 50.0% |
| Cerclage wire group | n | 13 | 2 | 15 |
| | % Line | 86.7% | 13.3% | 100.0% |
| | % Total | 43.3% | 6.7% | 50.0% |
| Total | n | 19 | 11 | 30 |
| | % Line | 63.% | 36.7% | 100.0% |
| | % Total | 63.3% | 36.7% | 100.0% |

**Table 2.** *The distribution of results for group 1 and group 3*

| Groups | | Results | | Total |
|---|---|---|---|---|
| | | Unsuccessful | Successful | |
| CCC group | n | 6 | 9 | 15 |
| | % Line | 40.0% | 60.0% | 100.0% |
| | % Total | 22.2% | 33.3% | 55.6% |
| PLA Tool | n | 0 | 12 | 12 |
| | % Line | 0.0% | 100.0% | 100.0% |
| | % Total | 0.0% | 44.4% | 44.4% |
| Total | n | 6 | 21 | 27 |
| | % Line | 22.2% | 77.8% | 100.0% |
| | % Total | 30.0% | 70.0% | 100.0% |

The correlation between the distribution of results for group 1 and group 2 was found to be statistically significant ($c^2$=7.033, P=0.008) *(Table 1)*. The correlation between the distribution of results for group 1 and group 3 was also statistically significant ($c^2$=6.171, P=0.002), proving that the higher success rate in group 3 was statistically significant *(Table 2)*.

## DISCUSSION

A new practical and reproducible method for CCC is definitely needed in order to facilitate and make it safer for the beginning surgeon as well as for the experienced surgeon in an era when premium multifocal IOLs are being widely used. Creating a capsulorhexis of 5.5-6.0 mm size reproducibly is in fact difficult also for the experienced surgeon. To solve this problem, various techniques and instruments have been developed most of which are either high cost (as in FLACS, precision pulse capsulotomy-PPC) or lack clinical practicability [12-15].

Various animal eyes are used in educational and experimental studies throughout the world. In those studies, pig eyes are used more often, while cow, goat, sheep and rabbit eyes are other alternatives [3,5,22-24]. In our study, sheep eye was chosen because it is similar to human eye with respect to anterior chamber depth, axial length, equatorial diameter, and is easily obtained in our area. Corneal diameter of sheep eye is more than twice that of human eye. That's why the improvised instruments to be tested (cerclage wire and PLA) are of larger size accordingly. An instrument to be used in a human eye would be of smaller size, although this very instrument could be used with minimal changes in the semi-circle diameter to suit the human lens size. The design of the instrument is more important than the scaling, because the square shape and smoothness of the bottom surface of the PLA device uniformly supports the underlying capsule surface providing a better guiding contour for the advancing rhexis front.

In one study, the size of corneal incision was too small to insert the tested instrument -which had a circular shape- into the anterior chamber, thus the researchers decided to enlarge the corneal incision to allow for a successful capsulorhexis [8]. However, for small-incision cataract surgery the developed hand tool should be of proper size. In our study, a semi-circular shape was proper to entry into the anterior chamber through a smaller corneal incision.

In conventional capsulorhexis, the capsular edge may tear away toward periphery due to centripetal zonular forces, which adversely affects the success of capsulorhexis. To eliminate these forces in such instances, several prevention and correction methods are recommended [25-28]. Because of the round structure of cerclage wire used in Group 2, the instrument did not have enough contact area with the lens surface and the capsule was torn towards the periphery mostly in the direction of 8 to 10 o'clock, most probably due to insufficient pressure against the capsular surface. In the PLA instrument group, a semi-circle diameter of 205° and the contact surface that is flatter and larger eliminated the effects of capsular forces and thus, allowed for more successful capsulorhexis.

This study shows that, with the aid of the cost-efficient PLA hand tool and the bimanual technique described, a reproducible CCC of consistent size and shape could be achieved even by a beginning surgeon more easily compared to the classical CCC technique. The development studies will be continued to adjust the instrument sizes according to anterior chamber width, lens capsule thickness and pupil size in various species and determine its effectiveness upon live tissue.

### Financial Disclosure and Funding

All authors will be named as the inventors, in a patent application with the Near East University covering the details in this report. This work was supported by the Near East University [grant numbers: CE028-2016].

### Acknowledgements

The authors would like to thank İlker Etikan, PhD for his statistical analyses of this study.

## REFERENCES

**1. Dooley IJ, O'Brien PD:** Subjective difficulty of each stage of phacoemulsification cataract surgery performed by basic surgical trainees. *J Cataract Refract Surg,* 32, 604-608, 2006. DOI: 10.1016/j.jcrs.2006.01.045

**2. Gimbel HV, Neuhann T:** Development, advantages, and methods of the continuous circular capsulorhexis technique. *J Cataract Refract Surg*, 16, 31-37, 1990. DOI: 10.1016/S0886-3350(13)80870-X

**3. Izak AM, Werner L, Pandey SK, Apple DJ, Izak MGJ:** Analysis of the capsule edge after Fugo plasma blade capsulotomy, continuous curvilinear capsulorhexis, and can-opener capsulotomy. *J Cataract Refract Surg,* 30 (12): 2606-2611, 2004. DOI: 10.1016/j.jcrs.2004.05.020

**4. Kayıkcıoglu O, Egrilmez S, Emre S, Erakgun T:** Human cataractous lens nucleus implanted in a sheep eye lens as a model for phacoemulsification training. *J Cataract Refract Surg,* 30, 555-557, 2004. DOI: 10.1016/j.jcrs.2003.08.018

**5. Mohammadi SF, Mazouri A, Jabbarvand M, Rahman AN, Mohammadi A:** Sheep practice eye for ophthalmic surgery training in skills laboratory. *J Cataract Refract Surg,* 37, 987-991, 2011. DOI: 10.1016/j.jcrs.2011.03.030

**6. Bozkurt Oflaz A, Ekinci Köktekir B, Okudan S.** Does cataract surgery simulation correlate with real-life experience? *Turk J Ophthalmol*, 48, 122-126, 2018.

**7. El Chehab H, Agard E, Dot C:** Evaluation of surgical simulation sessions of the French society of ophthalmology. A new surgical instruction method. *J Fr Ophtalmol*, 40 (8): 636-641, 2017.

**8. Powers MA, Kahook MY:** New device for creating a continuous curvilinear capsulorhexis. *J Cataract Refract Surg*, 40, 822-830, 2014. DOI: 10.1016/j.jcrs.2013.10.041

**9. Soylak M:** Novel device for creating continuous curvilinear capsulorhexis. *SpringerPlus,* 5:2053, 2016. DOI: 10.1186/s40064-016-3736-6

**10. Tassignon MJ, Rozema JJ, Gobin L:** Ring-shaped caliper for better anterior capsulorhexis sizing and centration. *J Cataract Refract Surg*, 32 (8): 1253-1255, 2006. DOI: 10.1016/j.jcrs.2006.02.067

**11. Wallace RB:** Capsulotomy diameter mark. *J Cataract Refract Surg*, 29 (10): 1866-1868, 2003. DOI: 10.1016/S0886-3350(03)00354-7

**12. Fugo RJ, Del Campo DM:** The Fugo blade: the next step after capsulorhexis. *Ann Ophthalmol*, 33 (1): 12-20, 2001. DOI: 10.1007/s12009-001-0066-1

**13. Kranitz K, Takacs A, Mihaltz K, Kovacs I, Knorz MC, Nagy ZZ:** Femtosecond laser capsulotomy and manual continuous curvilinear capsulorrhexis parameters and their effects on intraocular lens centration.

*J Refract Surg,* 27, 558-563, 2011. DOI: 10.3928/1081597X-20110623-03

**14. Palanker D, Nomoto H, Huie P, Vankov A, Chang DF:** Anterior capsulotomy with a pulsed-electron avalanche knife. *J Cataract Refract Surg*, 36, 127-132, 2010. DOI: 10.1016/j.jcrs.2009.07.046

**15. Hooshmand J, Abell RG, Allen P, Vote BJ:** Thermal capsulotomy: Initial clinical experience, intraoperative performance, safety, and early postoperative outcomes of precision pulse capsulotomy technology. *J Cataract Refract Surg*, 44 (3): 355-361, 2018. DOI: 10.1016/j.jcrs.2017.12.027

**16. Fedorovich NE, Alblas J, Hennink WE, Öner FC, Dhert WJ:** Organ printing: The future of bone regeneration? *Trends Biotechnol,* 29 (12): 601-606, 2011. DOI: 10.1016/j.tibtech.2011.07.001

**17. Kondor S, Grant CG, Liacouras P, Schmid MJR, Parsons LM, Rastogi VK, Macedonia C:** On demand additive manufacturing of a basic surgical kit. *J Med Devices,* 7 (3): 030916, 2013. DOI: 10.1115/1.4024490

**18. Rankin TM, Giovinco NA, Cucher DJ, Watts G, Hurwitz B, Armstrong DG:** Three-dimensional printing surgical instruments: Are we there yet? *J Surg Res,* 189 (2): 193-197, 2014. DOI: 10.1016/j.jss.2014.02.020

**19. Serra T, Mateos-Timoneda MA, Planell JA, Navarro M:** 3D printed PLA-based scaffolds: A versatile tool in regenerative medicine. *Organogenesis*, 9 (4): 239-244, 2013. DOI: 10.4161/org.26048

**20. Lunt J:** Large-scale production, properties and commercial applications of polylactic acid polymers. *Polym Degrad Stab*, 59 (1-3): 145-152, 1998. DOI: 10.1016/S0141-3910(97)00148-1

**21. Nuutinen JP, Clerc C, Virta T, Törmälä P:** Effect of gamma, ethylene oxide, electron beam, and plasma sterilization on the behaviour of SRPLLA fibres *in vitro. J Biomater Sci Polym Ed*, 13 (12): 1325-1336, 2002. DOI: 10.1163/15685620260449723

**22. Nagy Z, Takacs A, Filkorn T, Sarayba M:** Initial clinical evaluation of an intraocular femtosecond laser in cataract surgery. *J Refract Surg*, 25 (12): 1052-1060, 2009. DOI: 10.3928/1081597X-20091117-04

**23. Sengupta S, Dhanapal P, Nath M, Haripriya A, Venkatesh R:** Goat's eye integrated with a human cataractous lens: A training model for phacoemulsification. *Indian J Ophthalmol*, 63 (3): 275-277, 2015. DOI: 10.4103/0301-4738.156937

**24. Tahi H, Fantes F, Hamaoui M, Parel JM:** Small peripheral anterior continuous curvilinear capsulohexis. *J Cataract Refract Surg*, 25 (6): 744-747, 1999. DOI: 10.1016/S0886-3350(99)00041-3

**25. Mohammadpour M:** Rescue of an extending capsulorrhexis by creating a midway tangential anterior capsular flap: A novel technique in 22 eyes. *Can J Ophthalmol*, 45 (3): 256-258, 2010. DOI: 10.3129/i09-260

**26. Mohammadpour M:** Management of radial tears during capsulorhexis. *Tech Ophthalmol*, 4 (2): 56-60, 2006.

**27. Mohammadpour M, Erfanian R, Karimi N:** Capsulorhexis: Pearls and pitfalls. *Saudi J Ophthalmol,* 26 (1): 33-40, 2012. DOI: 10.1016/j.sjopt.2011.10.007

**28. Little BC, Smith JH, Packer M:** Little capsulorhexis tear-out rescue. *J Cataract Refract Surg*, 32 (9): 1420-1422, 2006. DOI: 10.1016/j.jcrs.2006.03.038

Kafkas Univ Vet Fak Derg
24 (5): 775-779, 2018
DOI: 10.9775/kvfd.2018.19754

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Short Communication

# Prevalence, Antimicrobial Resistance and Molecular Characterization of *Salmonella* spp. and *Listeria monocytogenes* Isolated from Chicken Carcass

Beren BASARAN KAHRAMAN [1] Ghassan ISSA [2] Tolga KAHRAMAN [3]

[1] Department of Microbiology, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcilar, Istanbul - TURKEY
[2] Avrupa Vocational School, TR-34010 Zeytinburnu, Istanbul - TURKEY
[3] Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcilar, Istanbul - TURKEY

**Article Code: KVFD-2018-19754 Received: 13.03.2018 Accepted: 07.06.2018 Published Online: 07.06.2018**

**How to Cite This Article**

**Basaran Kahraman B, Issa G, Kahraman T:** Prevalence, antimicrobial resistance and molecular characterization of *Salmonella* spp. and *Listeria monocytogenes* isolated from chicken carcass . *Kafkas Univ Vet Fak Derg,* 24 (5): 775-779, 2018. DOI: 10.9775/kvfd.2018.19754

## Abstract

This study aimed to investigate the prevalence of *Salmonella* spp. and *Listeria monocytogenes,* their antimicrobial resistance profile. *L. monocytogenes* was not isolated from any of the samples. *Salmonella* spp. was detected from 32 (8%) out of the 400 collected samples. Antimicrobial resistance was most frequently observed to nalidixic acid (100%), tetracycline (93.75%), erythromycin (90.6%), streptomycin (84.3%), followed by kanamycin (62.5%). Also, 37.5% of *Salmonella* isolates were phenotypically confirmed as ESBL producers. Multiple drug resistance was defined 93.75% of the isolates. Among the *Salmonella* isolates, all of them harbouring *qnrB* and *qnrS* genes and, 37.5% of them presented $bla_{TEM}$ gene..

***Keywords:*** *Antimicrobial resistance, Carcass, Chicken, Listeria monocytogenes, Salmonella*

# Tavuk Karkaslarından İzole Edilen *Salmonella* spp. ve *Listeria monocytogenes* Prevalansı, Antimikrobiyal Direnci ve Moleküler Karakterizasyonu

## Öz

Bu çalışmada *Listeria monocytogenes* ve *Salmonella* spp. prevalansı ve antimikrobiyal direnç profillerinin araştırılması amaçlandı. *L. monocytogenes* örneklerden izole edilmedi. *Salmonella* spp. toplanan 400 örneğin 32 (%8)'sinden saptandı. Antimikrobiyal direnç en sık nalidiksik asit (%100), tetrasiklin (%93.75), eritromisin (%90.6), streptomisin (%84.3), ardından kanamisin (%62.5)'de belirlendi. Ayrıca, *Salmonella* izolatlarının %37.5'i ESBL pozitif olarak fenotipik yöntemlerle doğrulandı. Çoklu ilaç direnci, izolatların %93.75'inde tanımlandı. Salmonella izolatlarının hepsinin *qnrB* ve *qnrS* geni, %37.5'inin ise $bla_{TEM}$ geni taşıdığı belirlendi.

***Anahtar sözcükler:*** *Antimikrobiyal direnç, Karkas, Listeria monocytogenes, Salmonella, Tavuk*

## INTRODUCTION

The poultry meat is one of the most favourite meat products being consumed worldwide. *Salmonella species and Listeria monocytogenes* are major foodborne pathogenic bacteria, and contaminants of raw poultry meat [1].

*Salmonella* species are Gram-negative, non-spore forming, non-lactose fermenting rod-shaped bacteria, and a member of the family Enterobacteriaceae. The genus Salmonella which is classified into two species, *Salmonella enterica* (type species) and *Salmonella bongori* based on differences in their 16S rRNA sequence analysis, includes more than 2600 different serotypes and most of these serotypes have the ability to adapt within a variety of animal hosts, including humans [1,2]. *L. monocytogenes* is gram-positive, rod-shaped, beta-hemolytic, motile, facultative intracellular bacteria, capable of surviving

**İletişim (Correspondence)**
+90 212 4737070/17360
beren@istanbul.edu.tr

under refrigeration conditions, low pH and in high salt concentration [3].

The European Food Safety Authority (EFSA) reported 94.530 laboratory confirmed Salmonellosis cases in humans and *S. enteritidis and S. typhimurium* accounted for almost 80% of human cases acquired [1]. *S. enteritidis* and *S. typhimurium* are the most commonly reported serovars in the European Union (EU), being associated with 52.3% and 23.3% of all confirmed human salmonellosis, respectively [2]. With it, Salmonella was most frequently isolated in poultry, in 2016 [1]. The incidence of listeriosis is low worldwide, however, *L. monocytogenes* can cause severe and lethal infections, ranging from 20% to 30% (septicaemia, encephalitis and meningitis) during vulnerable periods of life (older adults, pregnant women and immunocompromised patients). Chicken products can be contaminated with *L. monocytogenes* during processing, or cross-contamination during preparation, cooking, and serving food for other foods [3].

Over recent decades, antibiotic resistance undoubtedly represents a global public health problem. Several reports and books have been published about antibiotic resistance problem and the reasons behind the increasing rates. It was highlighted that poultry meat may play a major role in transmission [4-6]. The inappropriate, uncontrolled and excessive use of antibiotics in the treatment of infections in humans and veterinary medicine may be the reason for high rates of resistance, in poultry [7].

The European Commission emphasize the requirement of co-ordinated research effort about antimicrobial resistance. With this aim in mind, the current study aimed to investigate the prevalence, antimicrobial resistance and molecular characterization of *Salmonella* spp. and *L. monocytogenes* in chicken carcass.

# MATERIAL and METHODS

## Sample Collection

A total of 400 chicken carcasses were collected from various retail markets in different districts of Istanbul (Ataşehir, Avcılar, Bakırköy, Başakşehir, Beşiktaş, Beylikdüzü, Beyoğlu, Eminönü, Fatih, Gaziosmanpaşa, Kadıköy, Kartal, Maltepe, Pendik, Şişli, Üsküdar) between July 2014 and December 2016. Fresh packaged chicken carcasses were transported to the laboratory under cold chain and analysed within 2 h.

## Isolation and Species Identification

The detection of pathogens was performed following official methods: *Salmonella* spp. (ISO 6579:2002) [8] and *L. monocytogenes* (ISO 11290-2:2005) [9]. Suspected colonies were identified by API-20E for *Salmonella* spp. and by API-Listeria for *L. monocytogenes*. For confirmation and identification of the genus and species of the Salmonella isolates, multiplex PCR (mPCR) was performed [10]. Primers, band weight and references used are showed in *Table 1*.

## Antimicrobial Susceptibility Testing

Isolates were tested for antibiotic susceptibilities by the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) standards against 12 different antimicrobials in 7 antimicrobial classes, including those used to treat human listeriosis and salmonellosis: ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), erythromycin (15 µg), imipenem (10 µg), kanamycin (30 µg), meropenem (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), sulfamethoxazole/trimethoprim (1.25/23.75 µg) and tetracycline (30 µg).

Extended-spectrum beta-lactamases (ESBL) production was analysed by the double disk diffusion test containing cefotaxime and ceftazidime with and without clavulanic acid. An increase in the zone diameter of 5 mm or more when either of the antimicrobial agents was combined with clavulanic acid was considered evidence of ESBL production. The results were based on CLSI breakpoints [11].

Multiple drug resistance was defined as simultaneous resistance to clinically relevant drugs of at least three different classes. Moreover, the multiple antibiotic resistance (MAR) index was calculated using the formula: a/b, where 'a' represents the number of antibiotics to which a particular isolate was resistant and 'b' the total number of antibiotics tested, for all Salmonella isolates. As quality controls, *Escherichia coli* ATCC 25922 were tested in each run.

## Detection of Antimicrobial Resistance Genes

All of the isolates were analysed for the presence of plasmid-mediated quinolone resistance (PMQR) genes and some of the genes encoding β-lactam resistance. The PMQR genes (*qnrA, qnrB, qnrS, qnrC, qnrD, qepA*, and *aac(6')-Ib-cr*) were

***Table 1.*** *Primers and band weight used in the mPCR*

| Bacteria | Gene | Primer Sequence (5' to 3', as synthesized) | Size (bp) |
|---|---|---|---|
| *Salmonella* spp. | *invA* | AAA CGT TGA AAA ACT GAG GA<br>TCG TCA TTC CAT TAC CTA CC | 199 |
| *S.* Enteritidis | *sdf* | AAA TGT GTT TTA TCT GAT GCA AGA GG<br>GTT CGT TCT TCT GGT ACT TAC GAT GAC | 299 |
| *S.* Typhimurium | STM4492 | ACA GCT TGG CCT ACG CGA G<br>AGC AAC CGT TCG GCC TGA C | 759 |

detected with a polymerase chain reaction (PCR) assay using previously described primers and protocol [12]. Also, all phenotypically ESBL isolates were screened for genes encoding β-lactam resistance (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{OXA}$, and *bla*$_{CMY}$) using previously described methods [13,14].

## RESULTS

*Listeria monocytogenes* was not isolated from any of the samples analysed. *Salmonella* spp. were recovered from 32 (8%) out of the 400 collected carcass samples by conventional culture technique. All of the Salmonella serovars were confirmed, and also, the predominant serovar was identified as *S. enteritidis* (71.8%, 23/32) followed by *S. typhimurium* (28.1%, 9/32) with mPCR assays.

The antimicrobial resistance was most frequently observed to nalidixic acid, tetracycline, and erythromycin, streptomycin, followed by kanamycin. No resistance to carbapenems, phenicol and sulphonamide was recorded *(Table 2)*. Moreover, 12 out of the 32 (37.5%) isolates were phenotypically confirmed ESBL producers and multiple drug resistance was defined 30 out of the 32 (93.75%) isolates. Antibiotic resistant profiles and multiple resistance index (MAR) of Salmonella isolates from chicken carcasses are presented in *Table 3*.

***Table 2.*** *Antimicrobial susceptibility testing results of Salmonella isolates*

| Classes | | Antimicrobial Agents | Number of Resistant Isolates (n=32) | Percentage of Resistance (%) |
|---|---|---|---|---|
| Beta-Lactams | Penicillins | *Ampicillin* | 4 | 12.5 |
| | Cephalosporins | *Cefotaxime* | 12 | 37.5 |
| | Carbapenems | *Meropenem* | - | - |
| | | *Imipenem* | - | - |
| Aminoglycosides | | *Streptomycin* | 27 | 84.3 |
| | | *Kanamycin* | 20 | 62.5 |
| Quinolones | | *Nalidixic Acid* | 32 | 100 |
| | | *Ciprofloxacin* | 4 | 12.5 |
| Tetracyclines | | *Tetracycline* | 30 | 93.75 |
| Phenicols | | *Chloramphenicol* | - | - |
| Macrolides | | *Erythromycin* | 29 | 90.6 |
| Sulfonamides | | *Sulfamethoxazole/Trimethoprim* | - | - |

***Table 3.*** *Antibiotic resistant profiles and multiple resistance index (MAR) of Salmonella isolates from chicken carcasses*

| Isolates No | Serovar | Resistance Phenotype Profile | Phenotypic ESBL Results | Genotypic PCR Results | MAR Index |
|---|---|---|---|---|---|
| 1, 10 | *S.* Enteritidis | E, NA, TE | - | qnrB, qnrS | 0.250 |
| 2, 23 | *S.* Enteritidis | E, NA | - | qnrB, qnrS | 0.166 |
| 3, 4, 5, 13, 14, 15 | *S.* Enteritidis | CFX, E, K, NA, S, TE | + | *bla*$_{TEM}$, *qnrB, qnrS* | 0.500 |
| 6 | *S.* Enteritidis | K, NA, S, TE | - | qnrB, qnrS | 0.333 |
| 7, 17 | *S.* Enteritidis | AMP, CFX, E, K, NA, S, TE | + | *bla*$_{TEM}$, *qnrB, qnrS* | 0.583 |
| 8, 16, 18 | *S.* Enteritidis | E, K, NA, S, TE | - | qnrB, qnrS | 0.416 |
| 9, 11, 12, 19, 20, 22 | *S.* Enteritidis | E, NA, S, TE | - | qnrB, qnrS | 0.333 |
| 21 | *S.* Enteritidis | CIP, NA, TE | - | qnrB, qnrS | 0.250 |
| 24 | *S.* Typhimurium | CFX, K, NA, S, TE | + | *bla*$_{TEM}$, *qnrB, qnrS* | 0.416 |
| 25 | *S.* Typhimurium | K, NA, S, TE | - | qnrB, qnrS | 0.333 |
| 26 | *S.* Typhimurium | AMP, CFX, CIP, K, NA, S, TE | + | *bla*$_{TEM}$, *qnrB, qnrS* | 0.583 |
| 27, 29, 32 | *S.* Typhimurium | E, K, NA, S, TE | - | qnrB, qnrS | 0.416 |
| 28 | *S.* Typhimurium | CFX, CIP, K, NA, S, TE | + | *bla*$_{TEM}$, *qnrB, qnrS* | 0.500 |
| 30 | *S.* Typhimurium | AMP, CFX, E, K, NA, S, TE | + | *bla*$_{TEM}$, *qnrB, qnrS* | 0.583 |
| 31 | *S.* Typhimurium | CIP, NA, S, TE | - | qnrB, qnrS | 0.333 |
| Average: 0.400 | | | | | |

*AMP, Ampicillin; CFX, Cefotaxime; CIP, Ciprofloxacin; E, Erythromycin; K, Kanamycin; NA, Nalidixic Acid; S, Streptomycin; TE, Tetracycline*

All of the *Salmonella* isolates were positive for the presence of *qnrS* and *qnrB* genes. None of the tested isolates carried *qnrA, qnrC, qnrD*, *qepA,* or *aac(6')-Ib-cr.* Also, 12 out of the 32 (37.5%) isolates carried only $bla_{TEM}$ genes. None of the isolates presented $bla_{CTX-M}$, $bla_{SHV}$, $bla_{OXA}$, and $bla_{CMY}$ genes.

## DISCUSSION

European Food Safety Authority [1] accounted 2.536 laboratory confirmed Listeriosis cases in humans and also *L. monocytogenes* prevalence reported as 0.8% from broiler meat samples. Several reports have highlighted that poultry meat may play a major role in transmission [1,3]. In the current study, *L. monocytogenes* was not isolated from any of the samples. This promising result might be the consequences of applied biosecurity measures.

Worldwide, various prevalence rates of *Salmonella* spp. ranged from 0% to 100% was reported in poultry [2,4,5]. The authors emphasize that differences of Salmonella prevalence in chicken meat could be based on the geographical differences, sampling techniques, detection methods, slaughterhouse hygiene and cross-contamination of products [5,6]. Regarding previous studies in Turkey, *Salmonella* spp. was reported in 34% of packaged fresh raw chicken samples using cultural technique and PCR [4]. When comparing the reported prevalence among our country during recent years, the results of the present study are considerably lower. In addition, the authors reported that *S. typhimurium* was predominant ones recovered from chicken samples [4]. Contrary, in the presented study, *S. enteritidis* was predominant, followed by *S. typhimurium*.

The emergence of antimicrobial resistance is not a new phenomenon, nor an unexpected one. Nowadays, incidence of antimicrobial resistance in *Salmonella* spp. isolated from especially poultry products, has increased. In the current study, 93.75% of the Salmonella isolates was classified as multidrug resistant. Our results were relatively high according to some studies [15,16] while showed similarities to others [4,6].

In the current study, all of the *Salmonella* isolates were resistant to nalidixic acid, and most of them to tetracycline, erythromycin, streptomycin and kanamycin. These results substantiate other authors' findings [1,2,6]. The broad use of these classes of antibiotics in poultry may be the reason for this crucial problem.

Plasmid-Mediated Quinolone Resistance determinants are widely distributed among *Enterobacteriaceae*, including *Salmonella*, worldwide. Regarding previous studies, the most commonly identified resistance determinants are *qnrA, qnrB, qnrS,* and *aac(6')-Ib-cr* genes, in Turkey [17]. In the present study, PMQR mechanisms in all of the Salmonella isolates were identified as *qnrS* and *qnrB*. None of the tested isolates carried *qnrA, qnrC, qnrD*, or *qepA, aac(6')-Ib-cr.*

Plasmid-Mediated Quinolone Resistance determinants are often combined with extended-spectrum beta-lactamases (ESBLs) which are less prevalent in Salmonella. Prevalence of ESBL-producing *Salmonella* species isolated from humans and animals has been reported in many parts of the world [5,18]. On the contrary, foodborne ESBL-producing Salmonella has been rarely published. In the current study, 12 of the 32 (37.5%) isolates showed resistance against extended spectrum cephalosporin's phenotypically and carried only $bla_{TEM}$ genes, which have been stated to be one of the most widely distributed β-lactamase. These results might be consequences of the study limits, only some of the resistance determinants studied, so any another resistant gene could not be determined.

Globally, the author's highlight that poultry meat is a potential hazard for public health and the essential precautions should be taken to ensure improving the quality. In the current study provides baseline information on the highlights the widespread presence of the emerging foodborne pathogens and resistance profiles in poultry meat. Further multidisciplinary studies and novel strategies in the spirit of 'One Health' are needed.

## REFERENCES

**1. EFSA (European Food Safety Authority):** The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA J*, 15 (12): 5077, 2017. DOI: 10.2903/j.efsa.2017.5077

**2. Mąka L, Maćkiw E, Ścieżyńska H, Pawłowska K, Popowska M:** Antimicrobial susceptibility of *Salmonella* strains isolated from retail meat products in Poland between 2008 and 2012. *Food Control*, 36 (1): 199-204, 2014. DOI: 10.1016/j.foodcont.2013.08.025

**3. Oliveira TS, Varjão LM, da Silva LNN, Pereira RDCL, Hofer E, Vallim DC, de Castro Almeida RC:** *Listeria monocytogenes* at chicken slaughterhouse: Occurrence, genetic relationship among isolates and evaluation of antimicrobial susceptibility. *Food Control*, 88, 131-138, 2018. DOI: 10.1016/j.foodcont.2018.01.015

**4. Yildirim Y, Gonulalan Z, Pamuk S, Ertas N:** Incidence and antibiotic resistance of *Salmonella* spp. on raw chicken carcasses. *Food Res Int*, 44 (3): 725-728, 2011. DOI: 10.1016/j.foodres.2010.12.040

**5. Bae DH, Dessie HK, Baek HJ, Kim SG, Lee HS, Lee YJ:** Prevalence and characteristics of *Salmonella* spp. isolated from poultry slaughterhouses in Korea. *J Vet Med Sci*, *75* (9): 1193-1200, 2013. DOI: 10.1292/jvms.13-0093

**6. Abd-Elghany SM, Sallam KI, Abd-Elkhalek A, Tamura T:** Occurrence, genetic characterization and antimicrobial resistance of *Salmonella* isolated from chicken meat and giblets. *Epidemiol Infect*, 143 (5): 997-1003, 2015. DOI: 10.1017/S0950268814001708

**7. Bilge N, Vatansever L, Sezer Ç:** Antibiotic resistance of *Salmonella* spp. isolated from raw chicken wings. *Kafkas Univ Vet Fak Derg*, 24 (3): 431-435, 2018. DOI: 10.9775/kvfd.2017.19134

**8. ISO, The International Organization of Standardizations:** Microbiology of food animal feeding stuffs-horizontal method for the detection *Salmonella* spp. International Standard, ISO 6579:2002.

**9. ISO, The International Organization of Standardizations:** Microbiology of food animal feeding stuffs-horizontal method for the detection *Listeria monocytogenes* International Standard, ISO 11290-2:2005.

**10. Saeki EK, Alves J, Bonfante RC, Hirooka EY, Oliveira TCRM:** Multiplex PCR (mPCR) for the detection of *Salmonella* spp. and the differentiation of the Typhimurium and Enteritidis serovars in chicken meat. *J Food Safety*, 33 (1): 25-29, 2013. DOI: 10.1111/jfs.12019

**11. CLSI:** Clinical and Laboratory Standards Institute Performance standards for antimicrobial susceptibility testing: Twenty-fourth informational supplement. CLSI document M100-S24. Wayne, PA: CLSI, 2014.

**12. Ciesielczuk H, Hornsey M, Choi V, Woodford N, Wareham DW:** Development and evaluation of a multiplex PCR for eight plasmid-mediated quinolone-resistance determinants. *J Med Microbiol*, 62, 1823-1827, 2013. DOI: 10.1099/jmm.0.064428-0

**13. Saladin M, Cao VTB, Lambert T, Donay JL, Herrmann JL, Ould-Hocine Z, Verdet C, Delisle F, Philippon A, Arlet G:** Diversity of CTX-M β-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. *FEMS Microbiol Lett*, 209, 161-168, 2002. DOI: 10.1111/j.1574-6968.2002.tb11126.x

**14. Dallenne C, Da Costa A, Decré D, Favier C, Arlet G:** Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. *J Antimicrob Chemother*, 65 (3): 490-495, 2010. DOI: 10.1093/jac/dkp498

**15. Arslan S, Eyi A:** Occurrence and antimicrobial resistance profiles of *Salmonella* species in retail meat products. *J Food Prot*, 73 (9): 1613-1617, 2010. DOI: 10.4315/0362-028X-73.9.1613

**16. Ziech RE, Lampugnani C, Perin AP, Sereno MJ, Sfaciotte RAP, Viana C, Soares VM, Pinto JPAN, Bersot LDS:** Multidrug resistance and ESBL-producing *Salmonella* spp. isolated from broiler processing plants. *Braz J Microbiol*, 47 (1): 191-195, 2016. DOI: 10.1016/j.bjm.2015.11.021

**17. Nazik H, İlktaç M, Öngen B:** Prevalence of qnrA, qnrB, qnrS and aac(6')-Ib-cr (in qnr-positive isolates) among the ESBL-positive and/or ciprofloxacin-resistant isolates in Turkey. *J Chemother*, 21 (2): 219-221, 2009. DOI: 10.1179/joc.2009.21.2.219

**18. Wu H, Xia X, Cui Y, Hu Y, Xi M, Wang X, Shı X, Wang D, Meng J, Yang B:** Prevalence of extended-spectrum β-lactamase–producing *Salmonella* on retail chicken in six provinces and two national cities in the People's Republic of China. *J Food Prot*, 76 (12): 2040-2044, 2013. DOI: 10.4315/0362-028X.JFP-13-224

Kafkas Univ Vet Fak Derg
24 (5): 781-782, 2018
DOI: 10.9775/kvfd.2018.20421

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Letter to the Editor

# Eisenmenger's Syndrome in a Cat with Ventricular Septal Defect (Ventriküler Septal Defektli Bir Kedide Eisenmenger Sendromu)

Zeki YILMAZ [1,a] Meriç KOCATÜRK [1,b] Pınar LEVENT [1]
Ahmet SARIL [1] Hakan SALCI [2,c] Saim SAĞ [3]

[1] Department of Internal Medicine, Faculty of Veterinary Medicine, Uludag University, TR-16059 Bursa - TURKEY
[2] Department of Surgery, Faculty of Veterinary Medicine, Uludag University, TR-16059 Bursa - TURKEY
[3] Department of Cardiology, Faculty of Medicine, Uludag University, TR-16059 Bursa - TURKEY

[a] ORCID: 0000-0001-9836-0749; [b] ORCID: 0000-0002-2849-1222; [c] ORCID: 0000-0001-6548-8754

**Article Code: KVFD-2018-20421 Published Online: 13.07.2018**

**How to Cite This Article**

**Yılmaz Z, Kocatürk M, Levent P, Sarıl A, Salcı H, Sağ S:** Eisenmenger's syndrome in a cat with ventricular septal defect. *Kafkas Univ Vet Fak Derg,* 24 (5): 781-782, 2018. DOI: 10.9775/kvfd.2018.20421

## Dear Editor,

Ventricular septal defect (VSD), a congenital cardiac malformation, is characterized by an abnormal communication between two ventricular chambers. Congenital heart defects (VSD, ASD and PDA) cause left-to-right (LR) cardiac shunt, and then right-to-left (RL) shunt when right ventricular pressure exceeds the left ventricular (LV) pressure, resulting in pulmonary artery hypertension (PH). That cardiac shunt switches to a cyanotic RL shunt from LR shunt due to PH, which is defined as Eisenmenger's syndrome, has a high risk factor for mortality [1]. Endothelin (ET) plays a key role of pulmonary vasoconstriction in the pathophysiology of Eisenmenger's syndrome. There is limited information on the diagnostic steps of Eisenmenger's syndrome in cats [1,2], and also no available data on the use of ET receptor antagonists in the treatment of this syndrome in dogs and cats. Thus, this case report aims to share practical knowledge on size detection of the VSD by two non-invasive techniques; echocardiography and three-dimensional computed tomographic angiography (3D-CTA) and treatment possibilities in non-operable feline patients with Eisenmenger's syndrome.

A British shorthair cat (1.5 year, female, and 2.5 kg) was referred from a small animal clinic to Animal Hospital with a history of lethargy, exercise intolerance, and respiratory distress during exercise for 3 months. Before admission, the cat was treated with furosemide for 5 days due to pulmonary oedema. In physical examination, body temperature and heart and respiratory rates were within reference ranges. Cyanotic mucous membranes were observed. Systolic cardiac murmur - grade 4/6 was auscultated over left and right 2-4 intercostal spaces. Thoracic radiography revealed enlarged heart size silhouette and pulmonary artery bulging with an alveolar pattern lung tissue appearance. ECG examination showed rS complexes and right axis deviation suggestive for right-sided cardiac enlargement. Complete blood cell count was non-specific (Hct: 43.4%, reference: 24-45%). Serum biochemistry profile showed pre-renal azotemia (BUN: 36 mg/dL, reference: 10-30 mg/dL; Cr: 1.3 mg/dL, reference: 0.3-1.8 mg/dL) and hyponatremia (137 mmol/L, reference: 142-164 mmol/L) due to diuretic administration.

Standard echocardiographic images showed severe right (2.22 cm) and left atrial enlargement (1.79 cm, reference 8-13 cm) with an increased left atrial-to-aortic diameter ratio (2.4, reference <1.5) on the right parasternal long and short axis views. M-mode measurements of the LV at diastole and systole showed that all geometric and functional parameters were within reference ranges; LV dimensions - 1.41 cm (1.2-1.8 cm) and - 0.50 cm (0.5-1.0 cm), interventricular septal thickness - 0.41 cm (0.3-0.5 cm) and - 0.5 cm (0.5-0.9 cm), LV post wall thickness - 0.46 cm (0.3-0.5 cm) and - 1.0 cm (0.4-0.9 cm) and fractional shortening - 30% (30-55%), respectively. Pulmonary and aortic Doppler flow velocities were 1.9 m/s (≤1.2 m/s) and 0.88 m/s (≤1.2 m/s), respectively. A 2.6 mm VSD was identified with RL shunting on color Doppler, with a maximum velocity of 2.3 m/s (estimated peak pressure gradient of 21.6 mmHg). Normally, since the pulmonary blood flow (Qp) is equal to the systemic blood flow (Qs), their ratio (Qp:Qs) is equal to one. Qp:Qs shows the direction and magnitude of the shunting occurring LR (greater than one) or RL (less than one) [3]. In this case, echocardiographic estimation of Qp:Qs

**İletişim (Correspondence)**
+90 224 2940809
zyilmaz@uludag.edu.tr

(0.8) in the first day of diagnostic work-up was consistent with suspected shunt way. An agitated saline micro-bubble contrast study performed via the cephalic vein confirmed a RL shunt across the interventricular septum. Inlet VSD (atrioventricular septal defect) was confirmed by use of 3D-CTA *(Fig. 1)*, as reported in a previous case [4]. Based on these observations, the cat was diagnosed with VSD and suspected pulmonary vascular obstructive disease resulting in PH (Eisenmenger's syndrome). In this case, lack of polycythemia maybe due to relatively recent shunt reversal, in agreement with the acute decompensation before presentation, as reported earlier [2].

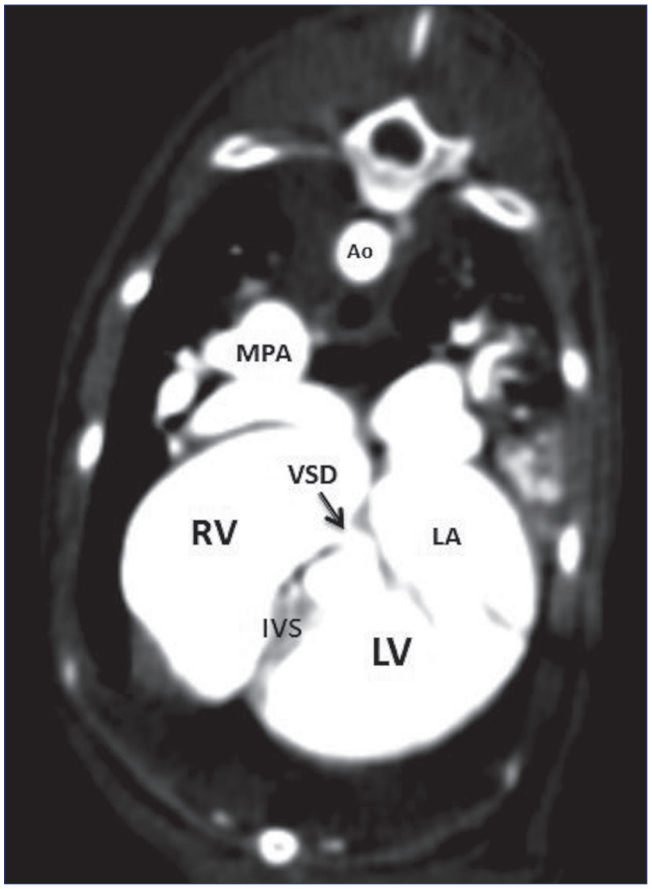



**Fig 1.** Three-dimensional computed tomographic angiography shows the presence of ventricular septal defect (VSD) just behind the atrioventricular valves between two ventricular chambers

Ao: aorta, RV: right ventricle, LA: left atrium, LV: left ventricle, IVS: interventricular septum, MPA: mean pulmonary artery

The cat was treated with an ACE-i drug (enelapril, 0.5 mg/kg 1x1, PO), diuretic (furosemide, 2 x 2 mg/kg, PO) and dietary salt restriction, to alleviate PH and reduce volume overload. Because exercise intolerance was stabile for two weeks despite to the treatment regimen, medical strategy was modified. Sildenafil (Revatio® 20 mg/tablet, Pfizer, 0.5 mg/kg, PO, q12 hr) was administered as an oral phosphodiesterase type V inhibitor acting preferentially to vasodilate arteries in the lung for two weeks, as suggested [1]. Since Eisenmenger's syndrome is associated with increased ET expression, patients may benefit from ET receptor antagonism. Thus, bosentan as an oral ET-1 receptor antagonist (Tracleer 125 mg/tablet, 3 mg/kg, PO, q12 h) was suggested with concomitant use of sildenafil citrate [5]. Although bosentan could not effective to produce dramatic changes in Doppler spectral pattern of flow through pulmonary artery and septal defect, it was well tolerated and improved the exercise capacity. Thus the cat was alert and not shown the clinical signs on the control examinations with two week intervals for 3 months.

In conclusion, high technological imaging systems such as 3D-CTA may help to confirm the diagnosis of congenital cardiac defects. VSD should be considered as a risk factor leading to PH (Eisenmenger's syndrome) in cats as reported in human medicine. Thus, in addition to traditional therapy (ACE-I and diuretic), sildenafil and/or bosentan may be used to alleviate clinical signs in these cases.

## REFERENCES

**1. Chow KS, Beijerink NJ, Ettinger S, Fliegner R, Beatty JA, Barrs VR:** Use of sildenafil citrate in a cat with Eisenmenger's syndrome and an atrial septal defect. *JFMS Open Rep,* 1 (1): 1-5, 2015. DOI: 10.1177/2055116915579680

**2. Buttgereit R, Betzinger S, Fonfara S:** Eisenmenger's syndrome in a domestic shorthair cat with bilateral abdominal cryptorchidism. *Kleintierpraxis*, 58 (5): 249-255, 2013. DOI: 10.2377/0023-2076-58-249

**3. Serres F, Chetboul V, Tissier R, Gouni V, Desmyter A, Sampedrano CC, Pouchelon JL:** Quantification of pulmonary to systemic flow ratio by a Doppler echocardiographic method in the normal dog: Repeatability, reproducibility, and reference ranges. *J Vet Cardiol,* 11 (1): 23-29, 2009. DOI: 10.1016/j.jvc.2009.04.001

**4. Kocatürk M, Saril A, Altinci C, Salci H, Yilmaz Z:** Diagnostic steps of a cat with patent ductus arteriosus. *Kafkas Univ Vet Fak Derg*, 24 (4): 631-632, 2018. DOI: 10.9775/kvfd.2018.20140

**5. Puza N, Papich MG, Reinero C, Chang CH, Yu DH, Sharp C, Declue A:** Pharmacokinetics and preliminary safety data of a single oral dose of bosentan, a dual endothelin receptor antagonist, in cats. *J Vet Pharmacol Ther*, 37 (2): 192-195, 2014. DOI: 10.1111/jvp.12083

## INSTRUCTION FOR AUTHORS

**1- Kafkas Universitesi Veteriner Fakultesi Dergisi** (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (ISSN: 1300-6045 and e-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given in the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal cowers all aspects of veterinary medicine and animal science.

In the interests of brevity and standalone readability, **Kafkas Universitesi Veteriner Fakultesi Dergisi** strongly discourages the submission of multi-part manuscripts. Authors who feel that their topic requires an exception should obtain approval from the editor before submission of a multi-part manuscript. If submitted, multipart papers can be assigned to different editorial board members and independent outside expert reviewers. It is necessary to load all parts of manuscript are required to be loaded into the online system at the same time.

**Kafkas Universitesi Veteriner Fakultesi Dergisi** is an *Open Access* journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

Manuscripts submitted for publication should be written in Turkish, English or German.

**2-** The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text.

The manuscript and its supplementary (figure etc.) should be submitted by using online manuscript submission system at the address of *http://submit.vetdergikafkas.org/*

During the submission, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Transfer Agreement Form signed by all the authors should be send to the editorial office.

**3-** Authors should indicate the name of institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, editorial board may also request the official document of the ethical commission report.

**4- Types of Manuscripts**

***Original (full-length) Manuscripts*** are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit not include tables and illustrations. Abstract should contain 200±20 words.

***Short Communication Manuscripts*** contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but each of the abstracts should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

***Preliminary Scientific Reports*** are short description of partially completed original research findings at interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

***Case Reports*** describe rare significant findings encountered in the application, clinic and laboratory of related fields. The title and summary of these articles should be written in the format of full-length original articles and the remaining sections should follow Introduction, Case History, Discussion and References. The length of the text should be no longer than 4 pages in total. The page limit not include tables and illustrations.

***Letters to the Editor*** are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 2 pages in total. The page limit includes tables and illustrations.

***Reviews*** are original manuscripts gather the literature on current and significant subject along with the commentary and findings of the author on the particular subject. The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should follow Introduction, text (with appropriate titles), conclusion, and references. The length of the text should be no longer than 15 pages in total.

**5-** The necessary descriptive information (thesis, projects, financial supports etc) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of title.

**6-** References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

*Example:* **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

*Example:* **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must be complied with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

**Follow the link below for EndNote style of Kafkas Universitesi Veteriner Fakultesi Dergisi;** *https://researchsoftware.com/downloads/journal-faculty-veterinary-medicine-kafkas-university*

**7-** The Latin expression such as species names of bacterium, virus, parasite and fungus and anatomical terms must be written in italic character keeping their original forms.

**8-** The editorial board has the right to perform necessary modifications and reduction on the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by editorial board, the article can only be published after the approval of the field editor and two referees specialized in the particular field.

**9-** All responsibilities from published articles merely belong to the authors. According to ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with broad database of academic publications.

**10-** There is no copyright fee for the authors.

**11-** A fee is charged from the authors to cover printing cost and other expenses. This payment information can be found at *http://vetdergikafkas.org/*

**12-** Reprints (in multiples of 50) of the article are sent to the authors for free.

## SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

**- Cover Letter (without author/authors name)**

• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction)

• Other information has been added that should be known by the editorial board (e.g.; list of other journal or conference papers (if any) published or submitted by you or any co-author)

All necessary files have been uploaded

**- Title page**

• Include title, running title (no more than 5 words)

• The author's name, institutional affiliation

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

**- Manuscript**

• Include title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

**- Supplemental files (where applicable)**

**Further considerations**

• Journal policies detailed in this guide have been reviewed

• Manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Acknowledgement and conflicts of interest statement provided