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# EDITORIAL

Dear Readers;

We are very pleased and honored to present the first issue of our journal for the year 2024. In this edition, we bring to you a total of 15 articles, comprising 13 research articles, and 2 case reports.

Dear readers; I would like to provide you with a brief overview of our journal's statistics for the year 2023. During that year, a total of 187 articles were submitted to our journal, with 36 being accepted. Throughout this process, our article rejection rate was determined to be approximately 80%. Of the 187 articles submitted to our journal, 166 were "Research Articles", 14 were "Case Reports", 5 were "Short Papers" and 2 were "Review Articles". Out of the 151 rejected articles, 13 were withdrawn by the authors, 25 were rejected following the "Referee Evaluation Process," and 113 did not pass the "Preliminary Evaluation Phase." In the Preliminary Evaluation Phase, the report prepared by the relevant field editors regarding the submitted articles is evaluated by the Editorial Board during their weekly meeting. It is at this juncture that decisions are made regarding whether to initiate the "Referee Evaluation Process" for the article. Articles rejected at this stage are returned to the authors along with the reasons for rejection. The Preliminary Evaluation Phase takes approximately 10 days.

Dear academicians, I wish to emphasize our eagerness to publish your articles of great value to you. We eagerly anticipate receiving your contributions to our journal. On this note, I hope that this issue of our journal will significantly contribute to the world of science, and I extend my sincerest regards to all of you.

Sincerely,

Dr. Levent ALTINTAŞ

Editor in Chief

Ankara Üniversitesi Veteriner Fakültesi Dergisi

# Influence of the Dietary Supplement of Protected Calcium Butyrate in Growing Japanese Quail Diets on Performance, Carcass Parameters, Blood Serum Biochemical Status, Meat Quality, and Jejunum Histomorphology

Erinç GÜMÜŞ[1,a,✉], Alev Gürol BAYRAKTAROĞLU[2,b], Kanber KARA[3,c], Neşe Hayat AKSOY[4,d], Yusuf CUFADAR[5,e]

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## Abstract

The effect of protected calcium butyrate (PCB) supplemented at different amounts on performance, carcass characteristics, blood biochemical values, jejunum histomorphology, and meat traits in Japanese quails were determined. One-day-old unsexed Japanese quails were divided into four groups with seven replicates of seven birds. A conventional corn and soybean meal-based diet was formulated, and all groups' diets were supplemented with 0, 0.5, 1.0 and 2.0 g/kg PCB respectively for 42 days. From the results, PCB supplementation significantly improved body weight (BW) on the 21st day, body weight gain (BWG) between 0 to 21 days, hot carcass yield (HCY), and relative weights of the hearth. Similarly, blood urea nitrogen (BUN), total cholesterol (TC), low-density lipoprotein (LDL) and villus height (VH) levels were lower in PCB-supplemented groups. Besides, PCB supplementation in Japanese quails decreased the villus-crypt rate (VCR) except for the control and the group fed with 2.0 g/kg PCB. This study showed that dietary PCB supplementation in Japanese quails' diet improved growth performance in young chicks and carcass yield, BUN, and lipid profile. On the other hand, the supplementation did not affect the antioxidant status, homocysteine, and folic acid values in blood and meat traits.

## Introduction

Butyric acid, which is produced by the colonic microbiome, is an important short-chained fatty acid (SCFA) in poultry nutrition due to its ability to improve intestinal absorption both stimulating epithelium proliferation and impairing health in the intestines of the poultry (1, 9, 17). Because free salt forms of organic acids are commonly dissolved and absorbed in upper digestive tract organs; salt forms of butyric acid, such as sodium and calcium butyrate, are commonly used instead of acid forms; (17, 19). Due to the low pH of upper digestive organs; butyrate salts it's quickly converted to the undissociated form, butyrate needs to be protected with fat or film coating or micro-encapsulation to improve efficiency in the intestines (9, 12).

Butyric acid is not only important in animal nutrition because of its boosting effect on performance, but it also has a protective effect on metabolism by anti-carcinogenic, anti-inflammatory, and antioxidant properties (9, 30). Although the antioxidant effect of

dietary butyrate implementation in poultry was stated by several researchers (8, 12, 29), it is not clearly understood the effect mechanism of butyric acid reactive oxidant species.

Folate or folic acid (FA) is a water-soluble vitamin that involves one on -carbon metabolism and is required for the homocysteine remethylation process to form methionine and in the biosynthesis of amino acids deoxynucleotides essential for DNA replication and repair (5, 25). The absorption of FA could be established both from the diet and from the bacterial synthesis in the colon. Although FA is absorbed both in the intestines and the colon, it appears to folate mostly taken in from the jejunum (20). The FA deficiency in metabolism is indicated to cause homocysteine accumulation in blood by decreasing cystathionine synthesis and inhibiting homocysteine remethylation. Thus, higher homocysteine levels in the blood also might induce to increase in malondialdehyde levels in tissues (25). In order to promote FA absorption, we hypothesized that dietary protected calcium butyrate may regulate the gut microbiota and jejunum histomorphology in a positive way. This, in turn, may have a positive impact on the blood homocysteine level and the oxidative status of Japanese quails.

Thus, this trial was conducted to investigate the effect of increasing doses of dietary protected calcium butyrate salt supplement on performance, carcass parameters, jejunum histomorphology, blood serum values, and meat quality in Japanese quails.

## Materials and Methods

***Animals and Management:*** The six-week feeding experiment included 196 one-day-old unsexed Japanese quail chicks (*Coturnix coturnix Japonica*) which were allocated randomly into four groups of 49 chicks in 7 replicates (7 birds in each replicate). The first group was determined as a control group and fed a basal diet (Table 1). The basal diet was formulated according to National Research Council to meet Japanese quails' nutritional needs (22). Protected calcium butyrate was added at levels of 0.5, 1.0, and 2.0 g/kg in the basal diet respectively for the other groups. The PCB used in the trial subsumed 70% butyrate and coated with palm oil by the microencapsulation method. The PCB levels were determined according to the study conducted by Elnesr et al. (9). All birds accessed feed and water ad libitum.

The quails were reared in the same type of plastic wire floor pens (30 x 80 x 18 cm) under the same conditions and management with 23 hours of artificial lighting per day. The room temperature was set at 35ºC for the first week and decreased to 2-3ºC every week until it was reduced to 24-25ºC.

***Performance Trial and Sample Collection:*** Live weights of the Japanese quails were evaluated on days 0, 21, and 42 of the trial individually with a 0.01 g scale. Supplied feed was recorded daily and feed intake was quantified by subtracting the remaining feed in feeders from the feed given during the period and dividing the total animals into replicates. Feed conversation ratio (FCR) and body weight gain (BWG) were calculated throughout the experimental period.

**Table 1.** Ingredients and chemical and calculated content of basal diet of growing Japanese quail.

| **Ingredients (%)** | |
|---|---|
| Barley | 10.00 |
| Vegetable Oil | 0.50 |
| Maize | 49.27 |
| Corn gluten meal | 1.72 |
| Soybean meal | 35.28 |
| Dicalcium phosphate | 0.73 |
| DL-Methionine | 0.15 |
| Coccidiostat | 0.08 |
| L-Lysine hydrochloride | 0.15 |
| Marble powder | 1.37 |
| Sodium bicarbonate | 0.10 |
| Salt | 0.40 |
| Vitamin and Mineral Premix[1] | 0.25 |
| **TOTAL** | **100.00** |
| **Chemical composition (%)** | |
| Dry Matters | 88.74 |
| Crude Protein | 21.60 |
| Crude Fat | 2.01 |
| Crude Ash | 7.11 |
| Crude Fiber | 5.93 |
| **Calculated composition** | |
| Sodium, (%) | 0.23 |
| Calcium, (%) | 0.85 |
| Phosphorus, (%) | 0.31 |
| Lysine, (%) | 1.34 |
| Total Met + Sis, (%) | 0.93 |
| ME (kcal/kg) [2] | 2.533 |

[1] 1 Kg Vitamin-Mineral Premix contains; 8,800 IU vitamin A, 2,200 IU vitamin $D_3$, 11 mg vitamin E, 44 mg nicotinic acid, 8.8 mg Calcium D-Pantothenate, 4.4 mg riboflavin, 2.5 mg thiamin, 6.6 mg vitamin $B_{12}$, 1 mg folic acid, 0.11 mg D-biotin, 220 mg choline, 80 mg manganese, 60 mg iron, 5 mg copper, 60 mg zinc, 0.20 mg cobalt, 1 mg iodine, 0.15 mg selenium.

[2] Metabolizable energy content of diets calculation was conducted according to the equation of Carpenter and Clegg (7).

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All quails fasted for 6 hours before the slaughter. 2 birds from each replicate were randomly weighed and killed by the cervical dislocation method. Blood samples were collected from the jugular vein of the animals. Hot undressed carcass and some organs including heart, liver, proventriculus (PV), gizzard, bursa of Fabricius (BF), and spleen were weighed and calculated relative weights by dividing them into live weights and multiplying by 100. Approximately 20 g of breast meat samples were collected from the hot carcasses after determining the carcass traits. Blood serum was collected after centrifugation for 15 minutes and 3.000 rpm. All samples were stored at -20°C until the day of analyses. Additionally, 1 cm of jejunum section was collected from the intestines for intestinal histomorphology evaluation.

***Intestinal Histomorphology:*** The jejunum samples cleaned with the physiological saline solution were steeped in formol solution for 24 h. Thereafter, tissue samples were dehydrated in 70% ethanol overnight and processed using an automatic tissue processor (Thermo-Fisher, MA, USA). The samples were embedded in paraffin wax and the blocks were kept at +4ºC overnight again. The paraffin blocks were cut at 6 µm thickness on a microtome and stained with Masson's trichrome. Microscopy was implemented using a light microscope (Leica DM 500, Leica Biosystems Nussloch GmbH, Germany). Villus height and crypt depth of each quail were evaluated by taking the average of five measurements with ImageJ software.

***Blood Chemistry:*** Blood urea nitrogen (BUN), glycogen, total protein (TP), albumin, globulin, Ca, P, triglycerides, total lipids, and their fractions were measured with commercial kits (Rel Assay Diagnostics, Gaziantep, Türkiye). Total antioxidant capacity and total oxidant status were analyzed by the diagnostic kits of the same company, spectrophotometrically. Homocysteine and folic acid levels in serum were measured with commercial kits (Wuhan USCN Business Co. Ltd, Wuhan, China) using an ELISA reader (ChroMate® 4300, Awareness Technology, Inc., Palm City, FL, USA).

***Meat Quality:*** Each breast meat sample was divided into 4 parts. The breast meat CIE L* (lightness), a* (redness), and b* (yellowness) values were determined by a Minolta colorimeter (CR-200, Minolta Co., Osaka, Japan). The tissue malondialdehyde (MDA) level was analyzed by a method based on the reaction with thiobarbituric acid (TBA) at 90–100ºC. In this process, 0.1 g of sample was homogenized with phosphate-buffered saline using a probe-ultrasonicator for 10 min (Hielscher-UP100H, Germany). The samples were mixed with two volumes of cold 10% (w/v) trichloroacetic acid for the precipitation of protein. The precipitates were pelleted by centrifugation and the aliquot of the supernatants were reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. The absorbance was read at 532 nm after cooling. Water holding capacity (WHC) was estimated as; 1.00 g of the breast meat sample was dried in a drying oven for 12 hours after being centrifuged for 4 min at 1.500 rpm. WHC was determined by the following formula: (weight after centrifugation – weight after drying) / initial weight × 100. The rest of the samples were grinned into mince and stored at +4ºC for a month. pH values of mince were measured on 1$^{st}$, 15$^{th}$, and 30$^{th}$ days with a portable pH/temperature meter (Milwaukee MW102, USA).

***Statistical Analysis:*** After all data was collected, the statistical analyses were performed using IBM Statistical Package for Social Sciences (SPSS) software for Windows version 26. One-way analysis of variance (ANOVA) is used to determine performance, carcass traits, jejunum micromorphology, blood chemical values, and meat quality of the control and treatment groups. Significance in the trial was based on $P<0.05$. Analysis and means were compared using the Duncan test.

## Results

Table 2 shows the effect of PCB on body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversation rate (FCR). In this study highest and lowest BW on the 21$^{st}$ day and BWG between the 1$^{st}$ to 21$^{st}$ days were 2.0 g/kg PCB supplemented group and control respectively ($P<0.05$). Nonetheless, FI and FCR, BW on the 42$^{nd}$ day, and BWG on 22-42 and 1-42 days values showed no difference between groups ($P>0.05$).

In this study, it was seen that dietary PCB supplementation increased average HCY and the best values were in the 0.5 g/kg PCB group among control and other experimental groups ($P<0.05$). Moreover, the quails fed with 2.0 g/kg PCB supplemented diet had the highest relative heart weight of other groups ($P<0.05$). On the other hand, liver, gizzard, PV, BF, and spleen were not changed by PCB ($P>0.05$) (Table 3).

The effect of dietary PCB supplementation on blood serum biochemical values in Japanese quails demonstrates in Table 4. BUN, TC, and LDL concentrations in blood serum changed with PCB ($P<0.05$). BUN concentration was decreased linearly with increasing levels of PCB supplementation in the current research. Besides, the PCB supplementation also decreased TC and LDL values in blood serum. There was no difference between control and dietary treatments in blood serum glycerol, TP, albumin, globulin, Ca, P, TG, HDL, homocysteine, FA, TOS, TAC, and OSI values ($P>0.05$).

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The influence of feeding different diets of PCB on jejunum histomorphology is shown in Table 5. No effect was observed in CD contents of jejunum among the control and treatment groups (P>0.05). On the other hand, PCB supplementation depressed VH values compared to control. Furthermore, the control and the group fed with 2.0 g/kg PCB had a greater V:C Rate than the groups that received 0.5 and 1.0 g/kg PCB respectively (P<0.05).

The effects of PCB supplementation to Japanese quail diets on meat quality parameters are shown in Table 6. Our results showed that dietary PCB has no effect on pH values on 1$^{st}$, 15$^{th}$, and 30$^{th}$ days, MDA, WHC, and L.A.B. parameters in breast meats (P>0.05).

**Table 2.** Body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion rate (FCR) of Japanese Quails supplemented with dietary PCB.

| **Parameters**[1] | **PCB Levels (g/kg diet)** | | | | ***Significance (P-value)***[2] | |
|---|---|---|---|---|---|---|
| | **0** | **0.5** | **1.0** | **2.0** | **L** | **Q** |
| BW, Day 0, g | 8.23±0.11 | 8.37±0.26 | 8.41±0.16 | 8.32±0.29 | 0.432 | 0.158 |
| BW, Day 21, g | 70.81±9.62$^{b}$ | 81.01±6.59$^{a}$ | 81.27±7.48$^{a}$ | 82.01±3.87$^{a}$ | 0.010 | 0.095 |
| BW, Day 42, g | 161.39±10.53 | 177.14±14.14 | 172.68±11.07 | 172.97±11.71 | 0.147 | 0.100 |
| BWG, 0-21, g | 62.58±9.61$^{b}$ | 72.63±6.51$^{a}$ | 72.85±7.38$^{a}$ | 73.69±3.80$^{a}$ | 0.010 | 0.101 |
| BWG, 22-42, g | 90.59±7.18 | 96.13±11.37 | 91.41±10.33 | 90.96±9.93 | 0.830 | 0.427 |
| BWG, 0-42, g | 153.16±10.53 | 168.76±14.16 | 164.27±11.08 | 164.65±11.70 | 0.115 | 0.105 |
| FI, 0-21, g | 236.82±18.45 | 254.22±43.15 | 241.65±20.15 | 259.65±25.12 | 0.256 | 0.978 |
| FI, 22-42, g | 364.63±38.08 | 387.74±43.76 | 395.76±60.86 | 396.71±40.93 | 0.199 | 0.537 |
| FI, 0-42, g | 601.44±43.37 | 641.96±68.67 | 637.41±74.91 | 656.36±45.26 | 0.125 | 0.637 |
| FCR, 0-21, g/g | 3.83±0.39 | 3.53±0.69 | 3.34±0.34 | 3.52±0.31 | 0.162 | 0.172 |
| FCR, 22-42, g/g | 4.02±0.21 | 4.06±0.52 | 4.33±0.40 | 4.37±0.24 | 0.194 | 0.992 |
| FCR, 0-42, g/g | 3.93±0.15 | 3.81±0.40 | 3.87±0.22 | 3.99±0.16 | 0.576 | 0.239 |
| Mortality rate, % | 7.14±3.72 | 10.71±3.26 | 8.93±5.92 | 8.93±3.57 | 0.853 | 0.678 |

[a – b] Means with different superscripts within the same line diverge significantly (P<0.05), according to Duncan's test.
[1] BW, body weight; BWG, body weight gain; FI, feed intake; FCR; feed conversation rate.
[2] Data were analyzed using linear and quadratic regression models of SPSS.

**Table 3.** Hot Carcass Yield (HCY), and relative organ yields of Japanese Quails supplemented with dietary PCB.

| **Parameters**[1] | **PCB Levels (g/kg diet)** | | | | ***Significance (P-value)***[2] | |
|---|---|---|---|---|---|---|
| | **0** | **0.5** | **1.0** | **2.0** | **L** | **Q** |
| HCY (%) | 57.12±4.01$^{b}$ | 61.54±2.67$^{a}$ | 60.41±4.92$^{a}$ | 59.19±2.81$^{ab}$ | 0.290 | 0.010 |
| Liver (%) | 2.72±0.70 | 2.54±0.62 | 2.70±0.71 | 2.50±0.66 | 0.554 | 0.897 |
| Heart (%) | 0.82±0.10$^{b}$ | 0.87±0.09$^{ab}$ | 0.92±0.15$^{a}$ | 0.95±0.10$^{a}$ | 0.003 | 0.632 |
| Gizzard (%) | 1.90±0.35 | 1.78±0.29 | 1.83±0.41 | 1.78±0.24 | 0.425 | 0.652 |
| PV (%) | 0.51±0.10 | 0.52±0.15 | 0.52±0.09 | 0.54±0.06 | 0.519 | 0.990 |
| BF (%) | 0.08±0.03 | 0.09±0.03 | 0.09±0.03 | 0.08±0.02 | 0.736 | 0.353 |
| Spleen (%) | 0.05±0.02 | 0.04±0.01 | 0.05±0.02 | 0.05±0.03 | 0.577 | 0.705 |

[a – b] Means with different superscripts within the same line diverge significantly (P<0.05), according to Duncan's test.
[1] HCY, hot carcass yield; PV, proventriculus; BF, bursa of Fabricius.
[2] Data were analyzed using linear and quadratic regression models of SPSS.

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**Table 4.** Biochemical values in blood serum of Japanese Quails supplemented with dietary PCB.

| Parameters[1] | PCB Levels (g/kg diet) | | | | *Significance (P-value)*[2] | |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 2.0 | L | Q |
| BUN (mg/dL) | 5.76±0.54[a] | 5.80±0.54[a] | 5.53±0.48[ab] | 5.18±0.59[b] | 0.007 | 0.223 |
| Glyc (mg/dL) | 345.17±26.57 | 345.42±24.03 | 323.42±36.69 | 337.33±37.58 | 0.274 | 0.460 |
| TP (g/dL) | 3.03±0.92 | 2.52±0.60 | 2.64±0.93 | 2.45± 0.50 | 0.105 | 0.475 |
| Alb (g/dL) | 1.75±0.28 | 1.64±0.31 | 1.59±0.34 | 1.58±0.24 | 0.162 | 0.580 |
| Glob (g/dL) | 1.29±0.66 | 0.88±0.34 | 1.05±0.63 | 0.87±0.32 | 0.105 | 0.452 |
| Ca (mg/dL) | 33.25±23.50 | 17.08±10.99 | 23.25±17.21 | 17.67±12.98 | 0.089 | 0.316 |
| P (mg/dL) | 9.23±4.22 | 7.87±1.58 | 7.57±3.30 | 6.88±1.73 | 0.058 | 0.697 |
| TC (mg/dL) | 263.00±76.94[a] | 176.17±38.79[b] | 231.00±67.12[a] | 222.50±55.99[ab] | 0.405 | 0.032 |
| TG (mg/dL) | 396.33±181.56 | 329.08±183.66 | 337.17±175.66 | 273.33±189.44 | 0.133 | 0.974 |
| LDL (mg/dL) | 77.08±43.70[a] | 30.25±17.39[c] | 58.75±39.09[ab] | 47.09±20.72[bc] | 0.158 | 0.071 |
| HDL (mg/dL) | 74.00±33.88 | 92.42±31.32 | 79.50±28.26 | 93.75±30.56 | 0.254 | 0.817 |
| HCST (ng/mL) | 4.19±1.36 | 4.65±1.50 | 4.44±1.30 | 3.70±1.47 | 0.383 | 0.160 |
| FA (pg/mL) | 3.09±1.99 | 2.15±1.30 | 3.25±0.86 | 3.15±1.20 | 0.130 | 0.081 |
| TOS (mmol/L) | 10.14±4.94 | 8.28±2.32 | 9.97±2.57 | 10.85±2.90 | 0.380 | 0.163 |
| TAC (mmol/L) | 1.54±0.32 | 1.64±0.39 | 1.67±0.31 | 1.63±0.52 | 0.556 | 0.552 |
| OSI | 0.71±0.43 | 0.52±0.15 | 0.60±0.11 | 0.76±0.40 | 0.571 | 0.052 |

[a – b] Means with different superscripts within the same line diverge significantly ($P<0.05$), according to Duncan's test.
[1]BUN, blood urea nitrogen; Gcly, glycogen; TP, total protein; Alb, albumin; Glob, globulin; Ca, calcium; P, phosphorus; TC, total cholesterol; TG, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HCST, homocysteine; FA, folic acid; TOS, total oxidative status; TAC, total antioxidant capacity and OSI, oxidative stress index.
[2] Data were analyzed using linear and quadratic regression models of SPSS.

**Table 5.** Jejunum histomorphology in Japanese Quails supplemented with dietary PCB.

| Parameters[1] | PCB Levels (g/kg diet) | | | | *Significance (P-value)*[2] | |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 2.0 | L | Q |
| VH (µm) | 644.86±93.83[a] | 548.36±94.75[b] | 563.90±63.23[b] | 614.50±127.51[ab] | 0.520 | 0.007 |
| CD (µm) | 95.19±12.00 | 100.47±13.26 | 98.17±12.36 | 101.63±13.99 | 0.275 | 0.792 |
| VCR | 6.39±0.59[a] | 5.79±0.67[b] | 5.43±0.71[b] | 6.47±1.05[a] | 0.905 | 0.000 |

[a – b] Means with different superscripts within the same line diverge significantly ($P<0.05$), according to Duncan's test.
[1]VH, villus height; CD, crypt depth; VCR, villus-crypt rate.
[2] Data were analyzed using linear and quadratic regression models of SPSS.

**Table 6.** Meat Quality Analysis in Japanese Quails supplemented with dietary PCB.

| Parameters[1] | PCB Levels (g/kg diet) | | | | *Significance (P-value)*[2] | |
|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 2.0 | L | Q |
| pH, 1st Day | 5.87±0.14 | 5.92±0.11 | 5.88±0.11 | 5.92±0.14 | 0.444 | 0.799 |
| pH 15th Day | 5.96±0.22 | 6.01±0.30 | 6.00±0.16 | 6.07±0.16 | 0.238 | 0.948 |
| pH 30th Day | 6.33±0.54 | 6.30±0.45 | 6.44±0.49 | 6.53±0.46 | 0.250 | 0.686 |
| WHC (%) | 59.21±2.95 | 60.03±1.78 | 59.61±5.47 | 60.80±2.89 | 0.348 | 0.857 |
| MDA (nmol/L) | 17.09±15.18 | 10.95±9.94 | 13.14±11.33 | 15.74±16.76 | 0.917 | 0.271 |
| Luminosity (L) | 34.44±2.99 | 36.14±3.54 | 35.06±3.48 | 36.18±2.93 | 0.329 | 0.752 |
| Color a | 6.51±1.99 | 6.85±2.53 | 6.99±2.43 | 6.15±1.80 | 0.748 | 0.360 |
| Color b | 5.80±1.96 | 5.56±1.58 | 5.35±1.72 | 5.26±2.71 | 0.492 | 0.898 |

[1]WHC, water holding capacity; MDA, malondialdehyde.
[2] Data were analyzed using linear and quadratic regression models of SPSS.

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## Discussion and Conclusion

It was reported that butyric acid-enhanced performance in livestock by improving the histomorphological structure of the intestines and increasing digestive enzyme secretions by stimulating primarily pancreas exocrine activity (1). In the present study, dietary PCB supplementation positively affected BW on the 21st day and BWG between 1-21 days, yet no effect was observed in BW on the 42nd day, BWG between 22-42 and 1-42 days, FI and FCR values statistically. Similarly, Panda et al. (23) suggested that 0.4% butyrate supplementation in broilers' diet had the best BWG results in 0-3 weeks period. Additionally, sodium butyrate in Japanese quail diets boosted live BW in 21 days and BWG between 1-21 days was mentioned by Elnesr et al. (9). The improvement of BW and BWG in the early period of the current trial may be due to the acidifying properties of organic acids in the digestive systems of young poultry whose endogenous acid production is insufficient (3).

Another important finding of this research was improved HCY in Japanese quails with dietary PCB supplementation. Similar to our findings, Abd El-Wahab et al. (1) reported that dressed carcass was greater in the groups that received dietary calcium butyrate in Japanese quails. Equivalently, several researchers also found an additive effect of butyrate on carcass yield in broilers (19, 23). Butyric acid can involve carcass characteristics through increasing performance by improving intestinal absorption capacity and enhanced microflora (19). Mátis et al. (19) also reported butyrate also affect positively muscle development and stimulate insulin. In the current study, butyrate addition to quail diets enhanced relative heart weight linearly. Contrary to our findings, no effect on heart weight was observed in butyrate supplementation studies in broilers (11, 19) and quails (1, 26). A higher heart weight ratio to body weight is related to elevated blood circulation to secure oxygen supply for increased organ weights and metabolism (21). In the current study, increased HCY might also improve relative heart weights in the groups fed with PCB-supplemented diets too.

Blood biochemical parameters in poultry could be used as an important indicator of the animals' current physiological status and nutritional condition. BUN level in blood serum was lower in groups fed with PCB in our study. Butyrate also reduced BUN concentration in broilers (15) and rats with renal ischemia-reperfusion injury (30). Higher BUN values are correlated with tissue destruction in kidneys due to the losing filtration ability of damaged glomerulus cells (28). Butyric acid is reported as a therapeutic agent to prevent renal dysfunctions because of its antioxidant, anti-inflammatory, and antiapoptotic effects (30). The addition of butyrate to animal diets is considered helpful in reducing cholesterol values in blood by regulating gene expression to decrease lipid bioactivities in the jejunum (27). In this study, total cholesterol and LDL levels were lower in the groups fed with PCB-supplemented diets. Yin et al. (27) also revealed that butyrate glyceride supplementation in broiler diets decreased total cholesterol, triglyceride, and LDL values in blood statistically. Similarly, cholesterol content was decreased with fed butyrate in quails (9) and broilers (3, 15, 29).

We hypothesized PCB could enhance folate absorption either by improving intestinal histomorphology or microflora, however, no significant alteration was observed both for FA and homocysteine levels in blood serum in the current study. Although no study was found on the effect of dietary butyrate supplementation on blood homocysteine levels, several research studies have been conducted on different SCFA responses. Similar to our findings, Gheflati et al. (10) expressed that dietary apple vinegar, rich with acetic acid, consumption didn't differ in homocysteine content in the blood of patients with type-2 diabetes. Thus, Lamarre et al. (16) described vitamin $B_{12}$ and folate deficiency caused to reduce methionine synthase activity and increase formate levels in rats. Hence, further studies are needed to assess the effect of butyrate on homocysteine concentration and FA production.

In the present study, the data on jejunum histomorphology showed that the supplement of PCB didn't differ in crypt depth on the 42nd day of the trial. It was also observed augmenting the amount of PCB in quail diets affected VH value negatively. Furthermore, the control group had a better VCR than the birds fed with 0.5 and 1.0 g/kg PCB supplemented diet and almost equal to the ones supplemented with 2.0g/kg PCB. Consistent with our results, different researchers reported no effect of butyrate on Jejunum histomorphology in broilers (12, 13, 17). Contrary, different studies indicate that dietary butyrate implementation has a beneficial effect on intestinal histomorphology in broilers (14, 23) and quails (9, 26). Adil et al. (2) indicated broilers fed with 2% and 3% butyric acid improved villus height in duodenum and jejunum yet did not differ statistically in ileum and crypt depth in all three parts of intestines. Despite the other researchers, Baltić et al. (6) mentioned medium chained fatty acids affect negatively villus height in the ileum. Antongiovanni et al. (4) pointed out dietary butyric acid supplementation depressed villus length in the jejunum and ileum of broilers. Another study conducted on broiler chickens showed that the birds fed with coarse ground diets had better villus height than the ones who received a fine ground diet even though, both groups were supplemented with BA in the experiment period (24). Divergences between different reports might be related to not only the amount of butyrate supplementation in the

diet, but also to slaughtering age, feed particle size in diet, enzyme supplementation, and intestinal part of the birds.

In our study, no significant effect of PCB was found on the oxidation process both in meat and blood serum. Similar to our findings, a trial in broiler chicken showed that coated sodium butyrate addition to birds' diet didn't significantly affect MDA values in broiler breast meat (11). A previous study in grass carp showed that dietary butyrate supplementation didn't significantly differ MDA and TAC values in hepatopancreas (18). Contrarily, several studies indicated MDA levels were depressed by dietary butyrate addition in broilers (12, 29) and quails (8). Liu et al. (18) stated indirect antioxidant effect of sodium butyrate depended on the supplement and was not sensitive in other tissues than the intestines which might be a possible explanation for our situation. Furthermore, WHC, meat pH, and meat LAB values weren't influenced by increasing amounts of PCB in the current trial. Meat quality could be associated with the antioxidant functions of butyric acid (29). The reason for the absence of difference in pH, color, and WHC values of the breast meat might be attributed to the fact that there is also no disparity in antioxidant levels in both meat and blood serum in our results.

According to our results for the present trial, it could be advised dietary PCB supplementation in Japanese quails improve body weight and body weight gain first half of the trial as well as HCY, relative heart weight, and reduce total cholesterol and HDL values in blood serum. Despite the majority of the other research studies, we couldn't find enhancing effect of PCB on jejunum histomorphology. In general, the results indicate that PCB at the dosage of 2.0 g/kg in quail feeds could be recommended to show butyric acid's positive impact on body weight, body weight gain, and carcass yield, especially for the first 21 days of the growing period.

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## Author Contributions

Experimental design was determined by EG. Performance was recorded by EG and YC. Carcass parameters were established by EG and AGB. Jejunum histomorphology process was conducted by AGB. NHA established biochemical analysis. Chemical analysis of the quails' feed was carried out by KK. Statistical analysis was performed by EG. EG also wrote the first draft of manuscript.

## Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

## Ethical Statement

The design of the experiment was approved by the local ethical committee of the Faculty of Agriculture in Selçuk University (Protocol No: 2019-001).

## Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

## Conflict of Interest

The authors declare that they have no competing interests.

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# Osteogenic differentiation of canine adipose derived mesenchymal stem cells on B-TCP and B-TCP/Collagen biomaterials

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## Abstract

Mesenchymal stem cells are adult stem cells that can differentiate into osteogenic, chondrogenic, adipogenic and myogenic lineages. In orthopedics and traumatology, mesenchymal stem cells, combined with biomaterials, are used mainly for treating bone fractures and diseases in humans and animals. This study aims to promote the growth, proliferation, and osteogenic differentiation of mesenchymal stem cells isolated from the adipose tissue of canines on B-TCP (Beta-tricalcium phosphate) and B-TCP/Collagen biomaterials. MTT analysis was performed to test the cell adhesion and proliferation on B-TCP and B-TCP/Collagen biomaterials used to mimic the extracellular matrix of three-dimensional bone tissue. Scanning electron microscope analysis was performed to show general surface characters of B-TCP and B-TCP /Collagen biomaterials. The osteoinductive capacities of the B-TCP and B-TCP/Collagen biomaterials were determined by alkaline phosphatase and Von Kossa stainings, and RT-PCR analysis. The ALP activity of the B-TCP/Col containing material was significantly higher than the B-TCP in the early days. In terms of gene expression, there were no significant differences except 14$^{th}$-day SPARC gene expression. The results of Von Kossa staining indicated that B-TCP/Col has above the desired level degradation capacity. As a result of this research, although it is advantageous in terms of alkaline phosphatase activity and osteogenic gene expression compared to B-TCP material, it is thought that B-TCP/Collagen biomaterial should be developed for use in bone tissue engineering due to its high degradation property.

## Introduction

Mesenchymal stem cells (MSCs) are adult stem cells that were first isolated from bone marrow (17, 18). After this isolation, they were separated from many tissues, especially tissues with abundant vascular connective tissue (6).

In 2001, Zuk et al. (53) showed that MSCs can also be obtained from adipose tissue. Adipose tissue derived mesenchymal stem cells (Ad-MSCs) are obtained by explant culture method or using collagenase enzyme (21, 33). Ad-MSCs have advantages over other sources of MSCs. It has been reported that Ad-MSCs have more osteogenic differentiation potential than mesenchymal stem cells obtained from bone marrow (9, 12, 28). On the other hand, unlike embryonic stem cells, there are neither ethical nor political concerns regarding Ad-MSC isolation since they can be isolated from the patient's adipose tissue (10, 13, 40). All these advantages make Ad-MSCs one of the most preferred sources of mesenchymal stem cells.

One of the most common uses of mesenchymal stem cells today is bone tissue engineering studies in orthopedics and traumatology, especially for treating bone, cartilage and tendon diseases (8). Various biomaterials can be used to provide structural and mechanical support during the healing process of large defects and multi-component fractures with insufficient bone regeneration capacity (13, 19, 20, 30).

One of the important developments in the field of biomaterials has been the use of bioceramics for bone replacements. Calcium phosphate ceramics have been

used as synthetic bone graft substitutes for over 30 years, as they have similarities with the inorganic composition of bones (46).

B-TCP is the most studied bioceramic in the treating bone fractures and diseases (46). According to the numerous in vivo and in vitro evaluations in the literature, B-TCP has excellent biocompatibility and osteoconductivity. It has also been shown to support the differentiation and proliferation of mesenchymal cells (7, 16, 44, 52). Using porous ceramic implants in bone tissue engineering can provide an environment for cells to grow and differentiate (3).

Collagen is a natural polysaccharide widely used in bone tissue engineering, because of its structural resemblance to natural bone tissue. It is a biocompatible material that the body can absorb. Moreover, collagen is proven to be minimally immunogenic and nontoxic material. Despite these advantages, collagen shows poor mechanical properties. Today, collagen is used in prosthetic implants and tissue engineering of many organs, including bone (1, 26, 36).

This study aims to compare the osteogenic differentiation capacities of canine Ad-MSCs biomaterials containing B-TCP and B-TCP/Collagen (B-TCP/Col). These biomaterials provide structural and mechanical support and create the microenvironment for stem cells during the healing process of large bone defects and multi-part fractures of the bone. It is thought that the biomaterial prepared as a combination of B-TCP and collagen will be advantageous in terms of adhesion, proliferation, compatibility and osteogenic differentiation. Comparing these biomaterials as microenvironments for stem cells may increase the treatment success of bone fractures and accelerate the post-operative healing process.

## Materials and Metods

***Isolation of Ad-MSCs:*** For the isolation of MSCs, inguinal adipose tissues were harvested from n= 5 dogs during ovariohysterectomy operation at Ankara University Faculty of Veterinary Medicine. This study was approved by Ankara University Animal Experiments Local Ethics Committee (2017-5-37). The explant culture method was preferred for mesenchymal stem cell isolation from adipose tissue. Adipose tissue ($1cm^3$) was divided into small pieces in a sterile petri dish under laminar flow and kept at 37 °C in an incubator with 5% $CO_2$ for 20 minutes. Then Modified Eagle Medium (Lonza) which contain 20% Fetal bovine serum (Biowest), 2% L-Glutamine, 1% Penicillin, Streptomycin and 77% Dulbecco's medium was added (53).

***Characterization of Ad-MSCs:*** At the end of the third passage, adipogenic, osteogenic, and chondrogenic differentiation and flow cytometry analyzes were performed for the characterization of canine Ad-MSCs.

For adipogenic differentiation, adipocyte differentiation basal medium and supplements (standard medium high-glucose DMEM (10% FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone, 10 µg/mL insulin, 0.5 mM indomethacin (Sigma-Aldrich, Switzerland); for osteogenic differentiation, osteocyte differentiation basal medium (DMEM-LG, 0.05 mM ascorbate-2-phosphate, 100 nM dexamethasone, and 10 mM sodium β-glycerophosphate (Sigma-Aldrich); and for chondrogenic differentiation, chondrocyte differentiation basal medium (high-glucose DMEM containing 6.25 µg/mL insulin-transferrin-selenious acid, 0.1 mM ascorbate2-phosphate, 10–7 M dexamethasone, 1.25 mg/mL bovine serum albumin, 5000 IU/mL penicillin, 50 µg/mL ascorbate 2-phosphate, and 100 nM dexamethasone and 10 ng/mL human transforming growth factor) were used. Adipogenic, osteogenic and chondrogenic differentiation were evaluated using Oil Red O, Von Kossa and Alcian Blue staining methods. The development of cells was observed with an inverted microscope (Olympus Cx45).

For flow cytometry analyzes, $1x10^6$ cells were placed in flow cytometry tubes and these cells were washed 3 times each in 3% Bovine serum albumin/PBS (BSA/PBS) solution. Stem cells were incubated with 0.1-10 µg/ml primary antibodies [CD 29 (P4611, Chemicon), CD 34 (ICO115, Novusbio, USA), CD 44 (G44-26, BD biosciences, USA), CD73 (P21589, Bioss, USA), CD 81 (J5-81, BD biosciences, USA), CD90 (OX-7, BD biosciences, USA) and CD 271 (C40-1457, BD biosciences, USA)]for 30 minutes at room temperature. Cells were washed 3 times in 3% BSA/PBS solution and then precipitated by centrifugation. For unconjugated primary antibodies, cells were incubated with secondary antibody for 30 minutes at +4 °C and washed 3 times in 3% BSA/PBS solution. After the cell sediments were dissolved in 1 ml of 3% BSA/PBS solution, the cell was analyzed in flow cytometry device (BD Accuri Plus flow cytometer) (48).

***Biomaterial fabrication, Cell seeding, and Osteogenic Differentiation Protocol:*** B-TCP (SupraBone, 0.5–1mm particle size, Figure1) and B-TCP/Col biomaterials were kindly donated by BMT Calsis Health Technologies (41). The pack of B-TCP/Col was split into 0.5 mm x 0.5 mm x 0.5 mm pieces (Figure1). Both biomaterials were placed in 24-well dishes and sterilized under UV light. Following sterilization, 200,000 canine Ad-MSCs reaching passage 3 were seeded onto each scaffold for further analysis. Before adding 2 ml of cell culture medium to each well, they were incubated for 4 hours at 37°C. During the 4-hour incubation, 20 µl of the medium was added to each cell scaffold every 30 minutes to prevent the materials from drying out. After cell cultivation, media were added to the biomaterials and incubated at 37°C in an incubator containing 5% $CO_2$ (25). To initiate osteogenic

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differentiation, osteogenic differentiation basal medium was added to each well after 1 day of incubation with cell culture medium.

**Figure 1.** Image of B-TCP (SupraBone, 0.5–1mm particle size) and Image of B-TCP/Col (0.5 mm x 0.5 mm x 0.5 mm).

***MTT Assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide):*** Colorimetric MTT test was performed on days 7, 14 and 21 to examine the growth and proliferation of cells on B-TCP and B-TCP/Col structured tissue scaffolds. Each 200,000 cells seeded biomaterials were kept in a solution (5 mg / mL) medium and MTT reagent (Biovision) was mixed at a ratio of 10:1 by volume for 4 hours at 37 °C in an incubator with 5% $CO_2$. Then, 100 µl of 1% SDS was added to each sample and kept at 37 °C in an incubator with 5% $CO_2$ and in an environment without light for 24 hours. Finally, 200 µl of the solution was taken and transferred to a 96-well plate. The absorbance was measured at 590 nm with a microplate reader (SpectraMax i3) (5, 32).

***Scanning Electron Microscopy (SEM):*** To analyze Ad-MSCs in B-TCP and B-TCP/Col scaffolds 250,000 cells were seeded in both materials. After 2 days of incubation at 37°C in the incubator, the biomaterials were fixed in 5% glutaraldehyde at 4°C for 24 hours. After washing with distilled water, dehydration was done by holding in 35%, 50%, 75%, 95% and absolute alcohols twice for 15 minutes. B-TCP and B-TCP/Col biomaterials were left to dry in a desiccator at room temperature after they were kept in 2 ml of Hexamethyldisilazane (HMDS). Dried biomaterials were coated with copper and examined under an SEM (EVO50 Zeiss) (31).

***Alkaline Phosphatase (ALP) Assay:*** Cell-seeded biomaterials on days 1, 7, 14 and 21 were fixed for 5 minutes using 10% neutral buffered formalin and washed with PBS. After washing, the biomaterials were dyed with the p-nitrophenyl phosphate (p-NPP) solution for 45 minutes in an incubator containing 5% $CO_2$ at 37 °C without light. After stopping the reaction with the stop solution, the absorbance values were recorded at 405 nm in a microplate reader (SpectraMax i3) (27, 32).

***Von Kossa Staining on Biomaterials:*** At the end of 7, 14 and 21 days, cell-seeded biomaterials were fixed for 30 minutes with 10% neutral buffered formalin. After washing with distilled water, silver nitrate solution (5%) was added onto the biomaterials and they were subjected to UV light for 60 min. Then they were rewashed, and mineralization was demonstrated by adding 1% sodium thiosulfate (32).

***RT-PCR (Real Time Polymerase Chain Reaction):*** Total RNA isolation was performed according to the manufacturer's protocol (Thermo GeneJet) on the 7th, 14th and 21st days of the osteogenic induction. Following isolation, RNA samples were transformed into cDNA. For this purpose, iScript cDNA synthesis kit (BioRad) was used. The relative gene expressions were determined by reverse-transcriptase polymerase chain reaction method using QPCR Green Master Mix Kit (Biotechrabbit). The reaction mixture was prepared to 20 µl total volume by using 10 µl master mix, 7.2 µl nuclease-free water, 0.4 µl forward primer, 0.4 µl reverse primer and 2 µl cDNA for each sample. The Cq results were obtained from RT-PCR device (CFX96 Touch Real-Time PCR Detection System) with protocol of 3 minutes at 95 °C, 15 seconds at 95 °C and 30 seconds at 60 °C for 40 cycles. Relative gene expression differences were calculated using the 2-ΔCt formulas and primers were designed for this study (Table 1). The beta actin (ACTB) gene was used for the normalization of the values (27).

***Statistical Analysis:*** Two-way analysis of variance was used to evaluate the effect of group and time on the measurements obtained from MTT, ALP and RT-PCR analyses. In the ANOVA model, group (B-TCP, B-TCP/Col and OC between-subject factor) and time (days 1, 7, 14 and 21 between-subject factor) and the interaction term of these two factors were included. Tukey test was used as an advanced test for the factors that were found to be significant. Analysis was done with GraphPad Prism software and data are presented as mean ± standard deviation. Statistical significance was expressed as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

**Table 1.** Primers used for RT-PCR analyses.

| | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| **RUNX2** | CCCAGAAGGCACAGACAGAA | CATCTGGCTCAGGTAGGACG |
| **SPARC** | TTCCTGTTGCCTGGCTCTAA | GGTTCTGGCAGGGGTTTTCA |
| **ACTB** | TCCATGAAACTACCTTCAACTCC | AACGCAACTAAAGTAACAGTCC |

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## Results

***Characterization of Ad-MSCs:*** For mesenchymal stem cell characterization, cells were induced for osteogenic, chondrogenic and adipogenic differentiation. Cell colonies were observed from day 9 in cells induced for osteogenic differentiation. The number of these colonies reached its highest level at the end of the second week. Von Kossa staining was performed to show osteogenic differentiation on day 14 and the calcium deposits were formed (Figure 2A). In the second week of chondrogenic differentiation, changes began to be seen in the morphology of the cells. On the 19th day, secreting proteoglycan specific to cartilages was observed. Therefore, Alcian Blue staining was performed to show cartilage differentiation on day 21 (Figure 2B). Adipose vacuoles were observed from day 14 in adipogenic differentiation. At day 21, adipogenic differentiation was demonstrated by staining the oil vacuoles in the cytoplasm of the cells with Oil red O staining (Figure 2C).

The cells isolated from adipose tissue were analyzed with seven antibodies for mesenchymal stem cell characterization and Figure 3A shows the control. It was shown by flow cytometry analysis that CD 44$^{+}$ (Figure 3G), CD 73$^{+}$ (Figure 3H), CD 81$^{+}$ (Figure 3F) and CD 90$^{+}$ (Figure 3E) were expressed from these antibodies and CD 29$^{-}$ (Figure 3B), CD 34$^{-}$ (Figure 3C) and CD 271$^{-}$ (Figure 3D) were not expressed.

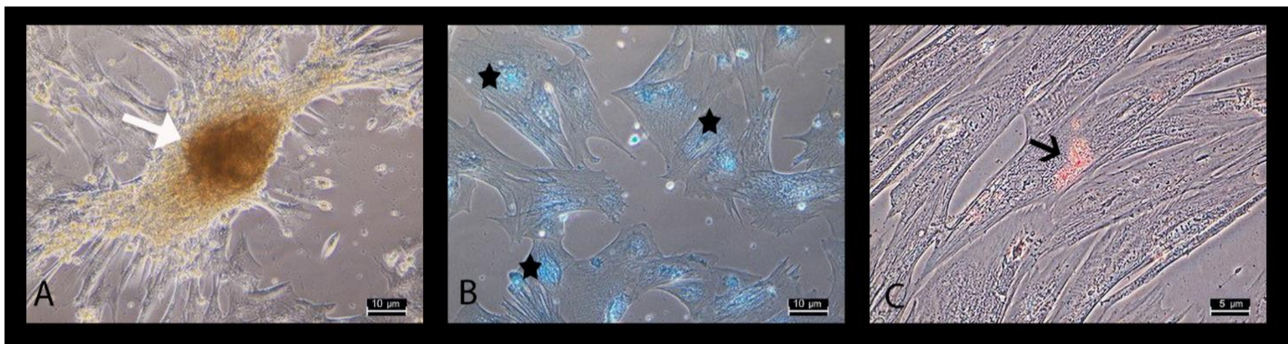


**Figure 2. A.** Osteogenic differentiation of Ad-MSCs. Von Kossa staining was used to detect calcium deposit at 14 days after osteogenic culture (white arrow), scale bar: 10 µm.**B.** Chondrogenic differentiation of Ad-MSCs. On the 21$^{st}$ day, chondrogenic differentiated cells were observed by Alcian blue staining (stars), scale bar: 10 µm. **C.** Adipogenic of differentiation of Ad-MSCs. On the 21$^{st}$ day, oil vacuoles were observed in the cytoplasm of the Ad-MSCs by Oil red O staining (black arrow) scale bar: 5µm.

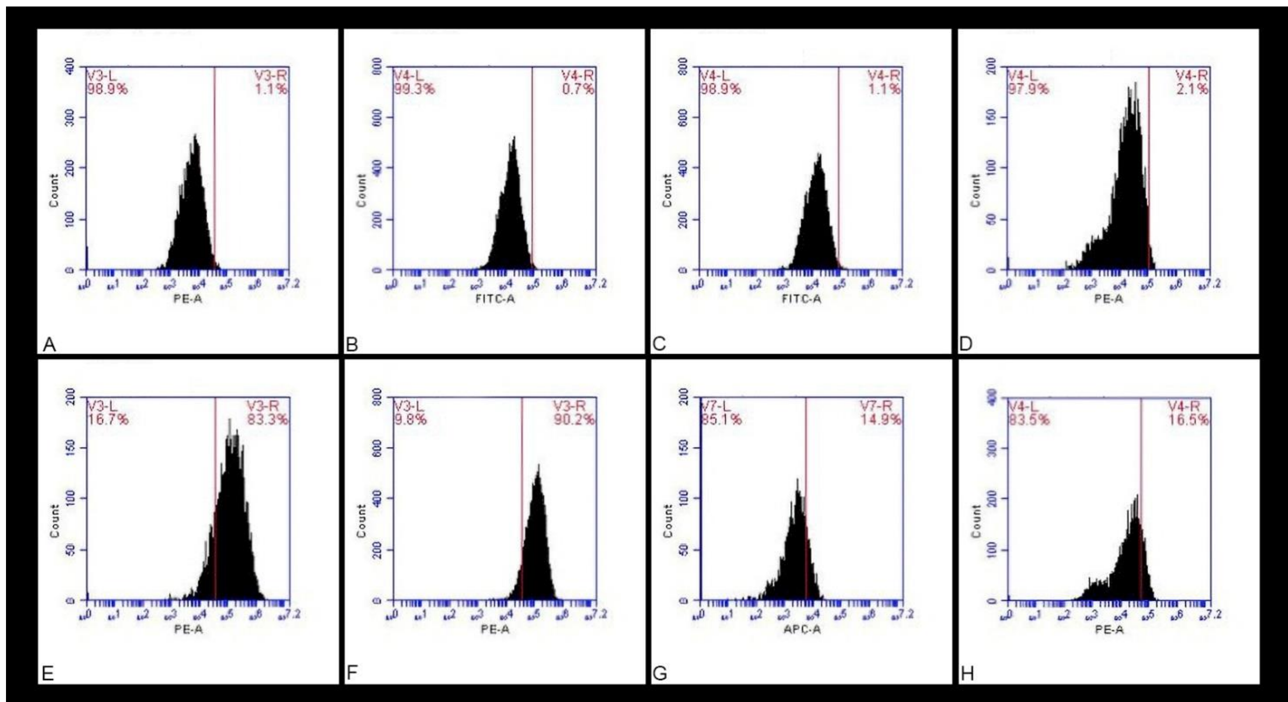


**Figure 3.** The expression of cell surface molecules in canine Ad-MSCs was detected by flow cytometry: **A.** Control, **B.** CD 29$^{-}$, **C.** CD 34$^{-}$, **D.** CD 271$^{-}$, **E.** CD 90$^{+}$, **F.** CD 81$^{+}$, **G.** CD 44$^{+}$, **H.** CD 73$^{+}$.

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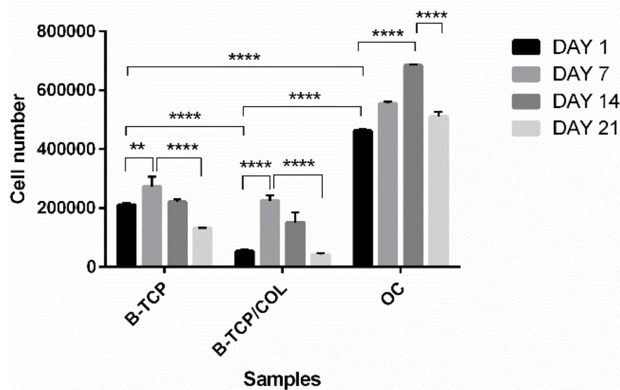


**Figure 4.** Ad-MSC cell numbers in cell-seeded B-TCP, B-TCP/Col and Only cell (OC) groups obtained from absorbance values of MTT test on days 1, 7, 14 and 21 (** P<0.01, **** P<0.0001).

***MTT Assay:*** Colorimetric MTT test was performed on the 1st, 7th, 14th and 21st days on B-TCP and B-TCP/Col based biomaterials and groups of only cells (OC) (Figure 4). According to the calibration curve, the cell numbers cultivated on biomaterials were calculated. Although the number of cells in the B-TCP material was significantly higher than the B-TCP/Col combination on day 1, there was no significant difference between the two materials on the other days. The cell number of the OC group was statistically higher than cell proliferation in both materials on days 1, 7, 14 and 21. The increase in cell number from day 1 to day 7 in both materials was statistically significant. The number of cells adhering to the materials decreased significantly from day 7 to day 21, while the proliferation of cells grown in tissue culture plates decreased from day 14 to day 21.

***Scanning Electron Microscopy (SEM):*** As a result of scanning electron microscopy, the general surface properties of cell seeded materials were photographed. Ad-MSCs attached to the porous surface of B-TCP was demonstrated in cell seeded biomaterials (Figure 5A). Biomaterials containing β-TCP/collagen were shown by SEM to have fewer pores than β-TCP. The collagen structure and location of Ad-MSCs were photographed in the cell-planted β-TCP/collagen-containing biomaterial (Figure 5B).

***Alkaline Phosphatase Assay:*** It was observed that ALP activity significantly increased from day 1 to 7 days in B-TCP and B-TCP/Col based biomaterials and groups of OC. Enzyme activity of ALP significantly decreased from day 14 to day 21 in all groups. Cells seeded in B-TCP/Col scaffolds possessed higher ALP enzyme activity on days 1 and 7 than B-TCP. ALP enzyme activity of cells seeded in B-TCP/Col scaffolds was significantly higher than OC groups on the 1st day. On the 14th and 21st days, there was no significant difference between the two materials. The ALP activity of wells with OC increased from day 1 to day 14 and decreased on day 21 (Figure 6).

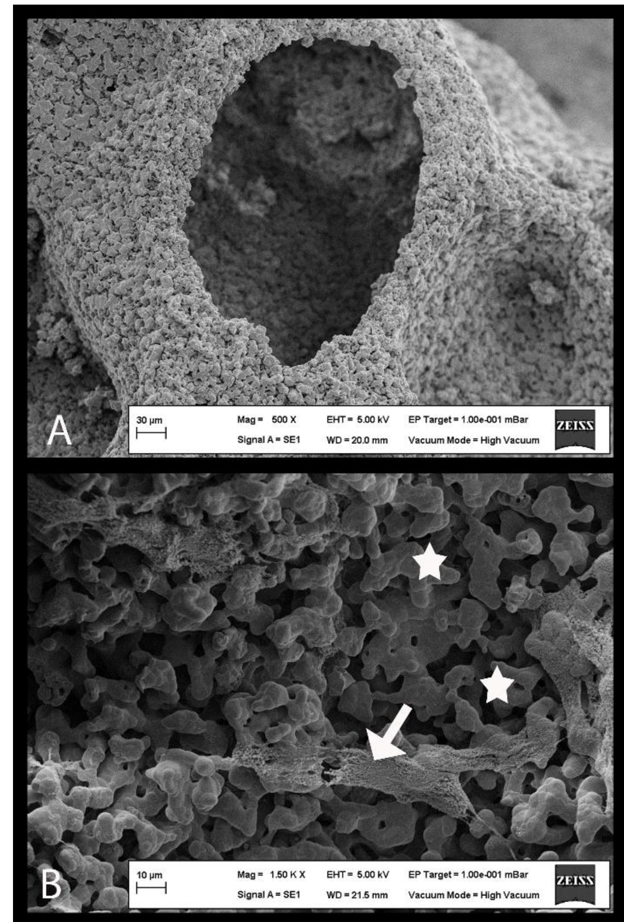


**Figure 5. A.** SEM image of Ad-MSCs seeded B-TCP material. Ad-MSCs covered the entire porous surface of the material. **B.** SEM image of Ad-MSC seeded B-TCP/Col material. The white arrow indicates pieces of material and stars point out cells.

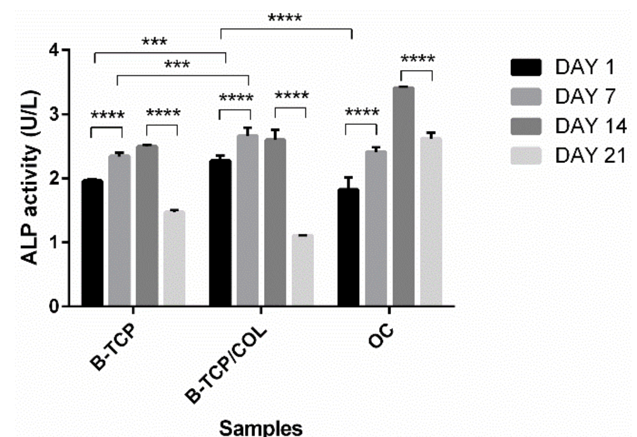


**Figure 6.** ALP activity of Ad-MSCs in cell-seeded B-TCP, B-TCP/Col and Only cell (OC) groups on days 1, 7, 14 and 21 (*** P<0.001, **** P<0.0001).

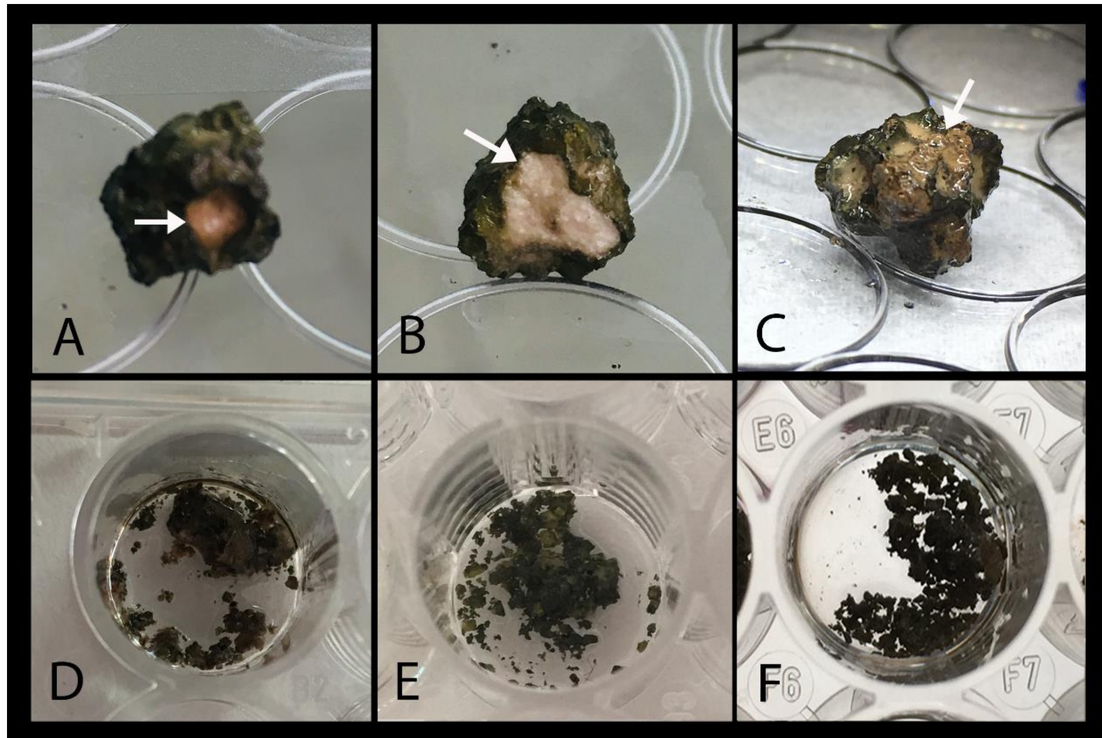


**Figure 7.** On days 7 (**A.**), 14 (**B.**) and 21 (**C.**), an increased amount of calcium deposition was demonstrated by Von Kossa staining as brown staining areas (arrows). However, on days 7, 14 and 21 (**D., E., F.**), the amount of calcium deposition could not be demonstrated because of the high biodegradation level of B-TCP/Col scaffolds.

***Von Kossa Staining on Biomaterials:*** Von Kossa staining results showed that the number of calcium deposits increased from the 7th day to the 21st day in the B-TCP and B-TCP/Col scaffolds (Figure 7). These deposits were demonstrated by the increase in areas of brown staining on the material after 60 minutes of UV light exposure. However, due to the high biodegradation rate of B-TCP/Col scaffolds, degradation started after the seventh day and calcium deposits could not be demonstrated.

***Real Time PCR***: The relative expressions of osteogenic marker genes; RUNX2 (Runt-related transcription factor 2) and SPARC (Secreted protein acidic and cysteine-rich) by cells inside B-TCP and B-TCP/Col containing materials and the group with OC were examined. RUNX2 gene expressions significantly increased from day 7 to day 14 in both biomaterials. Furthermore, from the 14th to the 21st day, the gene expressions of RUNX2 were significantly higher in B-TCP and B-TCP/Col containing materials and the OC group. 14th-day expressions of RUNX2 in both biomaterials were higher than in the OC group. There was no statistically significant difference between the two biomaterials regarding RUNX2 expressions. SPARC gene expressions significantly increased from day 7 to day 21 in B-TCP and B-TCP/Col containing materials and the OC group. Cell-seeded B-TCP biomaterial possessed significantly higher SPARC gene expression than the BTCP/Col and OC groups on day 21 (Figure 8).

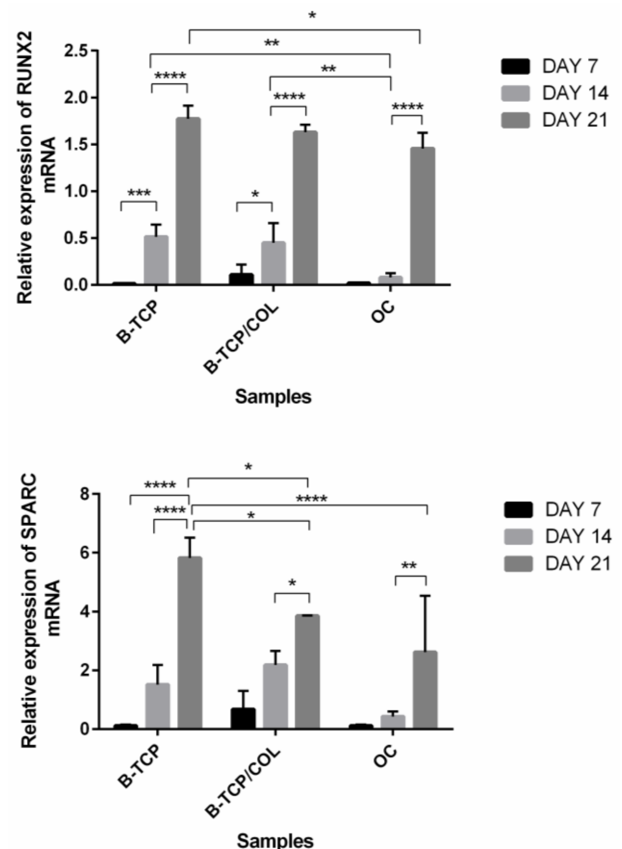


**Figure 8.** Relative mRNA expressions of RUNX2 and SPARC in cell-seeded B-TCP, B-TCP/Col and Only cell (OC) groups on days 7, 14 and 21. (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

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## Discussion and Conclusion

Tissue engineering provides a new approach to the therapy of damaged tissues. The development of biomaterials that can allow mesenchymal stem cell attachment, growth and proliferation are essential for creating tissue engineering scaffolds to support bone regeneration. The ideal biomaterial for bone tissue engineering should be osteoinductive, osteoconductive and biodegradable at the desired rate. Collagen is one of the natural polysaccharides that are biocompatible and biodegradable, the structure of which regenerates natural bone tissue glycosaminoglycans (36). In contrast, B-TCP which is a bioactive ceramic has a low capacity for biodegradation (4). Therefore, we used 3D porous scaffolds containing B-TCP and B-TCP/Col for use as bone repair substitutes. In this study, Ad-MSCs were seeded on mentioned biomaterials and their growth and differentiation capacities were compared on these materials.

Flow cytometry analysis characterized canine Ad-MSCs with antibodies CD 29, CD 34, CD 44, CD 73, CD 90, CD 81 and CD 271. The MSCs isolated from the canine adipose tissue expressed the stem cell markers CD $44^+$, CD $73^+$, CD $81^+$ and CD $90^+$, whereas the markers CD $34^-$ and CD $271^-$ were negative; these results are consistent with others (25, 39, 40, 42, 46). In the general Ad-MSCs characterization criteria, CD 29 is considered positive (18, 29, 37, 51). The previous characterization studies of Ad-MSCs revealed that CD 29 was also expressed in humans, horses, cats and dogs (28, 42, 45, 47, 49). However in this study the CD 29 surface antigen was not expressed. Canine Ad-MSCs isolation and characterization studies of Marx et al. (28) and Screven et al. (43) support the results obtained in this study. It is thought that this difference may arise from the differences between animal species and further research is needed for species-specific characterization.

MTT assay was performed to specify the number of viable cells on B-TCP and B-TCP/Col on the 1st, 7th, 14th and 21st days after seeding. The number of cells increased the first 7 days and reduced on the 21st day indicating that both biomaterials support the attachment, proliferation and osteogenic differentiation of cells. It is thought that the decrease in the number of cells on the 21st day in both materials and in the OC group is insufficient space for the cells to proliferate. The present findings are supported by previous studies made with different biomaterials containing B-TCP and MSC (11, 34, 50).

Alkaline phosphatase is an enzyme that refers to increased osteoblasts activity for new bone formation and growth. Therefore it is used as an early marker for osteogenic activity. The previous findings studied with cell seeded ceramic scaffolds and collagen containing biomaterials have indicated that ALP expression reaches a maximum level after 14 days in culture and declines up to day 21 (15, 22, 31). According to the present findings, the enzyme activity of B-TCP increased from 1 day to 14 days and it decreased on the 21st day. In B-TCP/Col structured biomaterial, ALP activity increased in the first week and decreased after the second week. It is thought that these findings can be the indicator of differentiation from osteoblasts to osteocytes (35).

To compare the qualitative assessment of mineralization of the extracellular matrix, Von Kossa staining was performed on the 7th, 14th and 21st days for both materials. It was determined that calcium deposits increased from 7 days to 21 days in the biomaterial containing B-TCP. However, since the degradation ability of the B-TCP/Col is high, the mineral dissolved after the 7th day and the mentioned calcium deposits could not be shown on the material. The high degradation ability of this material is due to collagen, a natural biomaterial it contains. This high degradation property of the material is a disadvantage for bone tissue engineering (39). Donzelli et al. (15) conducted an in vitro degradation study using MSC derived from rat bone marrow and collagen material. They predicted in vivo studies that collagen could be dissolved before bone tissue healed. Collagen was used in our study in combination with B-TCP to overcome this disadvantage of collagen. Kato et al. (23) demonstrated that the osteoconductivity and biodegradation property of B-TCP/Col composites are superior to B-TCP. However, it was seen that this combination could not sufficiently reduce the degradation properties of the material. Therefore, it is thought that degradation studies about B-TCP/Col should maintain.

RUNX2 also known as core-binding factor subunit alpha-1 is a protein that is an essential transcription factor for osteoblast differentiation (24). SPARC also called osteonectin is a glycoprotein initiating mineralization and promoting mineral crystal formation during bone formation (38). Because they are related to osteoblast activity, both genes are considered an early marker of bone regeneration. However, these genes have a significant role in the early and late phase of osteogenesis (14, 24). In studies with ceramic biomaterials and MSCs, expressions of the SPARC and RUNX2 genes were measured and it was shown that the expressions peak first days and then decrease (2, 50). In this study, Real-time PCR analysis was performed to determine osteogenic markers of RUNX2 and SPARC on days 7, 14 and 21 for materials and the OC control group. Expression of both genes increased in both biomaterials and control groups from 7th day to 21st day. The higher expressions of the RUNX2 and SPARC genes compared to the control group indicated that these materials have the osteoinductive capacity.

In the study, canine Ad-MSCs were propagated and differentiated to the bone on biomaterials containing B-

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TCP and B-TCP/Col. The proliferation and osteogenic differentiation capacities of Canine Ad-MSCs were compared in two different biomaterials. Cell adhesion, proliferation and differentiation capacities were tested by performing MTT, Alkaline Phosphatase, Von Kossa and RT-PCR analysis on biomaterials containing B-TCP and B-TCP/Col. It was concluded that both materials were successful in terms of cell attachment, proliferation and osteogenic differentiation. When comparing the biomaterials with each other, there was no significant difference in cell proliferation assay. However, the ALP activity of the B-TCP/Col containing material was significantly higher than the B-TCP. Although there were no significant differences in RUNX2, the SPARC gene expression of the B-TCP material on day 14 was higher than the other material. The results of Von Kossa staining showed that B-TCP/Col has above the desired level degradation capacity. The very high biodegradability of the B-TCP/Col combination is thought to make it possible to work in cartilage tissue engineering rather than bone tissue engineering. More research is needed to increase the absorption rate, mechanical properties and chemical stability of materials prepared in combination with collagen in the body.

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## Conflict of Interest

The authors declare no financial or personal conflicts of interest.

## Author Contributions

ÖÖÇ and AÖ conceived and planned the experiments. ÖÖÇ carried out the experiments. ÖÖÇ contributed to sample preparation. ÖÖÇ and AÖ contributed to the interpretation of the results. ÖÖÇ took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

## Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

## Ethical Statement

This study was approved by Ankara University Animal Experiments Local Ethics Committee (2017-5-37).

## Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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# Effect of Atorvastatin and *Lactobacillus acidophilus* on cholesterol metabolism in experimental hypercholesterolemia

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## Abstract

Hypercholesterolemia is a very common health problem in the world. In this study, it was aimed to investigate the effects of atorvastatin and *Lactobacillus acidophilus* probiotic on cholesterol metabolism, and formation of neurosterides and myelin. Five groups were formed in the study. Group-1 was fed with standard rat chow as a control group. Group-2 was designated as hypercholosterolemi group and fed with cholesterol added rat chow. Group-3 was fed with cholesterol and atorvastatin. *L.acidophilus* probiotic was given in the last four weeks of the experiment to Group-4. *L.acidophilus* and atorvastatin were given together for the last four weeks to the Group-5. At the end of the trial, some biochemical parameters were determined by autoanalyzer device and ELISA. LDL receptor (LDL-R), HMG-CoA reductase, GAPDH genes were determined by RT-PCR. In the case of adding cholesterol to the diet, total cholesterol in the serum, LDL-cholesterol level increased, HDL-cholesterol level decreased, liver enzyme activity increased, Ox-LDL level increased significantly in the brain, testosterone, progesterone, MBP level, nNOS activity were significantly increased. GAPDH gene gave bands at the same intensities in brain and liver of in all groups. When compared with GAPDH, band intensities of the LDL-R and HMG-CoA reductase genes were decreased. It was determined that the hypocholesteric effect of the combination of statin and probiotic is better and neurosterides have a positive contribution to the level of serotonin hormone. As a result, it was concluded that *L. acidophilus* probiotic supplementation with atorvastatin can be recommended as supportive product in the treatment of hypercholesterolemia.

## Introduction

Cholesterol is the precursor of cell membrane, myelin structure and oxysterol, and an important structural element of steroid hormones and bile acids. Brain is the richest organ in terms of cholesterol and contains 20% of the body's total cholesterol. 70-80% of the brain cholesterol was provided in the myelin sheath (12). Cholesterol regulates the tight relationship between neurons and glia. Cholesterol is an essential biomolecule in the formation of synapses and dendrites for normal brain development. Also, it is necessary for axonal development (10). In cholesterol deficiency, degeneration of synaptic and dendrite spine, neurotransmitter errors and decreased synaptic flexibilities could be formed. Defects in cholesterol metabolism cause structural and functional diseases in the central nervous system (6). These metabolic diseases, cholesterol biosynthesis, lipid and lipoprotein transport might be affected by different metabolic pathways such as molecules (12, 24). Hypercholesterolemia is a very common health problem in the world. Statin group drugs that inhibit the HMG-CoA enzyme involved in cholesterol synthesis are widely used in the treatment of hypercholesterolemia. Today, while these drugs are used to effectively reduce the level of

cholesterol in the plasma, there is not enough information about neurosteroids (estrogen, progesterone and testosterone) synthesized from cholesterol in the brain and their effects on myelin formation and cholesterol metabolism. Probiotics such as *Lactobacillus acidophilus* and similar lactic acid bacteriae also have a cholesterol-lowering effect.

The purpose of this study was to compare the effects of atorvastatin and *L. acidophilus* probiotic, which are both commonly used to lower the risk of coronary atherosclerosis following experimental hypercholesterolemia, on cholesterol metabolism and changes in serotonin, neurosteroids, nNOs, oxLDL, and MBP in the brain. The LDL receptor (LDL-R) genes, which are involved in absorption, and the HMG-CoA reductase gene, which is involved in cholesterol metabolism, were also examined in the study.

## Materials and Methods

***Study population and circumstances:*** The literature datas were considered as a guide to the selection of the animal species. The rat is the species that is most similar to humans in terms of physiology, anatomy, diet, pathology, and metabolism, according to researches done to explore cholesterol (17). G*Power software (version 3.1.9.7) was used for determining the sample size and analysing power in a study by using using a significance level of α = 0.05, 80% power, and an effect size of 0.50. Male Sprague-Dawley breed adult rats (n=50, 10-12 weeks-old, weighing 300-550 g) were obtained from Ondokuz Mayis University, Experimental Animals Application and Research Center. During the study, 22±2 °C room temperature, 60% humidity, 12/12 h light/dark environment was provided. During the investigation, ad libitum feedings were implemented for the experimental animals. The University Experimental Animals Application and Research Center administered regular rat food to produce hypercholesterolemia. A commercial feed mill added 2% cholesterol to the normal feed, mixed it thoroughly, and then re-pelleted it. All groups, excluding the control group, received prepared cholesterol meal as needed during the experiment.

***Preparation of probiotic suspensions:*** For the preparation of *L. acidophilus* suspensions, the lyophilized bacteria were diluted with De Man, Rogosa and Sharpe (MRS) broth. To test the bacteria's viability and purity, they were inoculated onto MRS agar. One milliliter of the culture was added to 500 ml of MRS broth after its viability and purity were confirmed, and it was then incubated at 35°C for 48 h. At the end of the incubation period, the suspension was inoculated to three MRS agar and incubated at 35°C for 48 h. Following incubation, the quantity of bacteria in the primary culture was determined by counting the number of bacterial colonies. Based on the calculations, 1X phosphate buffer solution (PBS) was used to suspend $10^{10}$ cfu/ml of bacteria, and *L. acidophilus* probiotic suspension was utilized as a treatment (33).

***Experimental plan:*** In the research, 5 groups with 10 animals were formed.

Group 1 (control, C): It was fed with standard pellet rat food for 8 weeks.

Group 2 (hypercholesterolemia group, H): 2% cholesterol added and pelleted standard feed was administrated during 8 weeks for forming hypercholesterolemia (32).

Group 3 (hypercholesterolemia+atorvastatin, HA): 2% cholesterol added and pelleted standard feed was administrated during 8 weeks, and in the last 4 weeks of the trial atorvastatin (Ator, Sanovel) (20 mg/kg/day, dissolved in 0.5 ml drinking water) was administered by oral gavage for treatment purposes (4).

Group 4 (hypercholesterolemia+probiotic, HL): 2% cholesterol added and pelleted standard feed was administrated during 8 weeks, and *L. acidophilus* probiotic ($2x10^8$ cfu/ml) was given by oral gavage for therapy in the last 4 weeks of the trial (31, 33).

Group 5 (hypercholesterolemia+atorvastatin+ probiotic, HAL): 2% cholesterol added and pelleted standard feed was administrated during 8 weeks. For the last four weeks of the trial, the combination of *L. acidophilus* probiotic and atorvastatin was given as described in Group 3 and 4.

At the end of eight weeks, after the rats were weighed, 10% ketasol (0.8-1.3ml/kg) and 2% basilazine (2-5 mg/kg) were administered via intraperitoneally. The animals were decapitated after blood was drawn from their hearts. After coagulation of the blood samples, they were centrifuged at 1550 x g for 10 min and their serums were extracted. The serums were divided into aliquots, and stored at -20 °C until used in the analysis. Following the decapitation, liver and brain tissue samples were taken and the tissues were kept at -80 ºC until analyzes.

***Extraction from brain tissue samples for ELISA:*** Brain tissue samples were extracted for using in ELISA kits in accordance with the instructions provided in the kit. To accomplish this, tissues were rinsed in PBS (0.01 mol/L, pH 7.0–7.2) in ice and dried. Use of a scalpel was used to weigh the tissues and cut them into extremely little bits. In a large glass tube, tissue fragments were placed, and PBS containing a protease inhibitor (5 g/ml aprotinin, 1 mM EDTA) was added to dilute the sample to 10 mg tissue/ml. The ultrasonicator was used to sonicate the regenerated tissues five times for 30 sec each. Following ultrasonication, the suspension was centrifuged at 5000 x g for 5 min to get the supernatant. Nanodrop Spectrophotometer was used to measure the quantity of protein in the supernatants (Thermo, 2000). Before being employed in an ELISA assay, extracted brain tissue supernatants were kept at -80°C.

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***Determination of some biochemical parameters in sera:*** Total cholesterol, HDL, LDL, total protein, albumin, ALT, AST amounts in serum were measured by spectrophotometric method using an autoanalyzer device (BS-120 Vet, Mindray).

***Determination of Serotonin, Estradiol, Testosterone, Progesterone, Myelin Basic Protein (MBP), Oxidized Low-Density Lipoprotein (Ox-LDL) and Neuronal Nitric Oxide Synthetase (nNOS) levels in whole brain:*** Serotonin, estradiol, testosterone, progesterone, MBP, nNOS and Ox-LDL levels in whole brain tissue supernatants were quantified by using the rat specific ELISA kits (cat.no. CSB-E13985r CUSABIO; CSB-E05110r CUSABIO; CSB- E05097r CUSABIO; CSB-E07282r CUSABIO; CSB-E08284r CUSABIO; CSB-E07932r CUSABIO; SEA815Ra Cloud-Clone) according to the manufacturer's instructions. A microplate reader (Infinite F50, TECAN) was used for absorbance measurements. The determined $OD_{450}$ values were calculated with the Magellan Standard Tracker (V7-2) software.

***Determination of LDL-R, HMG-CoA Reductase, GAPDH genes by Reverse Transcriptase-PCR (RT-PCR):*** Brain and liver tissue were used to determine LDL-R, HMG-CoA reductase, and GAPDH genes as described previously (16).

Macherey-Nagel brand NucleoSpin® RNA (740955.50) extraction kit was used for RNA extraction from tissues. The extraction process was carried out according to the method reported by the manufacturer. The concentrations of the extracted RNAs were measured spectrophotometrically with a Nano-Drop spectrophotometer and all RNAs were diluted to an equal concentration of 2 mg/ml. The obtained RNAs were stored at -80°C in order not to deteriorate their structure until use.

The extracted RNAs were translated into cDNA using the cDNA synthesis kit (iScript cDNA synthesis kit, Bio-Rad, 170-8891). This process was carried out according to the method specified by the manufacturer. The obtained cDNAs were stored at -80 ºC to be used in the PCR.

The oligonucleotide sequences and expected band sizes of LDL receptor, HMG-CoA reductase, GAPDH genes were presented in Table 1. PCRs were performed with the methods reported by Park et al (33). For the evaluation of the results, the GAPDH gene was taken as a reference and LDL receptor and HMG-CoA reductase mRNA presences and levels were compared among the groups.

***Statistical analyzes:*** SPSS statistical software for Windows (SPSS-PC, SPSS Inc., Chicago, Illinois, USA) was used for statistical analyzes. The differences and relationships among the groups were examined using Pearson correlation tests, Duncan's multiple range analysis, and one-way analysis of variance (ANOVA).

## Results

***Serum biochemical parameter levels:*** The mean and standard deviation of serum total cholesterol, HDL, LDL, total protein, albumin, AST and ALT levels of C, H, HA, HL and HAL groups are presented in the Table 2 and Figure 1 (mean ± SE).

**Table 1.** Oligonucleotide primer sequences used in RT-PCR (16).

| Target gene | | Oligonucleotide primer sequences | Expected amplicon size (bp) |
|---|---|---|---|
| LDL | F | ATT TTG GAG GAT GAG AAG CAG | 931 |
| | R | CAG GGC GGG GAG GTG TGA GAA | |
| HMG | F | GCG TGC AAA GAC AAT CCT GGA G | 245 |
| | R | GTT AGA CCT TGA GAA CCC AAT G | |
| GAPDH | F | GCC ATC AAC GAC CCC TTC ATT | 702 |
| | R | CGC CTG CTT CAC CAC CTT CTT | |

**Table 2.** The mean ± standard deviation (SE) of total cholesterol, HDL, LDL, total protein, ALT, AST measured in serum (the mean ± standard deviation (SE).

| Parameter | Group | | | | | |
|---|---|---|---|---|---|---|
| | C | H | HA | HL | HAL | P |
| **TP (g/dl)** | 6.01±0.11[ab] | 6.4±0.27[b] | 5.84± 0.09[a] | 5.96±0.11[ab] | 6.2±0.22[ab] | 0,095 |
| **Alb (g/dl)** | 3.09±0.04 | 3.15±0.15 | 3.18±0.03 | 3.18±0.03 | 3.15±0.04 | 0,001 |
| **TC (mg/dl)** | 51.5± 1.93[a] | 77.66±1.72[b] | 61.77±0.9[c] | 55.2±1.26[a] | 52.88±1.13[a] | 0,071 |
| **LDL (mg/dl)** | 5.41±0.44[a] | 22.85±1.55[b] | 15.41±0.81[c] | 9.22±0.99[d] | 6.64±0.02[ad] | 0,316 |
| **HDL (mg/dl)** | 39.23±2.56[a] | 28.28±1.02[b] | 34.44±1.12[ab] | 36.92±0.8[a] | 33.35±1.45[ab] | 0,007 |
| **ALT (IU/l)** | 54.12±2.94[ab] | 65±4.16[b] | 43.68±3.36[a] | 45.53±4[a] | 41.57±7.22[a] | 0,072 |
| **AST (IU/l)** | 146.45±5.73[ab] | 181.53±11.11[b] | 149.98±12.11[a] | 152.29±7.25[a] | 140.74±10.58[a] | 0,27 |

[a, b, c, d]: The differences between the groups indicated with different letters on the same line are significant (P<0.05).

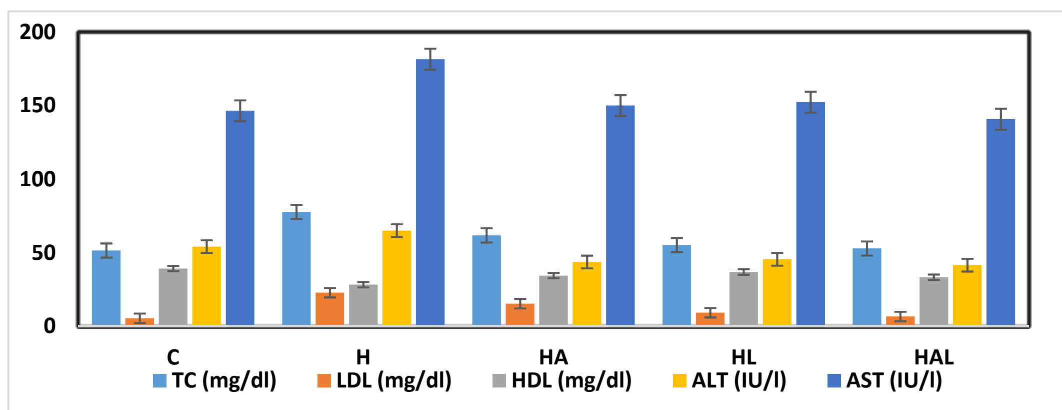


**Figure 1.** The results of serum biochemistry analyses.
TC: total cholesterol HDL: high density lipoprotein; LDL: low density lipoprotein; ALT: Alanin aminotransferaz; AST: Aspartat aminotransferaz.

**Table 3.** Correlation of total cholesterol, HDL, LDL, total protein, ALT, AST amounts between groups.

| | TP | Alb | HDL | LDL | TC | ALT | AST |
|---|---|---|---|---|---|---|---|
| **TP** | 1 | -0.063 | -0.102 | 0.015 | 0.177 | 0.280 | 0.159 |
| **Alb** | | 1 | 0.003 | 0.061 | 0.050 | -0.149 | 0.123 |
| **HDL** | | | 1 | -0.471** | -0.529** | -0.111 | -0.213 |
| **LDL** | | | | 1 | 0.812** | 0.376** | 0.398** |
| **TC** | | | | | 1 | 0.356* | 0.418** |
| **ALT** | | | | | | 1 | 0.346* |
| **AST** | | | | | | | 1 |

*(P<0.05), **(P<0.01).

***Serotonin, Estradiol, Testosterone, Progesterone, MBP, Ox-LDL and nNOS levels in whole brain:*** The amount of serotonin in the H group declined somewhat, began to climb significantly in the HA and HL groups (P>0.05), and increased significantly in the HAL group. The levels of estradiol in the H, HA, HL, and HAL groups were found to be higher than in the C group, and this difference was statistically significant (P<0.05). The testosterone level in the H group was found to be lower than in the C group, and this difference was significant. Progesterone level decreased in the H group compared to the C group, and this decrease was found to be significant (P<0.05). It was determined that the progesterone level increased in HA and HL groups compared to the H group, and this increase was significant (P<0.05). An increase was also found in the HAL group, but it was statistically insignificant (P>0.05). MBP level decreased in H group compared to C group and this decrease was evaluated as significant (P<0.05). A significant difference was determined between the HA and H groups (P>0.05). It was determined that the increase in the HAL group was higher than the HAL group and this was statistically significant (P<0.05). It was determined that the Ox-LDL level increased significantly in the H group compared to the C group, and this increase was significant (P<0.05). Ox-LDL levels were found to be significantly lower in HA, HL and HAL groups compared to C and H groups (P<0.05). The nNOS level was found to be lower in the H group than in the C group, and this decrease was found to be significant (P<0.05). It was determined that the nNOS level decreased significantly in the H and HA groups, and increased significantly in the HL and HAL groups (P<0.05).

***Correlation relations:*** There was a significant positive correlation between serotonin level and estradiol, testosterone, progesterone, MBP and nNOS levels (r=0.779**, r=0.922**, r=0.589**, r=0.618**, r=0.617**, respectively) and Ox-LDL, there was a significant negative correlation (r= -0.506**). A significant positive correlation was found between estradiol level and testosterone, MBP, nNOS (r=0.576**, r=0.629**, r=0.555**, respectively). Significantly positive correlation between testosterone level and progesterone, MBP, nNOS (r=0.684**, r=0.474**, r=0.455**, respectively), and significantly negative correlation with Ox-LDL (r= -0.515*) *) was determined. There was a significant negative correlation (r=-0.465**) between MBP and Ox-LDL. Significantly negative correlation (r=-0.784**) was determined between Ox-LDL and nNOS. The aggregated comparative evaluations of the ELISA results are presented in Tables 3 and 4.

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**Table 4.** Estradiol, testosterone, progesterone, serotonin, nNOS, Ox-LDL levels measured in whole brain tissue extraction (the mean ± standard deviation (SE).

| Parameter | Group | | | | | |
|---|---|---|---|---|---|---|
| | C | H | HA | HL | HAL | P |
| **Serotonin (ng/mg tissue)** | 7.5±0.24 [a] | 7.34±0.21[a] | 15.32±0.69[b] | 16.71±0.88[c] | 19.43±0.89[d] | 0.039 |
| **Estradiol (ng/mg tissue)** | 2.35±0.14[a] | 2.63±0.09[b] | 2.64±0.11[b] | 2.84±0.7[c] | 3.01±0.11[d] | 0.877 |
| **Testosterone (ng/mg tissue)** | 2.29±0.8[a] | 1.34±0.02[b] | 4.42±0.12[c] | 3.74±0.25[d] | 4.62±0.21[e] | 0.005 |
| **Progesterone (ng/mg tissue)** | 17.78±0.5[a] | 11.64±0.62[b] | 23.91±0.51[c] | 25.56±0.9[c] | 18.14±4.3[a] | 0.001 |
| **MBP (ng/mg tissue)** | 2.82±0.24[a] | 1.84±0.056[b] | 1.63±0.15[b] | 3.64±0.32[c] | 5.66±0.36[d] | 0.028 |
| **Ox-LDL (pg/mg tissue)** | 116.56±1.62[a] | 265.09±9.03[b] | 197±4.01[c] | 106.92±1.27[d] | 96.82±0.63[e] | 0.001 |
| **nNOS (ng/mg tissue)** | 1.53±0.11[a] | 1.34±0.05[b] | 1.34±0.03[b] | 1.79±0.09[c] | 1.88±0.13[d] | 0.260 |

[a, b, c , d, e]: Differences between groups, indicated by different letters on the same line, are significant. (P<0.05).

**Table 5.** Correlation of estradiol, testosterone, progesterone, serotonin, nNOS, Ox-LDL levels among the groups.

| | 5-HT | Estradiol | Testosterone | Progesterone | MBP | Ox-LDL | nNOS |
|---|---|---|---|---|---|---|---|
| **5-HT** | 1 | 0.779** | 0.922** | 0.589** | 0.618** | -0.506** | 0.617** |
| **Estradiol** | | 1 | 0.576** | 0.0229 | 0.629** | -0.253 | 0.555** |
| **Testosterone** | | | 1 | 0.684** | 0.474** | -0.515** | 0.455** |
| **Progesterone** | | | | 1 | 0.102 | -0.465** | 0.277 |
| **MBP** | | | | | 1 | -0.753** | 0.863** |
| **Ox-LDL** | | | | | | 1 | -0.784** |
| **nNOS** | | | | | | | 1 |

**0.001.

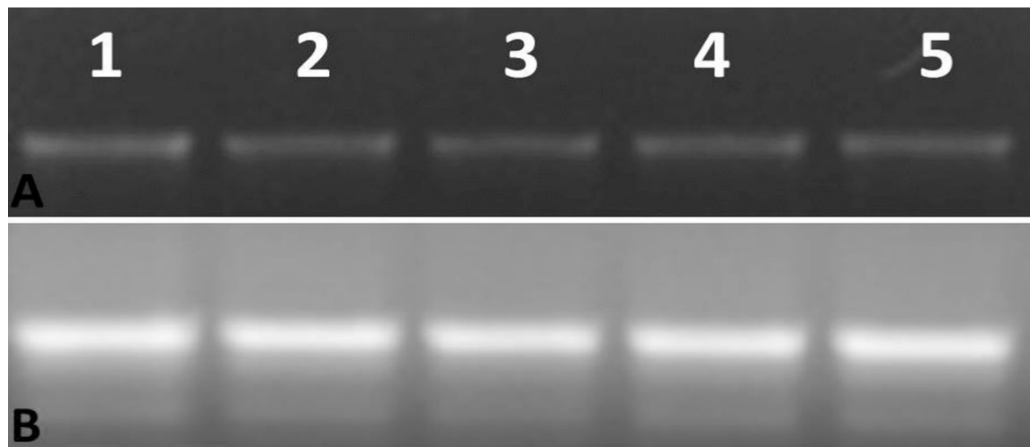


**Figure 2.** PCR results for the GAPDH gene.
A: brain, B: liver; 1: Group C, 2: Group H, 3: Group HA, 4: Group HL, 5: Group HAL.

***Determination of LDL receptor, HMG-CoA reductase, GAPDH by RT-PCR:*** RT-PCR was performed for determination of LDL receptor, HMG-CoA reductase, GAPDH genes. A 702 bp band was considered positive as a result of PCR performed for the determination of the GAPDH gene. The results obtained with the GAPDH gene were evaluated as the basic profile and the PCR results with other genes were evaluated by considering the intensity of the bands obtained as a result of the PCR for the GAPDH gene. As a result of PCR, it was determined that both brain and liver gave bands at the same intensities in all groups (Figure 2).

A 931 bp band was considered positive for PCR performed to determine the LDL receptor gene. Similar results were obtained as a result of PCR with RNAs extracted from brain and liver. Accordingly, as a result of PCR performed with RNAs from both organ extracts, band intensities were found to be less than that of the GAPDH gene. When the intensities between the groups were compared, the most intense band was determined in the C group. Band intensities were found to decrease as H, HA, HL, and HAL, respectively (Figures 3 and 4).

The presence of a 245 bp band was accepted as positive for the PCR performed to determine the HMG-

CoA reductase gene. Similar results were obtained as a result of PCR with RNAs extracted from brain and liver. Accordingly, as a result of PCR performed with RNAs from both organ extracts, band intensities were found to be less than that of the GAPDH gene. When the intensities between the groups were compared, the most intense band was determined in the HAL group. In the other groups, band intensities were found to decrease as HA, HL, C, and H, respectively (Figures 5 and 6).

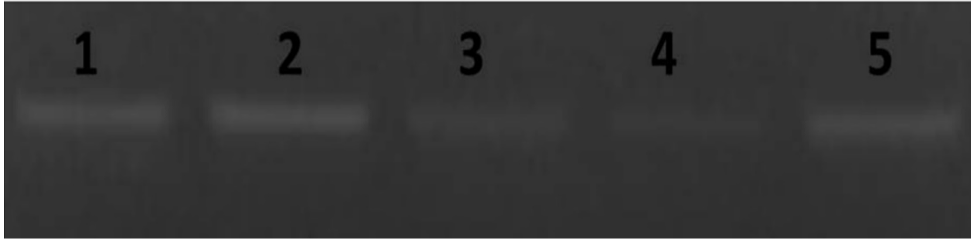


**Figure 3.** PCR results for LDL receptor gene in brain RNA extracts.
1: Group HA, 2: Group HL, 3: Group C, 4: Group H, 5: Group HAL.

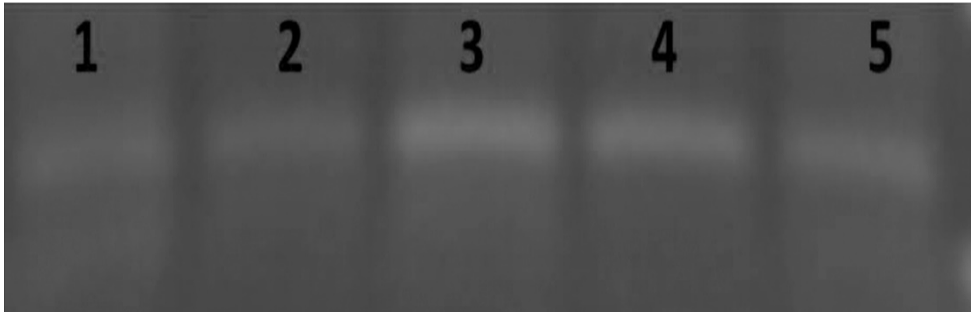


**Figure 4.** PCR results for LDL receptor gene in liver RNA extracts.
1: Group HA, 2: Group HL, 3: Group C, 4: Group H, 5: Group HAL.

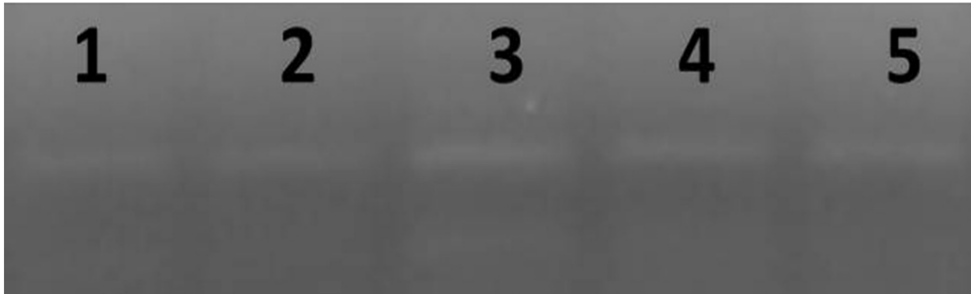


**Figure 5.** PCR results for HMG-CoA reductase gene in brain RNA extracts.
1: Group H, 2: Group C, 3: Group HL, 4: Group HA, 5: Group HAL.

**Figure 6.** PCR results for HMG-CoA reductase gene in liver RNA extracts.
1: Group H, 2: Group C, 3: Group HL, 4: Group HA, 5: Group HAL.

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## Discussion and Conclusion

Cholesterol homeostasis depends on cholesterol biosynthesis, removal of cholesterol from the circulation, dietary cholesterol intake, and excretion of cholesterol through feces and bile. Nutrition plays an important role in the control of cholesterol homeostasis. Cholesterol is essential for the body (an important component of the cell membrane, synthesis of bile acids), absorption of fats and fat-soluble vitamins, precursor in the synthesis of steroid hormones. Eating a high-cholesterol diet is the cause of hypercholesterolemia and cholesterol is also an undesirable culprit, leading to many conditions such as arteriosclerosis and ischemic heart disease, abnormal lipid oxidation or metabolism (32). Cholesterol levels increases in erythrocytes and endothelial cells as well as in serum in hypercholestemia. It has been reported that high cholesterol levels because an increase in oxidized free radical products in these cells (35). Hypercholesterolemia is a common health problem that concerns many people today.

The oxidation of lipids and the increase of reactive oxygen species (ROS), including superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$), peroxynitrite ($ONOO^-$), cause an increase in the amount of Ox-LDL in the cell. Oxidized LDL contributes to endothelial dysfunction by inhibiting nitric oxide (NO) production and also induces proatherogenic genes such as endothelial–leukocyte adhesion molecules and smooth muscle growth factors (37). In our study, total cholesterol and LDL levels in serum and Ox-LDL levels measured in brain tissue extract increased significantly ($P<0.05$), while HDL levels decreased ($P<0.05$) in the hypercholestemic group. It was determined that the hypocholestemic effect was higher in the group given atorvastatin and *L. acidophilus* together with cholesterol feed (HAL) compared to the other groups ($P<0.05$). Anderson et al. (3) found that when *L. acidophilus* contained fermented milk was used for 3 weeks to people with hypercholesteremia, the cholesterol level in the serum decreases by 1% and the risk of coronary heart disease is reduced by 3%. They reported a decrease of 6-10%. Agerbaek et al. (1) detected a significant decrease (3.5%) in serum cholesterol in the consumption of fermented milk containing *Enterococcus faecium* and *Streptococcus thermophilus* probiotics for 3 weeks. Schaafsma (39) reported that when *L. acidophilus* containing yogurt was administreted for 3 weeks, serum cholesterol level decreased by 4.5%. Gilliland and Walker (14) suggested that the bacterial metabolism of *L. acidophilus* contributes to the hypocholestemic effects of cholesterol and bile acids. Harrison and Peat (19) notified the addition of live cultures of *L. acidophilus* to the human intestine may produce a hypocholesterial effect. In the other studies, it was reported that total cholesterol and LDL levels increased significantly, while HDL cholesterol levels decreased in the group that added 1% cholesterol and 0.5% cholic acid to standard rat feed in parallel with our study (2, 18). Administrating the *Cleome arabica* extract (17), lycopene (38), 1% propolis with 1% cholesterol (2), Arginase enzyme (18), *Coptidis rhizome* (38) added groups, it was stated that the cholesterol level decreased and they had a hypocholestemic effect. The activity of the liver enzymes AST and ALT increased in the hypercholestemia group, while liver enzyme activity neared that of the control group in the group administered *L. acidophilus* probiotic and atorvastatin according to our findings (HAL). Hepatic enzymes in hypercholestemic groups were studied concurrently with ours. When lycopene was used, liver enzyme activity neared that of the controland polymethoxylate extract groups (34). In our study, it was indicated that liver enzyme levels were increased in the hypercholestemia group, and AST and ALT enzyme activity was decreased in the group given atorvastatin and *L. acidophilus* together with cholesterol feed for treatment, and these results suggested that it may have a potential protective effect against liver damage.

Cholesterol synthesis inhibitors (CSIs) are known as statins and are used to reduce LDL cholesterol levels (4). Statins, whose main effects are the inhibition of the rate-limiting enzyme HMG-CoA reductase, are also a pleotropic drug with many clinical efficacies (34, 40). Widespread use of statins not only affects CNS cholesterol metabolism, but also has important effects on CNS morphology and neuron functions (40). While *in vitro* studies conducted to better understand the effects of statin use on brain cholesterol metabolism and Alzheimer's disease have found positive associations, there are still contradictions in *in vivo* studies. Statins have positive effects on NO synthesis and endothelial function as well as lowering the lipid level in serum. This effect is caused by the phosphorylation of NO products and endothelial nitric oxide (eNO), resulting in an anti-angiogenic effect. Although the benefits of statins which are cholesterol-lowering drugs, are important on the heart, it has been reported that the progressive effect of the use of statins or the low or decreased cholesterol level may be associated with disorders in cerebral serotonin metabolism, and the drug should be discontinued in psychiatric diseases when there is a change in consciousness, emotion and behavior (aggression and violence).In a study, it was notified that the antidepressant property of atorvastatin is associated with the serotonergic system, and it can be used in alternative treatment by reducing the dose of antidepressant medication (27). In our study, the level of serotonin hormone fell slightly ($P>0.05$) in the hypercholestemic diet (H) group compared to the control group, but increased in the groups given solely

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atorvastatin (HA) and only *L. acidophilus* (HL). This rise was shown to be statistically more significant in the group given the *L. acidophilus* probiotic in combination (HAL) ($P<0.05$). Depression and mental illnesses can be caused by low serotonin hormone levels. In our study, it was concluded that co-administration of atorvastatin (HA) and *L. acidophilus* probiotic together with the aim of treating hypercholesemia had a positive effect as an antidepressant by increasing the serotonin hormone level better than giving it separately.

Nitric oxide is a vasodilator produced from L-arginine and plays an important role in regulating the functions of the cardiovascular, nervous and immune systems. There are three isoforms of NO. These are neuronal nitric oxide synthetase (nNOS), endothelial nitric oxide synthetase (eNOS), and inducible nitric oxide synthetase (iNOS). nNOS is intensely synthesized in the cerebellum, and it has been reported that nitric oxide produced plays a neurotransmitter role and causes neurotoxicity due to excessive stimulation of neurons by glutamate (9). It has been reported that nitric oxide products are decreased in response to oxidative stress, one of these complications associated with dyslipidemia (20). Hypercholestemia was increased the NO degradation by causing an increase in oxygen-derived free radicals in the endothelium (29). nNOS is specific for central and peripheral nervous system cells. It was informed that nNOS activity in the brains of patients with schizophrenia and depression was significantly lower than in the control group (5). In our study, nNOS levels decreased in the hypercholestemic diet (H) group compared to the control group, but increased in the hypercholestemic diet with *L. acidophilus* (HL) group, and this increase was statistically more significant in the group given *L. acidophilus* probiotic along with atorvastatin (HAL). It was concluded that taking atorvastatin and a probiotic like *L. acidophilus* at the same time can help prevent oxidative damage in hypercholesterolemia and neurological diseases by increasing vascular blood flow in the central nervous system and removing unwanted radicals and amyloid deposits from the environment.

Cholesterol is an integral part of myelin. The myelin layer consists oligodendrocytes and schwan cells. Oligodendrocytes surround neurons in the peripheral and central nervous system. Myelin layers are compact monolayers of Schwann cells and oligodendrocytes that form around the axons of neurons in the peripheral and central nervous system (CNS), respectively. It has been stated that demyelination and consequent deterioration of signal transmission can lead to severe neurological disorders and multiple sclerosis (MS) by causing focal deposition of cholesteryl esters in the brain and disruption of myelin structure. Some steroids are synthesized in the nervous system independently of peripheral endocrine glands and are named as 'neurosteroids'. The synthesis of neurosteroids in the nervous system starts from cholesterol. Experimental animal studies show that steroid synthesis in the CNS affects many functions of the brain and regulates protein synthesis through direct transcriptional changes via intracellular receptors (5). Neurosteroids were identified for the first time in male rat brain and reported as DHEA, pregnenolone and their sulfate esters (6). Other steroid hormones (estradiol and testosterone) were also detected in the brain of adult mammals (7). When neurosteroids are given to animals, they mostly show anticonvulsant, myorelaxant, anesthetic and anxiolytic effects (12). Estrogen, progestin, androgens, progesterone and dehydroepiandrosterone affect neuron function and play an important role in the nervous system, especially in aging (24). The levels of testosterone and progesterone hormones in brain tissue were found to be substantially lower ($P<0.05$) in the hypercholestemic diet group (H) compared to the control group in our study. The group given only atorvastatin (HA) and alone *L. acidophilus* (HL) with a hypercholestemic diet had a rise in testosterone levels, and this increase was statistically more significant in the group given atorvastatin and *L. acidophilus* probiotic combination ($P<0.05$). It was determined that the level of progesterone hormone in the brain decreased in the H group compared to the control group, and increased in the group treated with Atorvastatin (HA) and *L. acidophilus* (HL), this increase was greater than when given together (HAL).

Estradiol is produced by crossing the blood-brain barrier and endogenously from cholesterol in the brain. In addition to have an important role in the physiological and reproductive system, estradiol has been shown to be an important signaling molecule in the brain in recent studies. Estrogen has been shown to have beneficial effects on the entire bioenergetic system of the brain for the transport of glucose into the cell, glycolysis, tricarboxylic citric acid cycle, oxidative phosphorylation and ATP production (7). *In vitro* studies have shown that estrogen protects against DNA damage induced by hydrogen peroxide and arachidonic acid (28). In our study, estradiol level increased slightly in rats fed a hypercholestemic diet compared to the control group, and there was a significant increase in the group given only atorvastatin (HA) and only *L. acidophilus* (HL), and this increase was statistically significant in the group given atorvastatin and *L. acidophilus* probiotic together (HAL) was determined to be more important ($P<0.05$). It has been reported that estrogen in the central nervous system protects against the accumulation of beta amyloid proteins in neurons and increases glucose utilization by increasing cerebral blood

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flow (36). These results suggested that the combined use of atorvastatin and *L. acidophilus* in hypercholestemic patients may be effective in reducing neurological diseases such as Alzheimer's diseases. It has been notified that when the total cholesterol and LDL cholesterol increase in serum, LDL-R protein expression decreases when fed with a high-cholesterol diet (26). It has also been informed that a high-fat and cholesterol diet reduces LDL-R mRNA in the liver. It has been stated that there is a relationship between LDL receptor activity and the amount of LDL receptor protein, and that lack of LDL-R in rats may contribute to the increase in serum total cholesterol and LDL cholesterol (21). In most of the studies in the literature, it was reported that the use of lipodemic drugs affected LDLr expression (13). In another study with a hypercholestemic rat model, LDL-receptor was examined and liver LDL-R protein expression was found to be significantly decreased in the high cholesterol group compared to the other groups (22). Lactic acid bacteria are known to lower the cholesterol by increasing the excretion of bile acids. It has been reported that the reason why some *Lactobacillus* species lower cholesterol may be due to their collapse by not being able to bind with bile salts (25). Bile salt hydrolase is the enzyme that converts free bile acids into deconjugated form. It has been reported that the loss of bile salts with feces can reduce its level by using cholesterol for the formation of new bile salts (41). In a study, it was reported that after adding *Lactobacillus plantarum* to the control group, liver LDL receptor expression was affected and increased compared to the control group (23). In our study, the most intense band was determined in the HL group as a result of RT-PCR performed to determine the presence and level of LDLr mRNA. In the other groups, band intensities were found to be decreased as HAL, HA, C and H, respectively. The result obtained is in parallel with the studies in the literature, and it was determined that LDLr mRNA was at a higher level in rats fed with drugs and probiotic supplements used to reduce cholesterol levels. Goldstein and Brown (15) stated in their study using the northern blot method that when cholesterol is high, the amount of LDLr mRNA decreases and LDLr expression is regulated by negative recycling of cholesterol. As a result of the increase in cholesterol concentration in the cell, LDL cholesterol is released into the plasma, and as a result, it has been reported that LDLr and mRNA activity decreased in animals fed a high cholesterol diet (11). However; studies with different results are also available in the literature. In a study, it was notified that administration of Polysaccharide from fuzi for treatment in hypercholestemic rats had a reducing effect on LDL-receptor expression in the liver (22). HMG-CoA reductase is a restriction of cholesterol biosynthesis and is a peroxisomal enzyme bound to the endoplasmic reticulum. While this enzyme is expressed in all tissues, its expression level is higher in the liver. It plays a central role in cholesterol synthesis and regulation of cholesterol level in plasma. Ness et al. (30) reported that HMG-CoA reductase was affected very little as a result of feeding with a cholesterol diet, and the addition of cholesterol-lowering substance to the diet increased 15-20 times in both HMG-CoA reductase mRNA and activities. HMG-CoA reductase also has a limiting effect on the mevalonate pathway, which is an important pathway of cholesterol synthesis. In the literature, it has been reported that the inhibition of this enzyme in the liver with statins causes a decrease in cholesterol synthesis, an increase in LDL-R synthesis, and therefore a decrease in LDL and cholesterol levels in the circulation (8). The finding in rats fed a high-cholesterol diet differed from a previous report suggesting that increased cholesterol absorption associated with a high-fat diet resulted in increased free cholesterol levels in hepatocytes and consequent inhibition of HMG-CoA reductase expression. In a study investigating HMG-CoA reductase in a hypercholestemic rat model, HMG-CoA reductase mRNA expression was found to be significantly higher in the high-cholesterol group than in the control group and those treated with FPS (22). Lactic acid bacteria are known to lower cholesterol by increasing the excretion of bile acids. It has been reported that the loss of bile salts with feces can reduce its level by using cholesterol for the formation of new bile salts (41). In a study, it was notified that after the addition of *L. plantarum* to the control group, liver HMG-CoA reductase expression was affected and increased compared to the control group (23). In our study, the most intense band was determined in the HAL group as a result of RT-PCR performed to determine the presence and level of HMG-CoA reductase mRNA. In the other groups, band intensities were found to be decreased as HA, HL, C and H, respectively. As a result, it was determined that HMG-CoA reductase mRNA was higher in rats fed with drugs and probiotic supplements used to reduce cholesterol levels.

In conclusion, in the case of adding 2% cholesterol to the diet, total cholesterol in the serum, LDL-cholesterol level increases, HLD-cholesterol level decreases, liver enzyme activity increases, Ox-LDL level increases significantly in the brain, testosterone, progesterone, proteins in the myelin sheath. The important thing is that the level of MBP, which is expressed as the manager of myelin proteins, and nNOS activity decrease significantly, which may lead to the formation of neurological diseases in the brain. By lowering cholesterol levels better (hypocholestemic) than those in the given group, neurosteroid levels, which have a protective role in the brain, and by increasing the level of the hormone

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Serotonin, which has antidepressant properties by increasing its level, neurological diseases shaped by high cholesterol levels in the brain (for example, Alzheimer's) and learning and memory disorders shaped by myelin regulation. It is thought that it can be used in preventive treatment in cases and will shed light on further studies to be done.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

## Author Contributions

GÇ and AÇ conceived and planned the experiments. AÇ and TG contributed to bacterial sample preparation. GÇ, MÇ, BO, and SÇ carried out the experiments. GÇ and AÇ contributed to the interpretation of the results, and took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

## Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

## Ethical statement

The study was approved by the Animal Experiments Local Ethics Committee of Ondokuz Mayis University (Approval no: 2016/08).

## Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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# The beginning of veterinary surgery education in Türkiye and the historical development of this discipline in Ankara University Faculty of Veterinary Medicine

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ABSTRACT

Research on the history of the fields of science has special meanings and importance in the history of veterinary medicine. Various studies are carried out on this subject, both at the level of scientific articles and at the level of large-scale doctorate. In this article, the beginning of the veterinary surgery field in Türkiye and its historical development in Ankara University Faculty of Veterinary Medicine, which is the oldest faculty, are discussed. The main material of the study was the first-hand archival resources provided by the Rectorate, Dean's Office and Departments of Ankara University. Various books and journals related to the subject were also used. The data obtained were evaluated within the framework of the historical methodology and were written down chronologically. The processes of the modern surgery education, which started with the Prussian Veterinarian Godlewsky in the Ottoman period, continued within the Higher Agricultural Institute and later Ankara University in the post-Republican period, the changes in the structure of the department with the changes in the university laws, the educational status, the academic stages and the number of publications until April 2022 of faculty members have been determined. The data obtained can shed light on the evaluations to be made in this field. As a result, it has been tried to present a well-coordinated source on the history of the field. The inclusion of detailed data on the surgical units of all veterinary faculties in Türkiye would enable the holistic determination of the quality of surgery in veterinary medicine.

## Introduction

***Military and Civil Veterinary Schools Period:*** Veterinary medicine education in the Ottoman period started with the Prussian Veterinarian Godlewsky, who was appointed in the establishment of the Military Veterinary School in 1842. Godlewsky reported that the surgical courses, which were very important due to the students' devotion to empirical methods and their religious beliefs, could not be done very well (18). Veterinary classes were included in the Military School, where *serîriyyât* (clinic) courses were given in the 3rd and 4th grades by Dubroca, who was brought from France, and later by Lewis (1858). Since 1873, Hüseyin Hüsnü has taught "*fenn-i cerrâhiyye-i baytarî*" (veterinary surgery science) and Dezutter, who was brought from Belgium in 1884, was given the responsibility of clinical lessons. Surgery courses at the Military Veterinary School were given by Yusuf Ziya in 1887 under the name of "*ameliyyât-ı cerrâhiyye*" (surgical operations), and Necip Rıza Zobo served as his assistant. The courses named "*emrâz-ı hâriciyye*" (external diseases) and "*ameliyyât-ı cerrâhiyye*" were given by Hayreddin (1895) (6, 23, 27).

In the Civil Veterinary School (1889), the four-year curriculum was prepared taking into account the curriculum of the Alfort Veterinary School, and a veterinarian named Martel was brought from the Alfort Veterinary School for the clinical courses given in the 3rd and 4th grades (6, 14). Yusuf Ziya between 1889 and 1921 and Mehmet Ali between 1889 and 1897 took the courses on *"emrâzı-ı hâriciyye ve ameliyyât-ı cerrâhiyye"*

(external diseases and surgical operations). One year after the Civil Veterinary School was established, students began to be sent to Europe (9). Returning from France after completing his specialization, Abdullah was given "*emrâz-ı hâriciyye*" and "*fenn-i nalbandi*" (farrier science) courses, and he continued this duty until 1913. Hayreddin, who was appointed as the assistant of Abdullah, taught "*fenn-i nalbandi*" courses between 1906-1909 (14).

In 1909, 10 veterinarians were sent to Europe for specialization training at the Civil Veterinary School. Salih Zeki (Berker), who is among these veterinarians and received specialization training in the field of surgery at Alfort Veterinary School, returned to his homeland in 1911 and started to teach surgery courses (23, 27). In Higher Veterinary School (1921) which was established with the merging of Military and Civil Veterinary Schools, the *"ameliyyât-ı cerrâhiyye"* courses were given by Mustafa Santur and Yusuf Ziya, and the *"fenn-i vilâde"* (obstetrics) courses, which were presented within the surgery courses, were taught by Salih Zeki (Berker) since 1911 (6, 14). Many books[1] on veterinary surgery were written by these scholars during this period. Dinçer et al. (10) stated that the first copyrighted work that can be accessed from the 19th century is "*İlm-i Emrâz-ı Hâriciyye yâhud Cerrâhiyye*" (Science of External Diseases or Surgery) written by the Civil Veterinary Inspector Mehmed Ali in 1893 and "*Emrâz-ı Cerrâhiyye-i Baytâriyye*" (Veterinary Surgical Diseases) (1902) of Hayreddin for the 20$^{th}$ century.

With the establishment of the Turkish Republic in 1923, it was planned to focus on education among the prominent development plans of the new Türkiye, and veterinarians were sent to Europe for specialization with the principle that veterinary medicine and agriculture are the cornerstones of the country's economy (27). When these veterinarians returned the country, they were appointed as chief assistants at the Higher Veterinary School in Istanbul and started to apply modern clinical and laboratory methods. In this context, M. Tevfik (Başer) was sent to France Alfort Veterinary Faculty for his doctorate education in 1925. After three years of doctorate education, he returned the country and joined the surgical staff of the Higher Veterinary School[2]. Burhanettin (Öktem) started to work as a surgical assistant at the Istanbul Higher Veterinary School in 1933[3].

Various research is carried out on the historical development of the departments, which have special meanings and importance in terms of the history of veterinary medicine. Küçükaslan and Yiğit (21) reported the tags of the articles published on this subject in the bibliography they prepared. In this study, it is aimed to contribute to the "history of science fields" archive by considering the beginning of veterinary surgery education in Türkiye and its historical development in Ankara University Faculty of Veterinary Medicine (AUFVM).

## Materials and Methods

The material of the study consisted of the original documents in the Archives of Ankara University (AU) Rectorate and AUFVM Dean's Office, as well as the documents and photographs obtained from the Personnel Files and Biography Archive of History of Veterinary Medicine and Deontology Department. Archive files, books, journals and the internet database of the Council of Higher Education (7) were used for the data of academicians' publication numbers. The data obtained were evaluated within the methodology of history and transferred to the text in chronological order. Necessary institutional permissions were obtained to carry out the study.

## Results

***Higher Agricultural Institute Period:*** With the establishment of the Higher Agricultural Institute (HAI)[4] in Ankara in 1933, the Higher Veterinary School in Istanbul was transferred here as a "faculty" with all its staff and equipment and the school in Istanbul was closed (4, 26). *"Serîriyyât-ı Cerrahiye Enstitüsü"* (Institute of Surgery Clinic) was included in the eight "institutes" formed in the veterinary faculty. In the same year, Prof. Max Gebhardt became the director of the Institute, Salih Zeki (Berker) became associate professor[5], M. Tevfik (Başer) was appointed as the chief of the surgical branch[2] and Burhanettin (Öktem) was appointed as the surgical assistant[3]. After the departure of Max Gebhardt, he was replaced by Prof. Ludwig Lutz who was appointed as the Director of the Institute and continued his duty until 1937 (11). Bekir Sıtkı Artun, who completed his doctorate in Germany, was included in the staff of the school in 1934[6]. The courses taught in the field of surgery within the faculty which four years (eight semesters) curriculum was

[1] Mehmed Ali, İlm-i Emrâz-ı Hâriciyye Yâhud Cerrâhiyye, 1893; Yusuf Ziya, Ameliyyât-ı Cerrâhiyye-i Feres, 1898; Hayreddin Arif, Emrâz-ı Cerrâhiyye-i Baytarîyye, 1902; Yusuf Ziya, Ameliyyat-ı Cerrâhiyye-i Baytarîyye 1904; Yusuf Ziya, Fenn-i Eşkâl-i Hâriciyye-i Hayvânât-ı Ehliyye, 1909; Yusuf Ziya, Ameliyyât-ı Cerrâhiyye-i Baytarîyye, 1917; Mustafa Santur, Emrâz-ı Cerrâhiyye, 1917; Necip Rıza, Muhtasar Emrâz-ı Ayniyye-i Baytarîyye, 1920; Necip Rıza, Baytarî Emrâz-ı Cerrâhiyye-i Umûmiyye, 1925; Necip Rıza, Baytarî Emrâz-ı Cerrâhiyye-i Husûsiyye, 1925; Salih Zeki, Fenn-i Vilâde-i Baytari,1928; Salih Zeki, Gebe Hayvanlara Henüz Doğurmuş Olanlara ve Doğan Yavrulara Yapılması Lazım Gelen Tedbirler, 1928; Salih Zeki, Ehli Hayvanların Cerrahi Hastalıkları, 1931; Salih Zeki, Ehli Hayvanların Cerrahi Hastalıkları, 1932.

[2] Personnel File of Prof. M. Tevfik Başer, AUFVM Deanery Archive.

[3] Personnel File of Prof. Burhanettin Öktem, AUFVM Biography Archive of Veterinary History and Deontology Department. No: 563.

[4] Law No. 2291, Ankara HAI Law, No. 2432 Official Gazette dated 20 June 1933.

[5] Faculty members holding the title of "mudarris" in the Higher Veterinary School were given the title of "associate professor" at HAI without having a doctorate.

[6] Personnel File of Prof. Bekir Sıtkı Artun, AUFVM Deanery Archive.

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organized considering the programs implemented in Germany, and the staff is presented in Table 1 (2, 3, 11).

The first doctoral thesis in surgery was completed by Burhanettin Öktem in 1937 (24) in accordance with the "Doctoral Regulation"[7] issued at HAI in 1934. Afterward, Hüseyin Erk[8] in 1946 and Selim Tolkun[9] in 1947 completed their doctorate in this unit (19, 20, 28). The academic stages and the number of publications of the faculty members working at the HAI Veterinary Faculty Surgery Institute are presented in Table 2.

At HAI, activities of the unit continued with an examination room for small and large animals prepared in accordance with the needs of the field, an x-ray room, a classroom with the capacity of 50 students in which large animal operations are performed, instrument, medicine and sterilization rooms, outbuildings, five study rooms, and forty big animal boxes (22) (Fig. 1, Fig. 2). HAI Veterinary Faculty was affiliated to AU (1946) in 1948[10] which was established with the first University Law[11] of the Republic of Türkiye.

**Table 1.** Courses Taught in the Field of Veterinary Surgery at the Higher Agricultural Institute (1933-1934).

| Course Name | Term (4 years / 8 terms) | Lecturer |
|---|---|---|
| Clinical Diagnosis of External Diseases<br>Eye Diseases | 4 | Prof. Ludwig Lutz<br>Translator: Bekir Sıtkı Artun |
| Operation Knowledge | 5, 6, 7 | Branch Chief Tevfik Başer |
| Surgical Instruments Knowledge | 5 | Brach Chief Tevfik Başer |
| General Surgery | 5, 6 | Assoc. Prof. Salih Zeki Berker |
| External Diseases Clinic | 5, 6 | Prof. Ludwig Lutz<br>Assoc. Prof. Salih Zeki Berker<br>Branch Chief Tevfik Başer |
| Farriery and Foot Diseases | 6, 7 | Prof. Ludwig Lutz<br>Translator: Bekir Sıtkı Artun |
| Special Surgery | 7 | Assoc. Prof. Salih Zeki Berker |
| Reproduction Knowledge | 7 | Assoc. Prof. Salih Zeki Berker |

**Figure 1.** Views from the clinics of the Higher Agricultural Institute Veterinary Faculty (1933).

**Figure 2.** Views of surgical practices at the Higher Agricultural Institute and Ankara University Faculty of Veterinary Medicine (1930s, 1950s).

[7] Ankara HAI Doctoral Regulation. No. 2832 Official Gazette dated 18 October 1934.
[8] Hüseyin Erk's doctoral thesis is the first doctoral thesis written in the field of obstetrics and gynecology in Türkiye (18).
[9] Selim Tolkun's doctoral thesis is the first doctoral thesis written in the field of radiology in Türkiye (19, 28).
[10] Additional Law to the Universities Law No. 5234, No. 6951 Official Gazette dated 7 July 1948.
[11] Law No. 4936 Universities Law, No. 6336 Official Gazette dated 18 June 1946.

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**Table 2.** The Academic Stages and The Number of Publications of The Faculty Members[12] (1933-April 2022).

| Name-Surname | Title | Place of Birth/Year | Graduation/Year | Dr. | Assoc. Prof. | Prof. | Number of Publications | Current State |
|---|---|---|---|---|---|---|---|---|
| Salih Zeki Berker | Ord. Prof. | Trabzon/1884 | Civil Veterinary School/1908 | - | 1933 | 1936 | 26 articles, 13 books and brochures | Retired (1956) |
| Mehmet Tevfik Başer | Prof. | İzmir/1893 | Higher Civil Veterinary School/1920 | 1926 | 1937 | 1941 | 5 articles, 2 books and brochures | Retired (1958) |
| Burhanettin Öktem | Prof. | İstanbul/1904 | Higher Veterinary School/1927 | 1937 | 1943 | 1947 | 19 articles, 12 books and brochures | Retired (1974) |
| Bekir Sıtkı Artun | Prof. | İstanbul/1906 | Berlin Veterinary High School/1934 | 1934 | 1943 | 1947 | 8 articles, 7 books and brochures | Retired (1975) |
| Hüseyin Erk | Prof. | Kıbrıs/1914 | HAI/1937 | 1946 | 1950 | 1958 | 22 articles, 7 books and brochures | Left (1952)[13] |
| Selim Tolkun | Prof. | Bursa/1916 | HAI/1939 | 1947 | 1953 | 1968 | 19 articles, 3 books and brochures | Retired (1975) |
| Mustafa Temizer | Prof. | Elazığ/1923 | AUFVM/1947 | 1955 | 1961 | 1967 | 29 articles, 7 books and brochures | Left (1971)[14] |
| Hayrettin Anteplioğlu | Prof. | Van/1924 | AUFVM /1948 | 1955 | 1961 | 1967 | 43 articles, 7 books and brochures | Retired (1988) |
| Kadri Akın Finci | Assoc. Prof. | İstanbul/1931 | AUFVM /1957 | 1961 | 1968 | - | 10 articles, 2 books | Left (1973)[15] |
| Erdoğan Samsar | Prof. | Tokat/1935 | AUFVM /1958 | 1965 | 1972 | 1979 | 18 articles, 2 books and brochures | Retired (2002) |
| Nail Küçüker | Assoc. Prof. | Amasya/1928 | AUFVM /1954 | 1966 | 1972 | - | 4 articles, 1 book | Left (1974)[16] |
| Mehmet Ali Öztürk Tekeli | Prof. | Ankara/1935 | AUFVM /1959 | 1966 | 1971 | 1978 | 4 articles, 1 book | Retired (1991) |
| Mehmet Doğan Aslanbey | Prof. | Kütahya/1933 | AUFVM /1959 | 1966 | 1971 | 1978 | 6 articles, 1 book | Retired (2000) |
| Faruk Akın | Prof. | Bulgaristan/1938 | AUFVM /1962 | 1968 | 1974 | 1979 | 4 articles, 3 books | Retired (2005) |
| Necdet Güzel | Prof. | İzmir/1944 | AUFVM /1967 | 1975 | 1981 | 1988 | 15 articles,4 books | Left (1992)[17] |
| Arkun Candaş | Prof. | Çankırı/1942 | AUFVM /1969 | 1976 | 1981 | 1988 | 14 articles | Retired (2009) |
| Burhanettin Olcay | Prof. | Afyon/1945 | AUFVM /1971 | 1978 | 1989 | 1997 | 33 articles, 2 books | Retired (2012) |
| Bahattin Koç | Prof. | Eskişehir/1951 | AUFVM /1974 | 1979 | 1989 | 1997 | 42 articles, 3 books | Retired (2018) |
| Mehmet Sağlam | Assoc. Prof. | Manisa/1955 | AUFVM /1979 | 1987 | 2019 | - | 71 articles, 1 book | Left (2019)[18] |
| Zeki Alkan | Prof. | Balıkesir/1955 | AUFVM /1980 | 1987 | 1989 | 1997 | 21 articles, 1 book | Retired (2011) |
| Perran Gökçe | Prof. | Ankara/1962 | AUFVM /1984 | 1989 | 1995 | 2001 | 30 articles | Retired (2006) |
| Fatma Eser Özgencil | Prof. | Ankara/1961 | FUFVM /1985 | 1991 | 1999 | 2004 | 27 articles | Retired (2008) |
| Ümit Kaya | Prof. | Ankara/1965 | AUFVM /1988 | 1994 | 2001 | 2009 | 47 articles, 2 books | Working |
| Hasan Bilgili | Prof. | Ankara/1966 | AUFVM /1995 | 1995 | 2000 | 2006 | 96 articles, 5 book chapters | Retired (2019) |
| Ali Bumin | Prof. | Kırşehir/1964 | AUFVM /1987 | 1996 | 2002 | 2008 | 50 articles, 7 book chapters | Working |
| Ömer Beşaltı | Prof. | Urfa/1965 | AUFVM /1991 | 1997 | 2002 | 2008 | 79 articles, 1 book, 2 book chapters | Working |
| Oytun Okan Şenel | Assoc. Prof. | Ankara/1975 | AUFVM /1999 | 2004 | 2016 | - | 39 articles, 3 books, 1 book chapters | Working |
| İrem Gül Sancak | Assoc. Prof. | Ankara/1975 | AUFVM /1999 | 2010 | 2015 | - | 20 articles, 1 book, 4 book chapters | Left (2020)[19] |
| İrem Ergin | Assoc. Prof. | Ankara/1980 | AUFVM /2003 | 2010 | 2019 | - | 40 articles, 1 book,1 book chapter | Working |
| Murat Çalışkan | Assoc. Prof. | Samsun/1983 | AUFVM /2006 | 2013 | 2021 | - | 32 articles, 1 book | Working |

[12] The titles and publication numbers of the academicians who left and retired the faculty were recorded on the basis of the year they left.
[13] Transferred to the AUFVM Obstetrics and Gynecology Department.
[14] Transferred to the Elazığ Veterinary Faculty.
[15] Transferred to the İstanbul University Faculty of Veterinary Medicine.
[16] Transferred to the Çukurova University Faculty of Agriculture.
[17] Transferred to the Adnan Menderes University Faculty of Veterinary Medicine.
[18] Transferred to the Aksaray University Faculty of Veterinary Medicine.
[19] Went to the United States (US).

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**Figure 3.** Surgery instructors with faculty members and students at Ankara University Faculty of Veterinary Medicine (1948, 1950s).

***Ankara University Period:*** After the HAI Faculty of Veterinary Medicine was affiliated to AU, in the Institute of Surgery, which is among the eight institutes; Ord. Prof. Salih Zeki Berker, Prof. Tevfik Başer, Prof. Burhanettin Öktem, Prof. Bekir Sıtkı Artun, Asst. Hüseyin Erk and Asst. Selim Tolkun took charge (12). After the rearrangement of the curriculum of the faculty whose education period was set to five years in 1939, external clinical diagnosis was taught in 4th term, general surgery in 5th and 6th terms, surgical instrument knowledge in 5th term, operation knowledge in 5th, 6th, 7th and 10th terms, foot diseases and horseshoe technique in 6th, 7th and 8th terms, special surgery and obstetrics in 7th and 8th terms, eye diseases in 8th term, and clinical practice from 5th to last term (15). In the Faculty of Veterinary Medicine, the curriculum was reorganized in the 1949-1950 academic year so that the practices of the courses could be carried out in the stud farms in the 10th semester, and in 1955, students were split to practice in four fields (clinics, bacteriology, food control, zootechny) in this regard (14).

At the beginning of 1952, AUFVM switched from the "institute" system to the "kürsü" (department) system[20], and within this framework, *"I. Şirürji Kürsüsü ve Kliniği" (I. Surgery Department and Clinic)* and *"II. Şirürji Kürsüsü ve Kliniği"(II. Surgery Department and Clinic)* were established among 18 department[21] Ord. Prof. Salih Zeki Berker[21] was brought to the direction of the I. Surgery Department and Clinic and Prof. Burhanettin Öktem, Asst. Selim Tolkun and Asst. Hayrettin Anteplioğlu took role in this department (Fig. 3). Prof. M. Tevfik Başer[21] was brought to the direction of the II. Surgery Department and Clinic and Prof. Bekir Sıtkı Artun and Asst. Mustafa Temizer took part in this department. The veterinary obstetrics and gynecology field which was within surgery has also been separated from surgery as an independent department[20].

Regarding the postgraduate education before the Council of Higher Education, it was reported that 394 people registered for specialization and 39 people registered for doctorate at the AUFVM Animal Breeding and Health Sciences Specialization School (25). Although the doctorate information of six people[22] and specialized information of seven people[23] regarding surgery in this school were reached, the exact number could not be determined.

After the Universities Law No. 1750[24] came into effect in 1973, the issue of restructuring the departments in AU came to the fore. With the approval of the AU Senate, the two existing surgical departments (I. and II. Surgery Departments) were combined[25] as a single department under the name of "Surgery Department and Clinic" and Prof. Burhanettin Öktem was brought into the direction[26]. In addition to this, an independent "Orthopedics and Anesthesiology Department" was established[25] and Prof. Bekir Sıtkı Artun was brought to the direction[26].

When looking at the education and examination regulations[27] of the faculty in various periods, it is seen that the 10th term is devoted to exercises in the stud farms; practical training were placed in clinical courses and external clinical diagnosis was taught in 4th term for 2 hours/week, general surgery in 5th and 6th terms for 2 hours/week each, special surgery in 7th and 8th terms for 2 hours/week each, eye diseases in 9th term for 1 hour/week, operation knowledge in 6th term for 2 hours/week, 7th term

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[20] Decision No. 661 dated 15 January 1952 of AU Senate.
[21] Decision No. 662 dated 15 January 1952 of AU Senate
[22] Doctoral theses, AUFVM Deanery Archive. Necdet Güzel 1975, Arkun Candaş 1976, Burhanettin Olcay 1978, Bahattin Koç 1979, Cihat Ersümer 1980, Nuri Yavru 1982.
[23] Master's Theses, AUFVM Deanery Archive. Sedat Kalaycı 1972, Turan Özer 1975, Said İnan 1975, Erdoğan Nayman 1979, Murat Kuloğlu 1979, Fevzi Toprak 1979, Ahmet Uğur 1980.
[24] Law No. 1750, No. 14587 Official Gazette dated 7 July 1973.
[25] Decision No. 514 dated 30 April 1974 of AU Senate.
[26] Decision No. 111 of AUFVM Board dated 9 May 1974.
[27] AUFVM Education and Examination Regulations; 1951, 1960, 1968, 1974, 1977.

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for 3 hours/week, 8th term for 2 hours/week, foot diseases in 6th and 7th terms for 1 hour/week, orthopedy in 5th and 6th terms for 1 hour/week. In addition, it was determined that practical courses were carried out in Small and Large Animal Clinics, Anesthesiology and Physical Therapy Units, and radiological examinations were carried out in the X-Ray Unit [28].

With the organization implemented in the higher education system within the scope of the Higher Education Law[29] the "division" system was started. In 1982, "Diseases and Clinical Sciences Division", which is one of the three divisions, was established[30]. Under the head of department of Prof. Hayrettin Antepli̇oğlu, the "*Cerrahi Anabilim Dalı*" (Surgery Department) and its subunits, "*Cerrahi Bilim Dalı*" (Surgery Branch) and, "*Travmatoloji ve Ortopedi Bilim Dalı*" (Traumatology and Orthopedics Branch) were established[31]. Within the framework of the *"Principles on Education and Training Plan in Veterinary Faculties"* prepared by the Council of Higher Education in 1982, it was decided that the course hours in veterinary faculties would be 4132 hours[32]. In this context, 42 hours of theoric general surgery course in 3rd year, 42 hours of theoric special surgery and clinic and foot diseases and orthopedics courses each in 4th year, 42 hours of theoric and 52 hours of practical special surgery and clinic course in 5th year was arranged. In addition, clinical courses, including surgical practices, were arranged as 218 hours in the 4th year and 144 hours in the 5th year. For the implementation of these plans and principles, the 1982-1983 academic year was set aside as the adaptation process. As of the 1983-1984 academic year, this program has been made compulsory to be implemented in all veterinary faculties (26).

*"Minimal Courses and Minimal Hours to be Taught in Veterinary Faculties"* were determined at the meeting held on 28-29 March 1989 of the *"Inter-University Board Veterinary Sciences Education Council"*, which was formed in accordance with the Higher Education Law[29] in 1989. It has been approved that the introduction to external diseases course should be given 28 hours to the 3rd graders, 168 hours of the surgery course to the 4th grades, 28 hours of the veterinary orthopedics course to the fifth grades, and 112 hours of the clinical courses including the surgery courses to the 5th grades [33].

The issue of arranging the sub-units of the Surgery Department was brought to the agenda again in 1993, *"in terms of having sufficient faculty members, convenient physical structure, improving clinical services, education and research opportunities"*. In that regard, it was proposed to open four branches within Surgery Department under the names of "Veterinary Radiology, Veterinary Anesthesiology and Reanimation, Veterinary Surgery, and Veterinary Orthopedics and Traumatology"[34,35]. These branches were structured upon approval by the Higher Education Council[36]. Prof. Erdoğan Samsar as the Head of Department of Surgery, Prof. Faruk Akın as the Head of Surgery Branch, Prof. Doğan Aslanbey as the Head of Orthopedics and Traumatology Branch, Assoc. Prof. Bahattin Koç as the Head of Anesthesiology and Reanimation Branch and Assoc. Prof. Zeki Alkan as the Head of Radiology Branch has been appointed[37]. After 13 years, all sub-units of the Surgery Department were abolished in 2006, in line with the proposal to combine small subunits of departments within the framework of European Union Accreditation processes[38,39,40]. Since this date, the activities of these sub-units have been carried out in the Department of Surgery. With the regulation made in higher education in 2009, the number of divisions was increased to five, and the Department of Surgery took its place as one of the four departments[41] structured within the Division of Clinical Sciences[42].

The head of the department is Prof. Ali Bumin in AUFVM Surgery Department as of April 2022[43](5). In the department, a total of nine faculty member work including three professors, three associate professors and three research assistants. The courses given by these lecturers are presented in Table 3. The academic stages and the number of publications of 30 faculty lecturer working in the field of surgery in the faculty between 1933 and April 2022 are presented in Table 2, with their photos Fig. 4, 5, 6. The academics who make up the academic staff of the unit continue their postgraduate education tasks as well as research and publication activities. In this context, it has

[28] Letter 98 dated April 2, 1980 sent to the Dean's Office by the AUFVM Chair of Surgery Department and Clinic.
[29] Law No. 2547, No. 17506 Official Gazette dated 6 November 1981.
[30] Letter dated 16 August 1982 and numbered 2950 sent to AU Rectorate by the Council of Higher Education.
[31] AUFVM Dean's Letter dated 6 December 1982 and numbered 10134.
[32] Letter dated 18 August 1982 and numbered 220/2960 sent to the Dean of AUFVM by the Council of Higher Education.
[33] Letter 1, dated 7 April 1989, sent to all veterinary faculties by the Inter-University Board Veterinary Sciences Education Council.
[34] Letter of AUFVM Head of Division of Diseases and Clinical Sciences dated 6 July 1993 and numbered 74.
[35] Decision No. 1257 dated 20 July 1993 of the AU Senate.
[36] Letter dated 27 October 1993 and numbered 4384 sent by the Council of Higher Education to the AU Rectorate.
[37] AUFVM Deanery Archive, Personnel Files.
[38] Decision of AUFVM Faculty Board dated 19 April 2006 and numbered 05.
[39] Decision No. 2193 dated 16 May 2006 of the AU Senate.
[40] Letter dated 7 July 2006 and numbered 2211 sent to the AU Rectorate by the Council of Higher Education.
[41] Surgery, Obstetrics and Gynecology, Reproduction and Artificial Insemination, Internal Diseases.
[42] Letter dated March 2, 2009 and numbered 880 sent by the Council of Higher Education to the AU Rectorate.
[43] Prof. Ali Bumin has been serving as the Head of the Surgery Department since 2019.

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been determined that 88 doctoral dissertations and 85 master's theses have been completed in the field of veterinary surgery until April 2022 within the Institute of Health Sciences [44,45] (8).

Within the Animal Hospital of the Faculty, polyclinic services are provided for surgical diseases of all domestic and wild animals, and clinical applications are carried out on various animal species within the scope of undergraduate, graduate and doctoral programs. In this context, routine applications for diagnosis and treatment are carried out in Radiodiagnostic (Digital X-ray, Ultrasonography, Doppler Ultrasonography, Echocardiography, Magnetic Resonance Imaging, Computed Tomography), Endoscopic Examination (arthroscopy, laparoscopy, thoracoscopy, bronchoscopy, cystoscopy, gastroscopy, colonoscopy), Arthroscopic, Laparoscopic and Thoracoscopic Surgery, Neurology-Neurosurgery, Microsurgery, Veterinary Dentistry, Veterinary Ophthalmology, Physiotherapy-Rehabilitation Wildlife and Veterinary Orthopedics Units (5).

**Table 3.** Courses Taught in the Field of Surgery at Ankara University Faculty of Veterinary Medicine (2021-2022).

| Course Name<br>Elective: (E) Compulsory: (C) | Weekly Course Hours | | | | Grade |
|---|---|---|---|---|---|
| | Autumn | | Spring | | |
| | Theoric | Practical | Theoric | Practical | |
| Surgery (C) | - | - | 4 | - | 4 |
| Traumatology and Orthopedic Surgery (C) | - | - | 1 | - | 4 |
| Radiology (C) | - | - | 1 | - | 4 |
| Hoof Diseases Horseshoe Technique (C) | - | - | 1 | - | 4 |
| Anesthesiology and Reanimation (C) | - | - | 1 | - | 4 |
| Eye Diseases (C) | - | - | 1 | - | 4 |
| Professional Practice and Clinical Skills Methods (C)* | - | - | - | 4 | 2 |
| Physio-Therapy and Rehabilitation (E) | 1 | - | | - | 4 |
| Veterinary Neurology Neurosurgery (E) | - | - | 1 | - | 4 |
| Tooth Diseases (E) | 1 | - | - | - | 4 |

* The course is given in a multidisciplinary manner in the form of a module system.

**Figure 4.** Salih Zeki Berker, Mehmet Tevfik Başer, Burhanettin Öktem, Bekir Sıtkı Artun, Hüseyin Erk, Selim Tolkun, Mustafa Temizer, Hayrettin Anteplioğlu, Kadri Akın Finci, Erdoğan Samsar.

**Figure 5.** Nail Küçüker, Mehmet Ali Öztürk Tekeli, Mehmet Doğan Aslanbey, Faruk Akın, Necdet Güzel, Arkun Candaş, Burhanettin Olcay, Bahattin Koç, Mehmet Sağlam, Zeki Alkan.

**Figure 6.** A. Perran Gökçe, F.Eser Özgencil, Ümit Kaya* Hasan Bilgili, Ali Bumin*, Ömer Beşaltı*, Oytun Okan Şenel*, İrem Gül Sancak*, İrem Ergin*, Murat Çalışkan*.

* Current academic staff

[44] The completion year of the first master's thesis in the field of veterinary surgery at AUFVM is 1992.
[45] AU Health Sciences Institute Archive.

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**Figure 7.** Ankara University Faculty of Veterinary Medicine Surgery Clinic (1980, 2022).

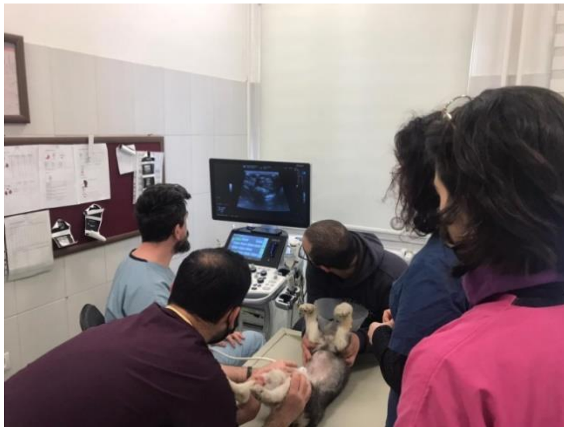

**Figure 8.** Views from Ankara University Faculty of Veterinary Medicine Surgery Clinic (2022).

There are large and small animal operating rooms and Anesthesiology and Reanimation unit for soft tissue surgery and orthopedic interventions. The operating rooms are equipped with world-class equipment. In these rooms, there are ceiling lighting, anesthesia devices, central gas system, aspirator system, bedside monitors and electrocautery devices. In addition, there are intensive care units and a patient preparation room for cats and dogs. In the department of surgery, there are six examination rooms where patients are routinely examined. In these examination rooms soft tissue, orthopedic, eye, ear and dental diseases are diagnosed and treated. Apart from these, there are neurophysiology laboratory, physiotherapy-rehabilitation unit, electrophysiology, gait analysis unit and radiodiagnostic unit (5) (Fig. 7, Fig. 8).

## Discussion and Conclusion

Although the initiation of veterinary medicine education with Godlewsky in 1842 can be accepted as the beginning of education-training practices in the modern sense, it is understood that surgical practices could not be carried out very efficiently. Afterward, the surgery courses in Military and Civilian Schools were first supported by lecturers brought from abroad, and then they were carried out with the lecturers who were sent to Europe to specialize and included in the School staff when they returned, which shows that an up-to-date education in the field of surgery is being tried.

Regarding taking Alfort Veterinary School as a reference for education, Melikoğlu Gölcü (22) evaluated the transfer of students in both veterinary medicine and other professions mainly to France as an attitude related to the foreign relations of the Ottoman State. The establishment of the first veterinary school in France and the outstanding achievements of Louis Pasteur and his team in the field of microbiology are stated among the reasons for choosing France (22).

Tüzdil (27) stated that the specialists who were sent to Europe for specialization training from the Civil Veterinary School in 1909 and who were recruited when they returned home strengthened the School once again, and stated that the main clinical studies could only be carried out after 1911 when these instructors returned from Europe. It was reported by Öktem (23) that besides these positive developments, there are also some deficiencies. Öktem's (23), memories when he was a student at the Higher Veterinary School regarding the clinical education and training in 1923 explains the

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situation of his time: *"There was no clinic building for internal and external diseases and sick animal treatment barns. However, the practice of the polyclinic was administered by the surgeons, internal diseases and pharmacology professors, two days a week, on guard duty. In our school, which was renamed as Yüksek Baytar Mektebi* (Higher Veterinary School) *when we were students, the courses are taught in a theoretical way; Fenn-i Vilâde* (Obstetrics), *Emrâz-ı Mevâşî* (Ruminant Diseases), *Emrâz-ı Dâhiliyye* (Internal Diseases) ... *were administered by taking notes or giving grades by our lecturers. ... the mobile practice was only possible in Botany and Fenn-i Eşkâl* (Morpology) *classes, not exceeding 2-3 times a year, while the polyclinic practice was held partially indoors, but rather in the garden in favorable weather."* When looking at the post-republican period, it is clearly seen how much value is given to education, agriculture and animal husbandry among the development plans of the country. The establishment of HAI, one of the modern education and training facilities in Ankara, the establishment of an academic structure under the institute system, the execution of educational activities under the leadership of German scholars, and the implementation of a modern education at undergraduate and graduate level show that integration has been achieved. As a matter of fact, Erk et al. (16), in their studies on the development of clinical teaching in veterinary medicine, reported that the main developments in the field of veterinary surgery were after the proclamation of the Republic and the scientific level of the surgical field rose during the Republican period. Erk et al. (16), emphasized that as of 1981, when they published their studies, clinical applications, use of x-ray and similar diagnostic tools and all kinds of operation opportunities in veterinary faculties in Türkiye have reached the level of veterinary education institutions in western countries. From these statements, it is seen that veterinary medicine education reached a higher level and gained an institutional structure in the periods after the proclamation of the Republic.

It was determined that the Department of Surgery was divided into four branches in 1993 under the names of Veterinary Radiology, Veterinary Anesthesiology and Reanimation, Veterinary Surgery, Veterinary Orthopedics and Traumatology, and these branches were closed in 2006.[38,39,40] AUFVM Faculty Board, taking into account the proposal of EAEVE to merge small subunits during accreditation studies, proposed the closure of these units. This proposal was accepted by AU Senate and Higher Education Institution[38,39,40].

Today, in the AUFVM, it is seen that the compulsory courses (Table 3) given at the undergraduate level in the field of surgery are administered in accordance with the current criteria (17) of the European Association of Establishments for Veterinary Education (EAEVE), which regulates education in Europe. The surgical unit in the faculty is equipped to meet today's EAEVE requirements in terms of equipment and infrastructure, and it strives to improve it (1). It can be considered that this is a parameter that shows the level of surgery education in AUFVM and that education is given at the level of European countries.

In addition to the hundreds of undergraduate and graduate students they have trained, academics of the AUFVM surgery depardment have contributed to the field by being members of numerous academic juries in the country[37]. They have undertaken important duties in education, research and publication and in the structuring of the surgical units of other veterinary faculties, as well as in the training of academicians and students who will work in these units[46]. In addition to the scientific research of academicians of the field, the numerical data of postgraduate studies of the last 40 years can shed light to the future evaluations for the field.

As a result, in this article, which deals with the beginning of the veterinary surgery field in Türkiye and its development in AUFVM, a compact resource on the history of the field has been presented. The inclusion of detailed data on the surgical units of all veterinary faculties in Türkiye will enable the holistic determination of the quality of surgery in veterinary medicine.

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## Conflict of Interest

The authors declared that there is no conflict of interest.

## Author Contributions

NY conceived and planned the study. NY and ÖK designed the study. All authors conducted literature review, archive research and writing the manuscript. All authors contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis and manuscript.

## Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

[46] Commission Report dated 4 January 1978 prepared with the Decision of the Faculty Board of AUFVM dated 28 December 1977 and numbered 285.

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Research Article

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# Clinical trial of the efficiency of three different compositions of acaricidal substances against varoosis in honey bee colonies

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## Abstract

This study aimed to evaluate and compare the varroacidal efficacy and mite mortality dynamic during autumn treatment of honey bee colonies in two experimental areas (Boychinovtsi-Northwestern Bulgaria and Zlatiya-Northeastern Bulgaria), treated with three available veterinary medicinal substances. The clinical studies were conducted on the efficiency of the three acaricidal combinations (AC) - one, based on 3.6 mg flumethrin/strip (AC-1) and two contents of essential oils (first one with composition: 5 g thymol plus 2 g peppermint oil/lamellae (AC-2), and the second one with composition: 4 g thymol plus 2 g peppermint oil/ lamellae (AC-3), in the autumn of 2017. We used the product containing coumaphos and an additive with oxalic acid for the control treatment. Methods for establishing the levels of *Varroa destructor* infestation in bees and in brood were used according to OIE Terrestrial Manual. After 35 days of AC-1 exposure, 94.5% and 87.82% efficiency were achieved in the apiaries in Boychinovtsi and Zlatiya, respectively. Efficiencies of the combinations tested (AC-2 and AC-3) for 45 days were detected high only in the Zlatiya apiary (97% and 95%), also 91% and 80% in the Boychinovtsi apiary, respectively. The results of the experiments showed the absence of resistance to the tested substances.

## Introduction

Varroosis, a parasitic disease caused by *Varroa destructor* mite, is one of the leading problems in honey bee pathology. *Varroa* mites feed on the hemolymph of pupae and adults, which can result in premature mortality (27). The impacts of Varroa mite infestation can be immediate and profound and very often the mite is a vector for a large number of honey bee viruses (30). *Varroa destructor* prevalence must be constantly monitored and control in the European honey bee *Apis mellifera* so that the colonies can develop normally and produce. The most commonly used treatment schemes involve the application of synthetic acaricides to honey bee colonies. Despite their efficacy, these substances create new problems for beekeeping in Europe, one of which is the emergence of resistant mites (1, 2, 9, 20, 21, 28). The acaricidal efficiency varies with factors such as climatic conditions, treatment period, treatment method, the strength of bee colonies, level of infestation, etc. The moderate use of a given agent is a better tactic than administering a higher dose (22).

According to European requirements (6) for acaricidal **veterinary medicinal products** (VMPs) used against ectoparasites in animals, their efficacy against *Varroa destructor* in bees must be more than 90%. On the other hand, the effectiveness of acaricides is influenced by the rapid adaptation and development of *Varroa* mites resistance in the case of prolonged application of the same products or those containing substances of the same chemical groups. Three different mutations at position 925 of the *V. destructor* voltage-gated sodium channel have been associated with the resistance to these compounds.

González-Cabrera et al. (12), show new evidence for the significant correlation of the mutation with resistance and conclude that it is likely that resistant mites have a reduced fitness. Under current conditions, the demands on the quality and safety of bee products are increasing, which has led to a significant increase in the proportion of organic beekeeping (7, 11, 26, 29). This necessitated the development of new products based organic acids, essential oils and plant extracts.

In some studies, the product with 5 g thymol plus 2 g peppermint oil/ lamellae showed an efficiency of over 90% (15, 16, 17). Strips with flumethrin as an active substance has a proven effectiveness over time 99% (15, 17).

Different methods have been described for the detection of *V. destructor* resistance to fluvalinate used in the laboratory or field (1-4, 21, 34). Milani and Vedova (23), using paraffin capsules in the laboratory, found that mite resistance in northern Italy decreased tenfold after five years of no pyrethroid use. Similar results, in which the susceptibility of resistant mite increased, have been reported by Elzen et al. (10). Gracia-Salinas et al. (13), following the laboratory method of Milani (21) on the sensitivity of 10 populations of *V. destructor* against fluvalinate, reported for the first time resistance of *V. destructor* in areas of northern Spain, where LC50 is 25-50 times higher than this for sensitive mites. The incorrect use of VMP with molecule, that contain pyrethroids has led to the development of resistance in *V. destructor* (1).

In Bulgaria, strips with flumethrin have been used for control of *Varroa destructor* in conventional beekeeping for more than 20 years, and products containing fluvalinate as an active ingredient have been used for a long time. These acaricides are representatives of the same chemical group - the synthetic pyrethroids. This gives us the reason by a clinical trials to verify the previous high efficacy - over 99%, of the flumethrin – strips, and to identify whether any resistance of *V. destructor* has been developed. Early detection of *Varroa* resistance to pyrethroids would sharply reduce losses in beekeeping.

This study aimed to evaluate and compare the varroacidal efficacy and mite mortality dynamic during autumn treatment of honey bee colonies situated at two experimental areas influenced by different environmental conditions, treated with three combinations of acaricidal substances and compared with untreated control of honey bee colonies. And check for possible development of *Varroa destructor* resistance to some of them.

## Materials and Methods

The experiments were carried out in the period July - October 2017. The guidelines used were those specified in the ordinance on the requirements for the data contained in the documentation for the issuance of a license for the use of VMP (25), technical guidelines for the evaluation of treatments for control of *Varroa* mites in honey bee colonies (14). Bee colonies from two settlements in the district of Dobrich (Northeastern Bulgaria) and in the district of Montana (Northwestern Bulgaria) were used as follows: apiary in the village of Zlatiya with sixty-two total number of bee colonies; apiary in town of Boychinovtsi with one hundred and eight total number of bee colonies. From each of these apiaries, forty bee colonies were selected. The main selection criteria are the strength of the bee colonies and the amount of the sealed bee worker brood.

***I. Methods:*** 1. Determination of colony development by generally accepted zootechnical methods for measuring strength of bees in kg and brood amount in number of brood cells.

2. Methods for establishing the levels of *Varroa destructor* infestation in bees and in brood were used according to OIE, 2013 (24).

3. Calculate the effectiveness of three acaricidal combinations (AC) using the following formula:

Effectiveness = mites killed by AC x 100

(% Reduction) killed by AC + remaining mites

- Mites killed by AC are all mites collected during exposure to the test product;

- The remaining mites are those that are not killed by the AC and fall off after treatment with the control product.

4. The presented results were processed statistically using the computer program "Statistics".

5. The treated colonies were monitored throughout the test period for normal development, the occurrence of adverse more harmful effects of the test substances on the queen, bees, and brood.

***II. Information on the combinations of acaricidal combinatons(AC) used in the trial:*** - AC (1): strips with composition: 3.6 mg flumethrin/strip

- AC (2): lamellae with composition: 5 g thymol, 2 g peppermint oil and excipients ad 51 g /lamellae

- AC (3): lamellae with composition: 4 g thymol, 2 g peppermint oil and excipients ad 51 g / lamellae

- Control product with composition: coumaphos - 32 mg/ml

- Zootechnical Feed additive for bees with Oxalic acid - composition: plant extracts, organic acids and inverted syrup.

The products were administered according to the manufacturer's instructions.

***III. Groups - bee colonies:*** Colonies in both experimental apiaries were grouped as follows:

E (1) - experimental group - 10 colonies treated with 4 strips of product AC- 1 for 35 days.

E (2) - experimental group - 10 colonies treated with 2 lamellae of product AC-2 for 45 days.

E (3) - experimental group - 10 colonies treated with 2 lamellae of product, AC-3 for 45 days.

K- control group - 10 bee colonies, untreated.

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***IV. Apiaries:*** In the apiary in Zlatiya, twenty of the colonies were settled in ten-frame Langstroth-Root (LR) hives, and twenty – and ten-frame Dadant-Blatt (DB) hives, evenly distributed between the experimental and control groups. In the apiary in Boychinovtsi, experiments were carried out on forty honey bee colonies settled in ten -frame Dadant-Blatt (DB) hives.

***V. Stages of the study:*** - Determination of *Varroa destructor* infestation level of bee colonies (bees and brood) of the experimental and control groups before treatment.

- The infestation level of the bees was determined by taking out a brood comb and approximately 200–250 bees were removed in a container with alcohol. After that the container was stir for 10 minutes. The bees were separated from the mites by means of a sieve with a mesh size of approximately 2–3 mm. Bees and fallen mites were counted. The level of infestation of bees with mites was calculated as a percentage.

- The infestation level of the brood is determined by removing pupae from 100 combs cells with a sealed brood and number of found mites was calculated as a percentage.

The experiments included hives with a level of infestation of 300 to 3000 mites per colony.

- Calculation the strength (in kg) and the amount of sealed brood (in number of cells) of the colony before treatment.

- Rapid test for 4 hours to establish resistance of *Varroa detructor* mite to AC-1 (flumethrin-strips), performed on bees from bee colonies in the experimental group E -1 (AC-1).

- Clinical tests of acaricidal activity against *Varroa destructor* – treatment with the selected substances and combinations. The release of thymol and peppermint oil included in the lamellae does not depend on environmental factors. The active substances are released gradually over 45 days.

- Control treatment with the VMP, containing coumaphos (32 mg/ ml) for bee colonies from experimental group E-1 (AC-1) and with feed additive based on oxalic acid for the bee colonies from the other groups ( E-2, E-3, K).

- Monitoring the number of fallen mites in the control and experimental groups.

During the experiment, 530 checklists with mites from each apiary were counted. Counting of mites, fallen into each of the hives included in the groups was provided on the 24$^{th}$ hour, 48$^{th}$ hour, 72$^{nd}$ hour and on the 4$^{th}$, 8$^{th}$, 10$^{th}$, 15$^{th}$, 20$^{th}$, 25$^{th}$, 30$^{th}$, 35$^{th}$, 40$^{th}$ and 45$^{th}$ day. For every apiary mite counts were carried out every of these dates during treatment (for E (1), and five bee colonies from K groups -11 reads; for E (2) , E (3) and bee colonies from K groups - 13 reads were recorded for each colony), and 24 hours after control treatment – 2 reads.

On day 35, a control treatment of group E (AC-1) was performed (10 colonies) were treated with coumaphos. At the same time five colonies from the untreated group (K) were treated with oxalic acid. On 45$^{th}$ day the control treatments of groups E (AC-2) and E (AC-3) were performed (10 colonies of each group) and the other five colonies of the control group (K) were treated with oxalic acid. We monitored the number of mites, fallen by the control treatment after 24 hours.

- Calculation the strength (in kg) and the amount of sealed brood (in number of cells) of the colony after treatment.

- Determination of *Varroa destructor* infestation level of bee colonies (bees and brood) of the experimental and control groups after the treatment is completed. The infestation level of bees and brood was calculated in the same way as before treatment.

- Calculation the efficiency of the relevant combinations.

***VI. Data analysis:*** The data obtained were processed variationally and statistically on Descriptive statistic – Normal distribution with software STATISTICA 12, Copyright © Stat Soft Inc. 1984-2014 (StatSoft, 2014). It was used to establish the reliability of the obtained statistical differences with One – Way ANOVA analysis (31).

## Results

***Apiary in Zlatiya:*** In the apiary in Zlatiya, the experiment used colonies settled in two types of hives – Dadant-Blatt (DB) and Langstroth Root (LR). The different hive types were distributed almost equally in each group. The strength of the colonies before and after the treatment is given on Table 1.

At the beginning of the experiment, most of the mites were concentrated in the sealed brood (Table 2).

After staying the products in the hives in group E-1 (AC-1) for 35 days, the mite infestation level on bees was 0.51±0.18%. In the experimental groups E-2 (AC-2) and E-3 (AC-3) after 45 days the infestation level on bees decreased to 2.05±0.94% for E (2), and in E (3) slightly increased to 3.09±1.05%. The differences being of low significance /$P \leq 0.05$/. Brood rearing in colonies in all groups was stopped. In the control /K / group, the mite infestation level for bees remained almost the same at 3.38±1.06%. The reliability between the experimental groups E-1 (AC-1) and E-3 (AC-3) was low - $P \leq 0.05$ /Table 2/. The resistance test showed an average of 2.3 mites fallen from the product with flumethrin (AC-1) and 0.6 remaining on the bees in the containers. The test proved a lack of resistance to flumethrin.

The data showed that the most significant number of mites dropped out in the E (2) group under the action of AC-2 which has a higher level of infestation/ -

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769.0±210.73 mites, while after the control treatment dropped out - 18.4 ± 4.08 mites or 2.3% of all mites. In the control treatment, in the experimental groups E-1 (AC-1) and E-3 (AC-3) dropped out 7% and 2.7% of all mites, respectively. In the untreated group (K), as a result of control treatment mites that dropped out were about 30%.

The effectiveness of the tested products is presented in Table 3.

The condition of colonies at the end of the trials was normal for the season, according to their initial strength (Table 2). No differences were found between DB and LR hives.

**Table 1.** Development of bee colonies - apiary Zlatiya.

| Group | n | Before treatment | | | | After treatment | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Strength -kg | | Brood -number cells | | Strength -kg | | Brood -number cells | |
| | | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max |
| E-1 (AC-1) | 10 | 2,18±0,17 | 1,00/3,00 | 7150±501,39 | 5300/10200 | 2,39±0,18 | 1,50/3,20 | 410±129,49 | 0/1100 |
| E-2 (AC-2) | 10 | 2,31±0,10 | 2,00/2,80 | 8110±955,04 | 4100/13100 | 2,19±0,17 | 1,50/3,00 | - | - |
| E-3(AC-3) | 10 | 1,82±0,09 | 1,50/2,25 | 7080±459,42 | 4600/9800 | 1,83±0,11 | 1,00/2,20 | 1700±556,78 | 600/2400 |
| K-control (untreated) | 10 | 1,82±0,13 | 1,20/2,60 | 6420±598,11 | 4100/10000 | 2,10±0,11 | 1,75/2,60 | 1100±561,25 | 100/2600 |
| **Significance of differences between groups** | | E-2/E-3**<br>E-2/K** | | | | E-2/E-3* | | E-1/E-3** | |

The statistical analysis tests the differences between treated groups (E-1 (AC-1), E-2 (AC-2) and E-3 (AC-3)) and control group (ANOVA).
* Indicates P≤0.05 significant level.
** Indicates P≤0.01 significant level, Strength of colony (bees in kg) and (Brood in number of cells) in the beginning and the end of experiment; n (replicates) = 10.

**Table 2.** Infestation level of brood and bees (%) - apiary Zlatiya.

| Group | Before treatment | | | | After treatment | | | |
|---|---|---|---|---|---|---|---|---|
| | IL brood -% | | IL bees -% | | IL brood - % | | IL bees - % | |
| | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max |
| E-1 (AC-1) | 0,67±0,67 | 0/6,67 | 1,50±0,46 | 0/3,90 | - | - | 0,51±0,18 | 0/1,45 |
| E-2 (AC-2) | 5,33±4,07 | 0/40 | 3,31±0,75 | 0,60/7,90 | - | - | 2,05±0,94 | 0/9,90 |
| E-3 (AC-3) | 2,00±1,42 | 0/13,33 | 1,64±0,72 | 0/6,90 | - | - | 3,09±1,05 | 0/11,40 |
| K-control (untreated) | 3,33±2,28 | 0/20 | 0,44±0,33 | 0/2,10 | - | - | 3,38±1,06 | 0/11,69 |
| **Significance of differences between groups** | - | - | E-2/K** | - | | | E-1/E-3*<br>E-1/K* | - |

The infestation level (IL) of brood and bees in the beginning and in the end of experiment.
The statistical reliability between the experimental groups E (1) and E (3) is low - P≤0.05.

**Table 3.** Efficiency (%) of the used combinations - apiary Zlatiya.

| Group | n | Fallen mites from the treatment (number)<br>x̄ ±Sx̄<br>Min/max | Fallen mites from the control treatment (number)<br>x̄ ±Sx̄<br>Min/max | All fallen mites (number)<br>x̄ ±Sx̄<br>Min/max | Efficiency (%)<br>x̄ ±Sx̄<br>Min/max |
|---|---|---|---|---|---|
| E-1 (AC-1) | 10 | 691,3±208,16<br>60/2337 | 54,9±10,25<br>9/94 | 746,2±210,17<br>82/2419 | 87,82±3,10<br>70,17/99,25 |
| E-2 (AC-2) | 10 | 769,0±210,93<br>148/2306 | 18,4±4,08<br>3/39 | 787,4±214,33<br>152/2345 | 97,26±0,53<br>93,40/98,98 |
| E-3 (AC-3) | 10 | 693,7±242,98<br>65/2579 | 19,2±7,05<br>1/79 | 713,9±249,00<br>74/2658 | 95,84±1,79<br>81,48/98,72 |
| K-control (untreated) | 10 | 81,1±19,19<br>18/211 | 31,56±8,96<br>1/78 | 110,9±25,41<br>19/232 | |
| **Significance of differences between groups** | | E-1/K**<br>E-2/K**<br>E-3/K* | E-1/E-2**<br>E-1/E-3**<br>E-1/K* | E-2/K**<br>E-3/K* | E-1/E-2**<br>E-1/K*<br>E-2/K***<br>E-3/K** |

Efficiency calculated on the basis of group are means of 10 replicates, n = 10.
Different number of stars indicate significant differences among the groups (E-1, E-2, E-3 and K-control): * P≤0.05; **P ≤0.01, *** P≤0,001.

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***Apiary in Boychinovtsi:*** The experiments in Boychinovtsi's apiary included colonies with similar pre-treatment strength in all groups - from about 1.85 kg to 1.95 kg of bees. During the treatment period, the colonies were in good condition with about 2 kg of bees (Table 4).

At the beginning of the experiments, mites were distributed on the bees and in the sealed brood in experimental groups (Table 5). The infestation level (in %) of brood and bees in Boychinovtsi is given on the Table 5.

The resistance test showed an average of 9.4 mites dropped from the action of flumethrin-strips and 0.6 remaining on the bees in the containers. The efficiency of the tested products is presented in Table 6.

The established efficiency of flumethrin showed that it has a high acaricidal activity, as for the apiary in Boychinovtsi, it was almost 95%, respectively 94.52±1.16 with a moderate degree of reliability in the differences (P≤ 0.01) compared to the untreated control group after staying the strips in the colonies for 35 days.

**Table 4.** Development of bee colonies - apiary Boychinovtsi.

| Group | n | Before treatment | | | | After treatment | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Strength -kg | | Brood -number cells | | Strength -kg | | Brood -number cells | |
| | | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max |
| E-1(AC-1) | 10 | 1,95±0,05 | 1,75/2,25 | 3640±395,87 | 1900/5300 | 2,00±0,08 | 1,50/2,25 | 0 | 0 |
| E-2(AC-2) | 10 | 1,90±0,04 | 1,75/2,00 | 4510±449,31 | 2200/6300 | 1,85±0,05 | 1,50/2,00 | 100±100,00 | 0/1000 |
| E-3(AC-3) | 10 | 1,85±0,04 | 1,75/2,00 | 4660±385,34 | 3200/7200 | 2,07±0,07 | 1,75/2,25 | 160±110,75 | 0/1000 |
| K-Control | 10 | 1,90±0,04 | 1,75/2,00 | 3550±368,56 | 1800/5700 | 2,17±0,04 | 2,00/2,25 | 100±100,00 | 0/1000 |

Strength (bees in kg) and Strength (Brood in number of cells) in the beginning and the end of experiment; n (replicates) = 10.

**Table 5.** Infestation level of brood and bees (%) - apiary Boychinovtsi.

| Group | Before treatment | | | | After treatment | | | | Reliability of start / end differences (bees) |
|---|---|---|---|---|---|---|---|---|---|
| | IL brood-% | | IL bees-% | | IL brood-% | | IL bees-% | | |
| | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max | x̄ ±Sx̄ | Min/max | |
| E-1 (AC-1) | 6,67±3,97 | 0/40 | 4,32±2,09 | 0,4/22,6 | 0 | 0 | 0,42±0,21 | 0/1,9 | NS |
| E-2 (AC-2) | 7,33±4,91 | 0/40 | 9,63±3,85 | 0,6/31,5 | 0 | 0 | 12,25±7,44 | 0/72,4 | NS |
| E-3 (AC-3) | 3,34±1,49 | 0/13,3 | 5,39±1,85 | 0/20,6 | 0 | 0 | 10,80±3,88 | 0,6/42,7 | NS |
| K-Control (untreated) | 0 | 0 | 3,20±1,54 | 0/16,6 | 0 | 0 | 17,68±9,93 | 1,4/102,0 | NS |
| **Significance of differences between groups** | E-3/K* | - | - | - | | | E-1/E-3* | - | |

The infestation level (IL – the calculated percentage of *Varroa destructor* mite of brood and bees before and after treatment.
Different number of stars indicate significant differences among the groups:* P≤0.05; NS: Not significant (P > 0.05).

**Table 6.** Efficiency (%) of the used combinations - apiary Boychinovtsi.

| Group | n | Fallen mites from the treatment (number) x̄ ±Sx̄ Min/max | Fallen mites from the control treatment (number) x̄ ±Sx̄ Min/max | All fallen mites (number) x̄ ±Sx̄ Min/max | Efficiency (%) x̄ ±Sx̄ Min/max |
|---|---|---|---|---|---|
| E-1 (AC-1) | 10 | 1313,9±619,54<br>277/6651 | 36,5±6,60<br>15/88 | 1350,4±624,70<br>317/6739 | 94,52±1,16<br>87,38/98,86 |
| E-2 (AC-2) | 10 | 643,8±288,78<br>114/2295 | 43,7±17,15<br>5/176 | 687,5±300,35<br>128/3099 | 91,61±1,43<br>82,97/97,37 |
| E-3 (AC-3) | 10 | 318,8±104,97<br>34/1046 | 54,9±15,31<br>5/138 | 373,7±116,49<br>71/1184 | 80,07±5,24<br>47,89/93,70 |
| K-Control (untreated) | 10 | 57,4±27,14<br>10/299 | 22,9±5,33<br>0/52 | 80,3±28,93<br>22/333 | |
| **Significance of the differences between the groups** | | E-3/K* | | E-3/K* | E-1/E-3*<br>E-1/K**<br>E-2/E-3*<br>E-2/K** |

Different number of stars indicate significant differences among the groups (E-1, E-2, E-3 and K-control): * P≤0.05; **P ≤0.01; Efficiency calculated on the basis of group are means of 10 replicates, n = 10.

The treated groups' observations showed that the substances do not adversely affect on the bees and brood. Self-replacement of the queen was not observed in the experimental and control groups. The development of the colonies at the end of the experiments was normal for the season, according to their initial strength (Table 4).

## Discussion and Conclusion

The results obtained from the clinical trials of AC-1, with strips containing flumethrin, show high efficacy against varroosis. After an exposure of 35 days, efficiency is achieved, 94.5% / Min/Max - 87.38 /98.86%/ in Boychinovtsi and 87.82% in Zlatiya, respectively.

In Zlatiya, the lower efficiency of E (AC-1) is due to some technical problems during the experiments. The hives were placed close together, and the control groups were intermixed between the experimental groups, which is why mite re-infestation is possible. In addition, robberies are occurred between some of the colonies in the control group. In several hives of the same group the strips had fallen to the bottom, some of them thrown out by bees.

We believe that due to the high efficiency (over 90%, and in some individual experimental colonies up to 99%), which the flumethrin showed in both apiaries it is a suitable acaricide for the *Varroa* mite prevention and control in the conditions of our country and can meet the needs of beekeeping practice. The lack of harmful effect on bee colonies and the lack of mite resistance make it an excellent substance for controlling varroosis in Bulgaria. Based on the results of the rapid test for resistance and efficacy, we can say that, for the time being, it cannot be claimed that the resistance of *V. destructor* to flumethrin has been established in the test areas. The other tested substances combinations – AC-2 and AC-3 also showed high efficiency. For the apiary in Zlatiya 97% for AC-2 and 95% for AC-3, respectively, and for Boychinovtsi 91% for AC-2 and 80% for AC-3, respectively. Despite the lower thymol content of the AC-3 (with 4 g thymol/lamellae), no highly reliable differences were found in the efficacy of the two combinations in any of the apiaries included in the experiment. Due to the fact that these combinations have no harmful effect on bees, their natural composition, and the lack of residues in honey, they are preferred for use to combat varroosis in organic beekeeping. In addition to acaricidal effects, the application of VMP, containing essentials oils, into hives often also causes antimicrobial effects, which can lead to an overall improvement in the health status of honey bee colonies (5, 8, 19, 33).

It can be concluded that an adequate *V. destructor* mite control must include a few measures, firstly good beekeeping management practice in combination with the appropriate use of authorized acaricidal substances. Different treatment regimes should also be applied with continuous parasitic mite mortality monitoring. Consequently, there is a need to review research that supports a combination of multiple strategies available for *Varroa* control (18). To avoid re-infestation varroacides should be applied after the main honey flow, on all apiaries of the same area, and in all honey bee colonies with mite infestations levels above the economic threshold (32). According to Almecija et al. (1) in the absence of tau-fluvalinate treatment (> 2 years), the susceptible genetic profile is present at 97% of the mites. This seems to imply that *Varroa* mites can regain their sensitivity to tau-fluvalinate quite quickly (after 2 years minimum without tau-fluvalinate treatment for their study. Knowledge of the reversion period for tau-fluvalinate can play a crucial role in the control against the establishment of *Varroa* mite resistance to pyrethroids.

The most used chemical acaricides must be included in rotation programs to decelerate the resistance of *Varroa destructor* mites to multiply used products and reduce the impact of increasing comb wax contamination.

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## Conflict of Interest

The authors declared that there is no conflict of interest.

## Author Contributions

KG, IZ and DS conceived and planned the experiments. KG and DS carried out the experiments. KG and DS contributed to sample preparation. KG, IZ and DS contributed to the interpretation of the results. IZ analyzed the data. DS took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

## Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

## Ethical Statement

This study does not present any ethical concerns.

## Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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# Molecular characterization of virulence genes in broiler chicken originated *Salmonella* Enteritidis and *Salmonella* Typhimurium

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## Abstract

*Salmonella* Enteritidis and *Salmonella* Typhimurium are the most common serovars observed in human salmonellosis while contaminated poultry products are the major source of *Salmonella* transmission to humans. Therefore, high pathogenicity of poultry originated *S.* Enteritidis and *S.* Typhimurium strains poses a serious risk to human health. In this study, we investigated the virulence genes of *S.* Enteritidis and *S.* Typhimurium strains isolated from litter and environmental samples of broiler chicken flocks. *SipA, sipD, sopB, sopD, sopE, sopE2, sitC, sifA, ssaR, spvC,* and *pefA* genes were investigated in a total of 137 strains consisting of 105 *S.* Enteritidis and 32 *S.* Typhimurium. Nine strains (6.6%) had all genes. No negative strain was detected for all genes. *SopE* was found in all strains (100.0%). *SitC* (89.1%), *ssaR* (83.9%), *sipA* (70.1%), *sipD* (73.0%), *sopE2* (68.6%), *spvC* (68.6%), and *pefA* (73.0%) were also highly prevalent. Noticeable differences were observed between serovars in terms of *sopE2, spvC* and *pefA* prevalence: 77.1%, 80% and 82.9%, respectively, of *S.* Enteritidis strains were *sopE2, spvC* and *pefA* positive while 40.6%, 31.3% and 40.6% of *S.* Typhimurium strains were positive. This finding indicates that *S.* Enteritidis is more frequent than *S.* Typhimurium in poultry populations due to its higher virulence. Based on virulence gene distribution, the strains were divided into 44 different virulence genotypes, with the major genotype 4 (15.3%) carrying 8 of the 11 genes. The majority of strains (75.9%) were positive for at least 6 genes. *S.* Enteritidis and *S.* Typhimurium strains were highly virulent and pose a threat as zoonotic infection agents.

## Introduction

*Salmonella* is a worldwide critical foodborne pathogen, responsible for more than 70% of foodborne outbreaks. Salmonellosis caused by non-typhoidal *Salmonella* is the second most common zoonotic infection worldwide, with 93.8 million cases and approximately 155.000 human deaths annually (1, 19). The genus consists approximately 2,600 serovars. According to the European Food Safety Authority (EFSA) 2019 zoonoses report, the most frequent serovars in human salmonellosis cases are *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) (50.3%) and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (20.1%). The most frequencies of *S.* Enteritidis and *S.* Typhimurium in broilers are 67.8% and 34.8%, respectively, and 26.7% and 13.5%, respectively, in layers (3). Contaminated poultry products (meat and eggs) are considered the major source of *Salmonella* transmission to humans. Therefore, the characterization of pathogenicity-associated genes in poultry originated *S.* Enteritidis and *S.* Typhimurium is critical for controlling human salmonellosis outbreaks (6, 22).

*Salmonella* pathogenicity depends on the presence of virulence genes that encode various proteins responsible for adhesion, recognition, invasion, internalization, iron acquisition, neutralization, proliferation, and survival. They are located on plasmids or in *Salmonella* pathogenicity islands (SPIs). Among the 24 SPIs, SPI-1 and SPI-2 play major roles in *Salmonella* pathogenesis. SPI-1 and SPI-2 are DNA regions of approximately 40 kb located on the bacterial chromosome (12, 17). SPI-1

contains several virulence genes, including *sipA, sipD, sopB, sopD, sopE* and *sitC*, which encode effector proteins. *SipA, SopB, sopD* and *sopE*, are involved in adhesion to and invasion of host cells whereas *sipD* is responsible for translocation and *sitC* is involved in proliferation and iron acquisition (1, 14). SPI-2 contains virulence genes *sifA* and *ssaR,* associated with internalization, replication, and survival within host cells (1). Some *Salmonella* virulence genes are associated with plasmids, such as *spvC* and *pefA*. *SpvC* is carried on the 94.7 kb serovar-specific plasmid that can be used for serovar identification. It is responsible for survival and proliferation within macrophages. *PefA* encodes the fimbriae that mediate adhesion to host cells (2, 17).

This study analyzed the virulence genes of broiler chicken originated *S.* Enteritidis and *S.* Typhimurium strains in order to improve the detection of correlations between virulence genotypes, serovars, and pathogenicity.

## Materials and Methods

***Salmonella* strains and conventional confirmation:** *Salmonella* strains were derived from the culture collection at Ankara University Faculty of Veterinary Medicine, Microbiology Department. The strains originated from litter (n=102) and environmental (n=35) samples of different broiler chicken flocks in Türkiye, 2012-2021. A total of 137 strains were used, including 105 *S.* Enteritidis and 32 *S.* Typhimurium. *Salmonella* serovar confirmation was performed by conventional serotyping using commercial *Salmonella* antisera (SSI, Denmark) and Kauffmann-White-Le Minor scheme (5). The strains were stored in 20% glycerol at -80°C until molecular characterization.

***Molecular characterization of virulence genes:*** The genomic DNA was extracted using by boiling the bacterial suspensions for 10 min at 100°C. DNA purifications and concentrations were measured by NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). Eleven different virulence genes, *sipA, sipD, sopB, sopD, sopE, sopE2, sitC, sifA, ssaR, spvC,* and *pefA*, were amplified by polymerase chain reaction (PCR) analyses (Table 1).

The PCR analyses were performed using the primers recommended by previous studies (Table 2). The reactions were conducted in a total mixture volume of 25 µl, comprising 0.2 µl Taq polymerase (2U/µl) (Thermo Scientific, USA), 0.5 µl 10 mM dNTPs, 1 µl of each 10 mM primer, 2.5 µl of 10xbuffer, 3 µl MgCl2, 14.8 µl nuclease free water, and 2 µl template DNA. The amplifications were conducted as follows: initial denaturation for 3 min at 95°C; 30 cycles of 60 s at 94°C; 20 s at 60°C; 1 min at 72°C; and final extension for 7 min at 72°C. The amplicons were analyzed by electrophoresis on 1.5% agarose gel (Thermo Scientific, USA) and visualized in G: Box Chemi gel imaging system (SynGene, India). The virulence genotypes were defined by the combinations of virulence genes for each strain.

**Table 1.** Gene locations, virulence genes, and virulence functions.

| Gene locations | Virulence genes | | Virulence functions |
|---|---|---|---|
| SPI-1 | *sipA* | *Salmonella* inner protein A | Host adhesion/invasion |
| | *sipD* | *Salmonella* inner protein D | Host adhesion/invasion |
| | *sopB* | *Salmonella* outer protein B | Host adhesion/invasion |
| | *sopD* | *Salmonella* outer protein D | Host adhesion/invasion |
| | *sopE* | *Salmonella* outer protein E | Host adhesion/invasion |
| | *sopE2* | *Salmonella* outer protein E2 | Host adhesion/invasion |
| | *sitC* | *Salmonella* iron transport C | Iron acquisition |
| SPI-2 | *sifA* | *Salmonella*-induced filament A | Filamentous structure formation |
| | *ssaR* | Secretion system apparatus R | Replication and survival |
| Plasmid | *spvC* | *Salmonella* virulence plasmid C | Replication and survival |
| | *pefA* | Plasmid-encoded fimbriae A | Host adhesion/invasion |

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**Table 2.** Virulence genes, primer sequences, amplicon sizes, and references.

| Virulence genes | Primer sequences (5′-3′) | Amplicons sizes (bp) | References |
|---|---|---|---|
| *sipA* | F-ATGGTTACAAGTGTAAGGACTCAG<br>R-ACGCTGCATGTGCAAGCCATC | 2055 | (12) |
| *sipD* | F-ATGCTTAATATTCAAAATTATTCCG<br>R-TCCTTGCAGGAAGCTTTTG | 1029 | (12) |
| *sopB* | F-GCTCTAGACCTCAAGACTCAAGATG<br>R-GCGGCCGCTACGCAGGAGTAAATCGGTG | 1987 | (11) |
| *sopD* | F-GAGCTCACGACCATTTGCGGCG<br>R-GAGCTCCGAGACACGCTTCTTCG | 1291 | (11) |
| *sopE* | F-ATTGTTGTGGCGTTGGCATCGT<br>R-AATGCGAGTAAAGATCCGGCCT | 376 | (22) |
| *sopE2* | F-TACTACCATCAGGAGG<br>R-GAATGTTTTATGTGACGCAG | 995 | (11) |
| *sitC* | F-CAGTATATGCTCAACGCGATGTGGGTCTCC<br>R-CGGGGCGAAAATAAAGGCTGTGATGAAC | 768 | (14) |
| *sifA* | F-ATGCCGATTACTATAGGCAATGG<br>R-TTATAAAAAACAACATAAACAGCCG | 1011 | (7) |
| *ssaR* | F-GTTCGGATTCATTGCTTCGG<br>R-TCTCCAGTGACTAACCCTAACCAA | 1628 | (7) |
| *spvC* | F-ACTCCTTGCACAACCAAATGCGGA<br>R-TGTCTCTGCATTTCGCCACCATCA | 571 | (2) |
| *pefA* | F-GCGCCGCTCAGCCGAACCAG<br>R-GCAGCAGAAGCCCAGGAAACAGTG | 157 | (14) |

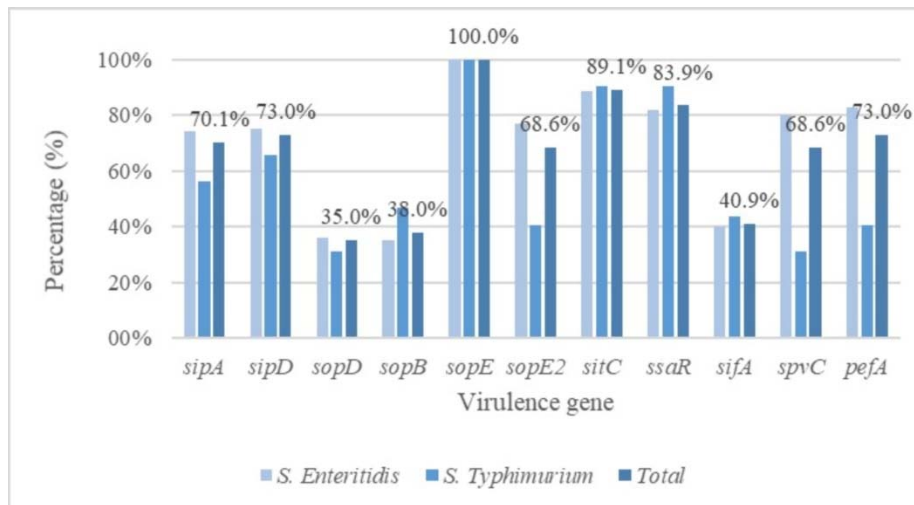


**Figure 1.** Percentages of the virulence genes in *S.* Enteritidis and *S.* Typhimurium strains.

## Results

Figure 1 presents the PCR findings for the 11 virulence genes. All virulence genes were found in nine strains (6.6%), specifically eight *S.* Enteritidis strains and one *S.* Typhimurium strain. No strain was negative for all genes. *SopE* was found in all strains (100.0%), followed by *sitC* (89.1%) and *ssaR* (83.9%) while *pefA* (73.0%), *sipD* (73.0%), *sipA* (70.1%), *sopE2* (68.6%), and *spvC* (68.6%) all had prevalence levels above 50%. The lowest prevalences were for *sopD* (35.0%), *sopB* (38.0%), and *sifA* (40.9%).

The virulence genotypes were investigated in all *Salmonella* strains. All strains were divided into 44 virulence genotypes (genotypes 1-44) (Table 3). Thirty-one different genotypes were detected in *S.* Enteritidis strains while 21 different genotypes were detected in *S.* Typhimurium strains. The dominant genotype (15.3%, 21/137) was genotype 4, namely *sipA, sipD, sopE, sopE2, sitC, ssaR, spvC* and *pefA* (+), and *sopD, sopB* and *sifA* (-). The next two most highly prevalent genotypes were genotype 21 (9.5%, 13/137) and genotype 1 (8.8%, 12/137).

**Table 3.** Combinations, numbers, and percentages of virulence genotypes.

| **Combinations of virulence genes** | | | | | | | | | | | **Number of strains** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Genotypes** | ***sipA*** | ***sipD*** | ***sopB*** | ***sopD*** | ***sopE*** | ***sopE2*** | ***sitC*** | ***sifA*** | ***ssaR*** | ***spvC*** | ***pefA*** | ***S.* Enteritidis** | ***S.* Typhimurium** | **Patterns (%)** | |
| **1** | + | + | + | + | + | + | + | + | - | + | + | 12 | 0 | 12 | 8.8% |
| **2** | + | + | + | + | + | + | + | + | + | + | + | 8 | 1 | 9 | 6.6% |
| **3** | + | + | + | + | + | + | + | - | - | + | + | 2 | 0 | 2 | 1.5% |
| **4** | + | + | - | - | + | + | + | - | + | + | + | 20 | 1 | 21 | 15.3% |
| **5** | - | + | - | - | + | + | + | - | + | + | + | 4 | 0 | 4 | 2.9% |
| **6** | - | - | - | - | + | + | + | - | - | + | + | 1 | 0 | 1 | 0.7% |
| **7** | + | - | - | - | + | + | - | - | - | + | + | 2 | 0 | 2 | 1.5% |
| **8** | - | + | - | - | + | + | + | + | + | + | + | 1 | 0 | 1 | 0.7% |
| **9** | + | + | - | + | + | + | + | + | + | + | + | 4 | 1 | 5 | 3.6% |
| **10** | + | + | + | - | + | + | + | + | + | + | + | 3 | 3 | 6 | 4.4% |
| **11** | - | - | - | - | + | + | + | + | + | + | + | 3 | 0 | 3 | 2.2% |
| **12** | - | - | - | - | + | + | - | - | + | + | + | 1 | 0 | 1 | 0.7% |
| **13** | - | + | + | + | + | + | + | + | + | + | + | 2 | 0 | 2 | 1.5% |
| **14** | + | + | + | + | + | + | + | + | + | - | + | 3 | 2 | 5 | 3.6% |
| **15** | + | + | + | + | + | + | + | - | + | - | + | 3 | 0 | 3 | 2.2% |
| **16** | + | - | + | + | + | + | + | - | + | - | + | 1 | 0 | 1 | 0.7% |
| **17** | - | + | - | - | + | + | + | + | + | - | + | 1 | 0 | 1 | 0.7% |
| **18** | - | + | - | - | + | - | + | - | + | + | - | 3 | 1 | 4 | 2.9% |
| **19** | - | + | - | - | + | - | + | - | + | - | - | 2 | 2 | 4 | 2.9% |
| **20** | - | - | - | - | + | - | - | - | + | + | + | 1 | 0 | 1 | 0.7% |
| **21** | - | - | - | - | + | - | + | - | + | - | - | 7 | 6 | 13 | 9.5% |
| **22** | + | - | - | - | + | - | + | - | + | + | + | 6 | 0 | 6 | 4.4% |
| **23** | + | - | - | - | + | - | + | - | + | + | - | 1 | 0 | 1 | 0.7% |
| **24** | - | + | - | - | + | - | + | - | + | - | + | 1 | 0 | 1 | 0.7% |
| **25** | + | - | - | + | + | - | + | - | + | + | + | 1 | 0 | 1 | 0.7% |
| **26** | + | + | - | - | + | - | + | + | + | + | + | 1 | 0 | 1 | 0.7% |
| **27** | + | + | - | - | + | - | - | - | + | + | - | 1 | 0 | 1 | 0.7% |
| **28** | + | + | - | - | + | + | - | - | + | + | - | 3 | 0 | 3 | 2.2% |
| **29** | + | + | - | - | + | + | - | - | + | + | + | 4 | 0 | 4 | 2.9% |
| **30** | + | + | + | - | + | + | + | + | + | - | - | 1 | 0 | 1 | 0.7% |
| **31** | + | - | + | + | + | + | + | + | - | - | + | 2 | 1 | 3 | 2.2% |
| **32** | + | + | - | + | + | - | + | + | + | - | - | 0 | 1 | 1 | 0.7% |
| **33** | - | + | - | + | + | + | + | - | + | - | + | 0 | 1 | 1 | 0.7% |
| **34** | + | - | - | - | + | + | + | - | - | - | - | 0 | 1 | 1 | 0.7% |
| **35** | + | - | - | - | + | - | + | + | + | + | + | 0 | 1 | 1 | 0.7% |
| **36** | + | + | - | - | + | - | + | - | + | - | - | 0 | 1 | 1 | 0.7% |
| **37** | + | + | - | - | + | - | + | + | + | + | - | 0 | 1 | 1 | 0.7% |
| **38** | + | + | + | - | + | - | + | + | + | - | - | 0 | 1 | 1 | 0.7% |
| **39** | + | + | + | - | + | - | - | - | + | - | + | 0 | 1 | 1 | 0.7% |
| **40** | - | - | + | - | + | - | + | + | + | - | - | 0 | 1 | 1 | 0.7% |
| **41** | - | + | + | + | + | - | + | - | + | - | - | 0 | 1 | 1 | 0.7% |
| **42** | - | + | + | - | + | - | - | - | - | - | - | 0 | 1 | 1 | 0.7% |
| **43** | - | + | + | - | + | + | + | - | + | - | + | 0 | 1 | 1 | 0.7% |
| **44** | + | - | + | + | + | + | + | + | + | + | - | 0 | 1 | 1 | 0.7% |
| **Total number of strains** | | | | | | | | | | | | 105 | 32 | 137 | |

## Discussion and Conclusion

Salmonellosis is one of the most common foodborne infections worldwide, transmitted to humans through infected animals and consumption of contaminated food, most commonly due to contaminated poultry products (18). Therefore, monitoring of poultry originated *Salmonella* pathogenicity is recommended to control human salmonellosis (6).

In this study, we analyzed the virulence genes of *S.* Enteritidis and *S.* Typhimurium strains isolated from litter and environmental samples of broiler chicken flocks. Eleven different virulence genes (*sipA, sipD, sopB, sopD, sopE, sopE2, sitC, sifA, ssaR, spvC,* and *pefA*) were investigated in 137 strains. The detected high prevalences for most genes were compatible with previous studies (1, 8). These genes are also commonly detected in clinical isolates. Some previous studies have reported poultry isolates that have similar virulence genes as human isolates and are human pathogens (4, 10, 14). *S.* Enteritidis and *S.* Typhimurium are the most common serovars observed in salmonellosis cases of humans, as reported by both the EFSA zoonoses report and recent clinical studies (3, 16, 20). This suggests that the high pathogenicity of poultry originated *S.* Enteritidis and *S.* Typhimurium strains poses a serious risk to human health.

Regarding specific bacterial genes, *sipA* and *sipD*, which are responsible for the invasion of host cells and significantly contribute to salmonellosis, were found in approximately 70% of all strains in our study. Shah et al. (12) reported expression levels of *sipA* and *sipD* that were respectively 16 and 11-fold higher in more invasive strains than less invasive strains. Thus, the high prevalences we found are worrisome in terms of pathogenicity.

The prevalence of *sopB*, which is responsible for adhesion to and internalization into host cells, had 38.0% prevalence in all strains. This contrasts with previous studies. Skyberg et al. (14) and Zou et al. (22) respectively reported 100.0% and 79.0% *sopB* prevalences in poultry originated *Salmonella* strains. Mezal et al. (10) reported 100.0% *sopB* prevalence in *S.* Enteritidis strains while Krawiec et al. (9) and Farahani et al. (4) respectively reported 94.45% and 99.6% *sopB* prevalence in *S.* Enteritidis strains. The low *sopB* prevalence detected in our study could be due to the presence of endemic strains.

All the strains in our study were *sopE* positive (100.0%). Since all strains studied have *sopE* and the major cause of human salmonellosis is poultry originated *S.* Enteritidis and *S.* Typhimurium, this finding indicates a significant risk for human health. Moreover, *sopE2* was positive in 68.6% of all strains, although the serovars differed significantly: while 77.1% of *S.* Enteritidis strains were *sopE2* positive, only 40.6% of *S.* Typhimurium strains were.

The other genes located in SPIs are *sitC, ssaR,* and *sifA*. We found *sitC, ssaR,* and *sifA* in 89.1%, 83.9% and 40.9% of all strains, respectively. Mezal et al. (10) reported that *sitC* belongs to SPI-1 and is associated with iron acquisition, which is thought to be important in pathogenesis. The high *sitC* prevalence in our study is similar to that reported by Mezal et al. (10) (91.6%), Krewiec et al. (9) (94.45%), and Farahani et al. (4) (97.9%). The SPI-2-located *ssaR* facilitates proliferation, survival in phagocytic cells and the spread of systemic infection. Almeida et al. (1) and Sever and Akan (8) also detected high prevalence of ssaR. SPI-2-located *sifA* are associated with filamentous structure formation (8, 21). The high positivity detected in our study is compatible with Mezal et al. (10). Furthermore, *sifA* has previously been detected in 93.3% and 97.9% of all *S.* Enteritidis strains, and 100% of all *S.* Typhimurium strains (4, 9, 10). In short, the high *sifA* prevalence detected in our study is compatible with previous studies.

*SpvC,* which is associated with survival in macrophages, infects reticuloendothelial system organs, such as spleen, lymph nodes, and liver. The other plasmid virulence gene, *pefA*, plays an essential role in adhesion to host cells. We detected *spvC* and *pefA* positivity in 68.6% and 73.0% of all strains, which are quite high rates compared with previous findings (14, 15). This may reflect differences in strain origins, and the pathogenicity of epidemic strains and serovars. We found lower *spvC* and *pefA* prevalence compared with virulence genes on the chromosome, except for *sopD* (35.0%), *sopB* (38.0%) and *sifA* (40.9%). Both genes are carried and transferred by virulence plasmids, which can be found on serovars or be strain specific. Therefore, *spvC* and *pefA* are likely to be less prevalent than the virulence genes carried by the chromosome.

We also detected a difference in the prevalence of *spvC* and *pefA* between serovars. While 80.0% and 82.9% of *S.* Enteritidis strains were *spvC* and *pefA* positive, respectively, 31.3% and 40.6% of *S.* Typhimurium strains were *spvC* and *pefA* positive, respectively. Yue et al. (20) also reported higher positivity for *spvB, spvR,* and *pefA* in *S.* Enteritidis strains compared to *S.* Typhimurium strains. Similarly, Siddiky et al. (13) reported that 92.0% of *S.* Enteritidis strains had *spvC* whereas only 28.0% of *S.* Typhimurium did. These findings suggest that the reason *S.* Enteritidis (50.3%) is more commonly observed than *S.* Typhimurium (20.1%) in poultry populations is due to the higher adhesion and invasion ability of *S.* Enteritidis. In other words, the higher prevalence of *S.* Enteritidis strains in poultry samples increases the possibility of virulence gene acquisition by horizontal transfer. This explains the higher prevalences of virulence genes in *S.* Enteritidis than *S.* Typhimurium strains.

Based on the distribution of 11 virulence genes, *S.* Enteritidis and *S.* Typhimurium strains were genotyped into 44 different virulence genotypes (genotypes 1-44). Thirty-one different genotypes were detected in 105 *S.* Enteritidis strains while 21 different genotypes were detected in 32 *S.* Typhimurium strains. The *S.* Typhimurium strains had greater diversity of genotypes (68.8%) than *S.* Enteritidis strains (29.5%). The major genotype 4 (15.3%, 21/137) was positive for all other genes except *sopD, sopB,* and *sifA*. The majority of strains (75.9%) were positive for at least six genes in 31 different genotypes.

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In this study, we analyzed the virulence genes of broiler chicken originated *S.* Enteritidis and *S.* Typhimurium strains. The findings contributed to understanding the pathogenicity and epidemiology of *Salmonella* strains in broilers. The high positivity detected for virulence genes provides clear evidence of the high pathogenicity of the serovars, which are common among both animals and humans. *S.* Enteritidis *and S.* Typhimurium strains were highly virulent and therefore pose a threat as a zoonotic infection. However, these findings need further support from molecular characterization and epidemiological analysis of virulence genes in human strains.

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## Author Contributions

SSI carried out the experiments, writing, and editing. MA contributed to the interpretation of the results and editing. SSI took the lead of writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

## Ethical Statement

This study does not present any ethical concerns.

## Conflict of Interest

The authors declared that there is no conflict of interest.

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# Shelf life extension of *Oncorhynchus aguabonita* fillets based on *Trachyspermum copticum* essential oil nanoemulsion coating during storage at 4°C

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## ABSTRACT

This study investigated the effects of *Trachyspermum copticum* essential oil nanoemulsion (TCEO-NE) coating on chemical, microbial and sensory changes of *Oncorhynchus aguabonita* fillets during storage at 4 °C. The components of *T. copticum* EO were identified using gas chromatography-mass spectrometry. TCEO-NE was prepared by ultrasonic method and its properties were determined. Fresh *O. aguabonita* fillets were immersed in TCEO-NE and stored at 4 °C. Chemical (pH, TVB-N, peroxide and TBARS), microbiological (total aerobic mesophilic bacteria, psychrotrophs, Enterobacteriaceae, lactic acid bacteria and *Staphylococcus aureus*) and sensory analyses (color, odor and taste) of fish fillets were evaluated on days 0, 2, 4, 6, 8 and 11. Thymol, limonene, and alpha-terpinene were the major compounds in *T. copticum* EO. The droplet size of TCEO-NE was 127.6 nm and PDI was 0.210. The control group exceeded the peroxide limit on day 6, while TCEO-NE 500.00 and 666.66 did so on day 8. The TBARS value in fish fillets was 0.57 mg MDA/kg on day zero which reached 4.76 mg MDA/kg in the control group and 2.90 mg MDA/kg in TCEO-NE 666.66 after 11 days at 4 °C. Aerobic mesophilic count in the control group exceeded the permissible level on day 6 and, in TCEO-NE 500.00 and 666.66, on day 8, therefore the shelf life of fish fillets was improved by two days. On the grounds of the favorable properties of TCEO-NE and its positive effects on chemical, microbial and sensory changes in fish fillets, it can be used as a natural food additive.

## Introduction

Fish and their products are rich sources of protein and contain large amounts of minerals, unsaturated fatty acids, water and fat-soluble vitamins, which make such products highly perishable with restrictions in storage even under refrigeration. Microbial and chemical spoilage in fish products occurs more quickly than in other animal products such as chicken and red meat (26, 67).

Various methods such as cold and freezing, heating and canning, drying, salting and adding preservatives can be used to prevent microbial and chemical spoilage in meat (17, 37, 78). Synthetic preservatives are used as a complementary method to increase the shelf life of food at low temperatures (16). Due to the increasing demand for food production, there is an urgent need to find and use safe food additives to improve food quality and shelf life.

Plant essential oils (EOs) are one of the food additives which increase the shelf life and improve the sensory properties of food by reducing or eliminating pathogens and preventing fat oxidation (72). EOs are secondary metabolites produced by aromatic plants. These substances are liquid and volatile compounds which are soluble in fats and organic solvents with lower density than water (12). The antimicrobial and antioxidant properties of EOs are well-known for many centuries (22, 74, 76). Substances such as carvacrol, eugenol and thymol belong to the phenolic group. The higher the number of phenolic groups, the greater the antimicrobial properties

of EOs (11). EOs can replace synthetic antioxidants and increase their shelf life by adding them to high-fat and perishable foods (46).

*T. copticum* is an annual plant of Apiaceae family that naturally grows in arid and semi-arid lands of India, Iran, Egypt, Afghanistan, Pakistan and Europe. The EO of this plant is rich in phenolic compounds such as thymol, cymene, β-pinene, γ-terpinene and sabinene (77). Chemical compounds of this EO have been identified previously (40, 45, 47, 49). Many traditional medicine books mentioned the antimicrobial and medicinal properties of *T. copticum*. Today, in traditional medicine, it is used to improve recovery weakness, cough, stomach pain, rheumatism, and also in treatment of various microbial infections and even for the treatment of abdominal tumors. Above all, *T. copticum* is also used as a flavoring and aromatizing agent in foods (8, 14).

EOs are hydrophobic compounds with very low solubility in water and are mostly soluble in non-polar and semi-polar solvents, alcohols, oils and waxes. They are sensitive to light, heat and air and easily oxidize, due to their molecular structure's double bonds and hydroxyl, aldehyde and ester (62). Another disadvantage of using EOs in food is the creation of a special taste and smell that the consumers may not like. The use of nanoemulsions of EOs allows for more solubility in water, increases the stability and protection of volatile compounds and improves the antimicrobial and antioxidant effects by boosting cell absorption and reducing the adverse impacts of EOs (18). Antimicrobial properties and food shelf life extension of essential oil nanoemulsions have been proved previously (64).

Golden trout (*Oncorhynchus aguabonita*) belongs to the Salmonidae. This fish is often referred to as the California golden trout and the native river trout, which live on the tributaries of the Kern River. Golden trout was chosen in this study due to its high consumption by the population in Iran, very little literature on this fish and the presence of unsaturated fatty acids and being more prone to oxidative spoilage (42).

This study aimed to investigate the effects of *T. copticum* EO nanoemulsion (TCEO-NE) coating on chemical, microbial and sensory properties of *O. aguabonita* fillet stored at 4 °C.

## Materials and Methods

***Preparation of EO and determination of its components:*** The EO of *T. copticum* was purchased on the market. The components of *T. copticum* EO were identified using gas chromatography (Thermoquest 2000, Manchester, UK) connected to a mass spectrometer (MSD5973). This device has an Hp5 capillary column and the data were obtained under the following conditions: initial temperature of 50 °C, the final temperature of 265 °C and injection temperature of 250 °C. Helium gas was used as a carrier gas at a rate of 1.1 ml/min with a separation ratio of 1:100. The mass spectrometer has an ionization energy of 70 electron volts and an interface temperature of 250 °C (54).

***Preparation of nanoemulsion:*** The ultrasonication emulsification method has been used to prepare a variety of essential oil nanoemulsions (10, 69, 70). Generally, oil in water nanoemulsions are very stable and can only be separated into two phases quickly using unique methods such as electric fields (34, 35). To make TCEO-NE at room temperature, first, the weight of *T. copticum* EO, double-distilled water, and a mixture of some surfactants (tween 80/ span 80) were calculated and were mixed together. It was transferred to an ultrasonic device to apply ultrasound and produce the nanoemulsion (model UP400S, maximum power 400 w, frequency 20 kHz, Hielscher, Germany). TCEO-NE was prepared at concentrations of 500.00 and 666.66 µl/ml with an ultrasound time of 300 seconds, an ultrasound cycle of 0.75% and an ultrasound intensity of 208 w/cm2 by ultrasound apparatus. These concentrations were chosen according to another study by the authors regarding the antibacterial effect of this nanoemulsion studied by disc diffusion assay, determination of MIC and MBC and bacterial growth kinetics (33).

***Determination of nanoemulsion properties:*** The average droplet size and polydispersity index (PDI) of TCEO-NE were obtained by Dynamic Light Scattering (DLS) method using nano series zetasizer (Nano ZS model, ZEN 3600, Malvern, UK) with a constant dispersion angle of 173°. In this study we used Shahavi et al. (70) method to prepare stable EO nanoemulsions. The measurements were repeated three times at 25 °C. Zetasizer Nano software (version 7.03) was used to collect and analyse the obtained data.

***Preparation of treatments:*** Live golden trouts (*Oncorhynchus aguabonita*) were purchased from a fish farm in Amol, then their head, tail and fins were removed and the contents of the abdomen were emptied and they were immediately transferred to the food hygiene laboratory of the Faculty of Veterinary Medicine in Amol University of Special Modern Technologies, Amol, Iran. Then, under full observance of hygienic principles, the fish was divided into 50 g fillets. The fillets were divided into three groups of control, TCEO-NE 500.00 and TCEO-NE 666.66. The control group was treated with double-distilled water and a mixture of some surfactants (tween 80/ span 80) while, each of TCEO-NE 500.00 and TCEO-NE 666.66 fillets were immersed in 200 ml TCEO-NE with concentrations of 500.00 µl/ml and 666.66 µl/ml for 3 min at room temperature, respectively. They were

then placed in zippered nylon bags sterilized by UV and labeled, then transferred to a refrigerator at 4 °C (19). All the tests were done two times. The TBARS value determination was performed at 0, 2, 4, 6, 8, and 11th day at 4 °C and also at the 30th day under freeze condition (55).

***Analysis of the approximate composition of fish fillets:*** The moisture content (2), ash (3), protein (4) and fat (5) of fish fillets in the control group on day zero were determined according to the method proposed by the Association of Official Agricultural Chemists.

***Chemical analyses:*** The pH values of fish fillets on days 0, 2, 4, 6, 8 and 11 were quantified by immersing the glass electrode of a digital pH meter (Mettler Toledo, Seven Easy, USA) in the homogenized solution of 10 grams of the ground beef with 90 ml of distilled water (75).

Total Volatile Basic Nitrogen (TVB-N) of the samples (mg/100 g fish meat) was determined according to (AOAC) (6). Peroxide value (meq/kg fish meat) was quantified in 1 g of fish meat based on (AOAC) (7). Thiobarbituric Acid Reactive Substances (TBARS) values of the samples were measured using 5 g of fish fillet and absorbance readings at a wavelength of 532 nm (44).

***Microbiological analyses:*** To perform microbial tests, 10 g of each sample was homogenized with 90 ml of 0.1% sterile buffered peptone water (i23029 Ibresco, Iran) for 3 min in sterile bags in a stomacher (Iul Masticator Classic 400 ml, 240 W, Barcelona, Spain). Then, making dilutions was performed with 0.1% peptone water and the dilutions were cultured in culture media (duplicate) and were counted after incubation at the required temperature and time (73). Total counting of aerobic mesophilic bacteria was done by surface culture method in Plate Count Agar (PCA) medium (105463 Merck, Germany) after incubation at 37 °C for 48 h (63). Psychrotrophs were counted by surface culture in PCA medium after incubation at 7 °C for 10 days (23). *Enterobacteriaceae* were counted by pour plate method in Violet Red Bile Glucose agar (VRBG) medium (i23193 Ibresco, Iran) and incubation was performed at 30 °C for 24 h (25). Lactic acid bacteria were counted by surface culture in MRS agar medium (110660, Merck, Germany). Incubation was performed at 30 °C for 48 h under anaerobic conditions (using anaerocult® A gas packs, Merck, Germany) (25). *S. aureus* was counted by surface culture in Baird Parker medium (i23013 Ibresco, Iran) after incubation at 37 °C for 48 h (27). Colony count results were reported as log cfu/g.

***Sensory evaluation:*** Sensory analysis was performed by a trained group of 6 students and staff of Amol University of Special Modern Technologies, unaware of the samples' nature. They were 2 women (21 and 35 years old) and 4 men (26, 27, 30 and 35 years old). To score the color index, the samples were randomly divided among them in an environment that was almost white without the evaluators being aware of the nature of the samples, and they used an 8-point scale for scoring (8 = very bright red, 7 = relatively light red, 6 = light red, 5 = low light red, 4 = bold red, 3 = light dark red, 2 = relatively dark red, 1 = dark red). To score the odor index, the samples were randomly divided among them in a well-ventilated environment without the evaluators being aware of the nature of the samples. Then they used a 9-point scale for scoring. Moreover, to score the taste index, first the fillets were fried in a small amount of frying oil and the oil was removed. Next, without the evaluators being aware of the type of samples, they were randomly divided between them and they used a 9-point scale to rate the fried fillets (0 = very undesirable, 9 = very desirable) (13, 32, 36, 51, 71, 73).

***Statistical analysis:*** Shapiro-Wilk test was performed to check the normality of the data. The linear procedure model test for Repeated Measures ANOVA and Bonferroni post hoc tests were performed to compare microbial and chemical change trends during each group's study period. For comparison between groups at any time, one-way ANOVA and Tukey post hoc tests were performed. Regarding the variables of color, odor and taste, non-parametric Friedman test was selected to compare the trend of their changes during the study period in each group and also Wilcoxon signed-rank test with Bonferroni correction was used to evaluate the two measured indices between different times. Moreover, Kruskal-Wallis non-parametric test was performed to compare groups at any time and Mann-Whitney U-test with Bonferroni correction was used to compare them in pairs. Results were expressed based on mean and standard deviation. Data analysis was performed using SPSS statistical software version 25 (SPSS Inc., Chicago, IL, USA). In all analyses, a significance level of less than 5% was considered.

## Results

The results regarding the identification of the components of *T. copticum* EO by GC/MS are shown in Table 1 and Figure 1. Sixteen different components of *T. copticum* EO were identified, accounting for 97.9% of the EO. Thymol (53.17%), limonene (21.12%) and alpha-terpinene (19.46%) were the three major compounds determined ($P<0.05$).

Droplet size and PDI of TCEO-NE using the DLS method were 127.6 nm and 0.21 (Figure 2). The average moisture, ash, protein and fat in *O. aguabonita* fillets on day zero were 73.66%, 1.30%, 17.50% and 1.36%, respectively.

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**Table 1**. The amount of compounds identified in *T. copticum* EO by GC/MS.

| No. | Constituent | Quantity (%) | Retention Time (min) |
|---|---|---|---|
| 1 | α-thujene | 0.31 | 10.86 |
| 2 | α-pinene | 0.17 | 11.95 |
| 3 | Limonene | 21.12 | 13.12 |
| 4 | Myrcene | 0.74 | 14.02 |
| 5 | α-terpinene | 19.46 | 15.16 |
| 6 | p-cymene | 0.21 | 16.25 |
| 7 | D-limonene | 0.63 | 17.48 |
| 8 | Cineol | 0.54 | 18.33 |
| 9 | γ-terpinene | 0.48 | 19.55 |
| 10 | Terpinolene | 0.17 | 20.47 |
| 11 | Thymol | 53.17 | 22.19 |
| 12 | α-terpineol | 0.21 | 25.88 |
| 13 | Carveol Acetate | 0.36 | 27.63 |
| 14 | Carvone | 0.17 | 29.46 |
| 15 | Carvacrol | 0.12 | 31.89 |
| 16 | Bergaptene | 0.04 | 34.19 |
| | **Total** | **97.9** | |

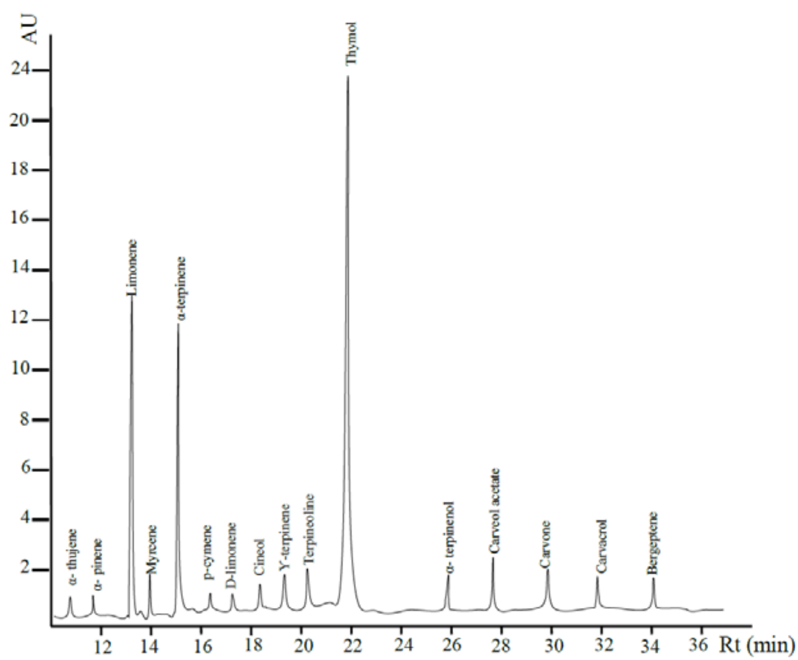


**Figure 1.** GC-MS chromatogram of *T. copticum* EO.

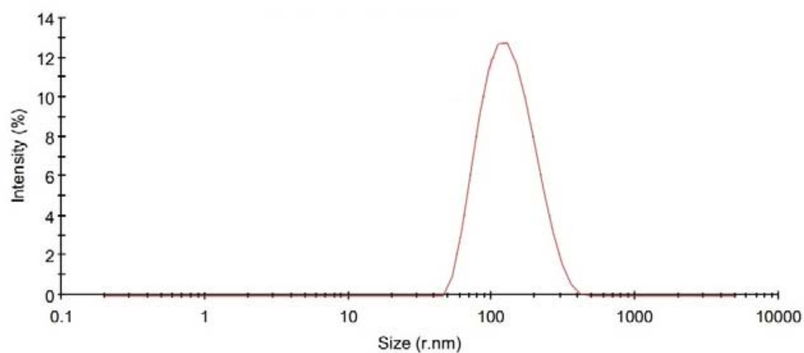


**Figure 2.** Particle size distribution of *T. copticum* essential oil nanoemulsion

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**Table 2**. Chemical properties of *O. aguabonita* fillet coated with *T. copticum* EO nanoemulsion stored at 4 °C.

| Parameter | Group \ Day | 0 | 2 | 4 | 6 | 8 | 11 |
|---|---|---|---|---|---|---|---|
| **pH** | Control | 7.00±0.06$^{a,A}$ | 7.18±0.03$^{a,AB}$ | 7.30±0.04$^{a,AB}$ | 7.39±0.07$^{a,AB}$ | 7.53±0.04$^{a,AB}$ | 7.78±0.03$^{a,B}$ |
| | TCEO-NE 500.00 | 7.00±0.06$^{a,A}$ | 7.10±0.01$^{b,AB}$ | 7.21±0.02$^{a,AB}$ | 7.30±0.05$^{ab,AB}$ | 7.43±0.02$^{a,AB}$ | 7.51±0.03$^{b,B}$ |
| | TCEO-NE 666.66 | 7.00±0.06$^{a,A}$ | 7.08±0.01$^{b,AB}$ | 7.12±0.03$^{b,AB}$ | 7.21±0.08$^{b,AB}$ | 7.26±0.06$^{b,AB}$ | 7.39±0.01$^{c,B}$ |
| **TVB-N (mg/100 g)** | Control | 14.64±0.07$^{a,A}$ | 19.83±0.03$^{a,B}$ | 26.35±0.47$^{a,C}$ | 36.39±0.06$^{a,D}$ | 38.53±0.18$^{a,E}$ | 40.24±0.18$^{a,E}$ |
| | TCEO-NE 500.00 | 14.64±0.07$^{a,A}$ | 17.58±0.35$^{b,B}$ | 21.07±0.03$^{b,C}$ | 28.73±0.03$^{b,D}$ | 36.74±0.06$^{b,E}$ | 38.84±0.11$^{b,F}$ |
| | TCEO-NE 666.66 | 14.64±0.17$^{a,A}$ | 16.81±0.12$^{c,B}$ | 18.37±0.06$^{c,C}$ | 27.80±0.02$^{c,D}$ | 35.91±0.14$^{c,E}$ | 37.71±0.23$^{c,F}$ |
| **Peroxide value (meq/kg)** | Control | 2.10±0.15$^{a,A}$ | 5.09±0.52$^{a,A}$ | 7.78±0.61$^{a,A}$ | 10.71±0.32$^{a,B}$ | 12.69±0.17$^{a,C}$ | 16.52±0.35$^{a,D}$ |
| | TCEO-NE 500.00 | 2.10±0.15$^{a,A}$ | 3.54±0.09$^{b,A}$ | 5.55±0.30$^{b,A}$ | 7.64±0.35$^{b,B}$ | 11.61±0.24$^{b,C}$ | 14.23±0.07$^{b,D}$ |
| | TCEO-NE 666.66 | 2.10±0.15$^{a,A}$ | 3.11±0.08$^{b,A}$ | 4.48±0.28$^{b,A}$ | 6.28±0.43$^{c,A}$ | 10.12±0.07$^{c,B}$ | 13.60±0.18$^{c,C}$ |

*Different lowercase letters show a significant difference at any time point between the three groups (P<0.05).
** Different uppercase letters show a significant difference in each group between six time point (P<0.05).

The results of pH in *O. aguabonita* fillets coated with TCEO-NE are shown in Table 2. The pH was 7.00 on day zero, which reached 7.78 (P<0.05) during an increasing trend on day 11 in the control group, while this increasing trend was less steep in the other two groups and in TCEO-NE 500.00 and TCEO-NE 666.66 reached 7.51 and 7.39, respectively. On all days except day zero, there was a significant difference between the pH of the control group and the pH of TCEO-NE 666.66 (P<0.05), while only on days 2 and 11, a significant difference was observed between the control group and TCEO-NE 500.00.

The results of TVB-N in *O. aguabonita* fillets coated with TCEO-NE are demonstrated in Table 2. TVB-N of fish fillets was 14.64 mg/100 g on day zero, which reached 40.24, 38.84 and 37.71 in the control group, TCEO-NE 500.00 and TCEO-NE 666.66, respectively on day 11. Regarding TVB-N content, no difference was observed between the groups on day zero (P>0.05). At other time points, there were significant differences among all three groups (P<0.05).

The results of peroxide value in *O. aguabonita* fillets coated with TCEO-NE are shown in Table 2. The highest and the lowest amount of peroxide at the end of the storage period were 16.52 and 13.60 meq/kg belonging to the control group and TCEO-NE 666.66, respectively.

Figure 3 shows the amount of TBARS in *O. aguabonita* fillets coated with TCEO-NE and stored at 4 °C. TBARS value in fish fillets of the control group on day zero was 0.57 mg MDA/kg of fish and after 11 days of storage at 4 °C reached 4.76 mg MDA/kg fish meat and 2.90 mg MDA/kg fish meat in the control group and TCEO-NE 666.66, respectively. These values were 5.88, 4.65 and 3.48 mg MDA/kg fish meat on day 30 of the storage period (freezing conditions) in the control group, TCEO-NE 500.00 and TCEO-NE 666.66, respectively.

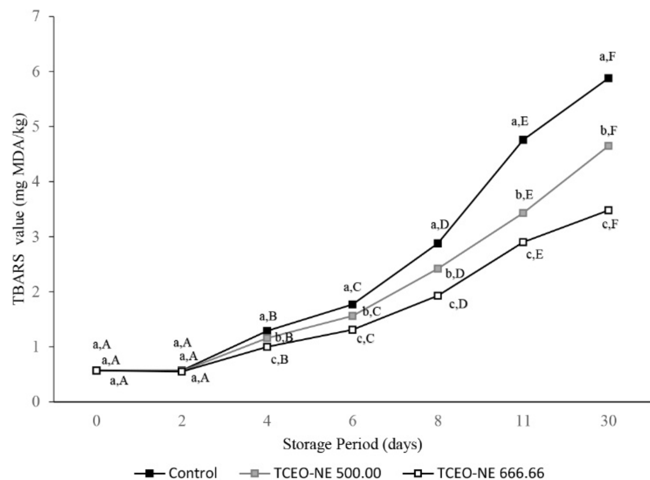


**Figure 3.** TBARS value of *O. aguabonita* fillet coated with *T. copticum* EO nanoemulsion stored stored at 4 °C and freezing condition.
* Different lowercase letters show a significant difference at any time point between the three groups (P<0.05).
** Different uppercase letters show a significant difference in each group between seven time points (P<0.05).

Figure 4 shows the total aerobic mesophilic count in *O. aguabonita* fillets coated with TCEO-NE and kept at 4 °C. This count increased in all groups during the storage period, with this trend being faster in the control group than in the other two groups. There was a significant difference between the control group and TCEO-NE

666.66 on all days except day zero. As can be seen in Figure 3, there was a significant difference among all groups on days 4, 6 and 8 (P<0.05).

Figure 4 shows the count of psychrotrophic bacteria in *O. aguabonita* fillet coated with TCEO-NE stored at 4 °C. Psychrotrophic bacteria count in fish fillets was 2.39 log cfu/g which reached 12.45 log cfu/g in the control group after 11 days of storage at 4 °C, while in TCEO-NE 666.66, this count reached 9.98 log cfu/g after 11 days of storage at 4 °C. There was a significant difference between the control group and the other two groups on all days except day zero (P<0.05), but TCEO-NE 500.00 and TCEO-NE 666.66 were not significantly different from each other.

*Enterobacteriaceae* count in *O. aguabonita* fillets coated with TCEO-NE, stored at 4 °C is shown in Figure 4. During the storage period, an increasing trend in the number of Enterobacteriaceae was observed in all groups. *Enterobacteriaceae* count in the control group reached 11.61 log cfu/g, while in TCEO-NE 500.00 and TCEO-NE 666.66 reached 10.58 log cfu/g and 8.41 log cfu/g at the end of the storage period, respectively. There was a significant difference among all groups regarding *Enterobacteriaceae* count on all days except day zero.

Changes in the number of lactic acid bacteria in *O. aguabonita* fillet coated with TCEO-NE and stored at 4 °C are shown in Figure 4. The difference of the number of lactic acid bacteria in the control group between day zero and day 11 was approximately 9 log cfu/g, but in TCEO-NE 666.66, it was approximately 5.5 log cfu/g. Significant differences were observed among the groups from day 6, so that on days 6, 8 and 11, all three groups had significant differences from each other (P<0.05).

Figure 4 shows the changes of *S. aureus* in the *O. aguabonita* fillet coated with TCEO-NE and stored at 4 °C. *S. aureus* count on day zero was 2.19 log cfu/g and in the control group, TCEO-NE 500.00 and TCEO-NE 666.66 reached 10.54 log cfu/g, 8.66 log cfu/g and 8.35 log cfu/g respectively, after 11 days of storage at 4 °C. *S. aureus* count in the control group was significantly different from the other two groups on all days except day zero, but no significant difference was observed between TCEO-NE 500.00 and TCEO-NE 666.66 (P<0.05).

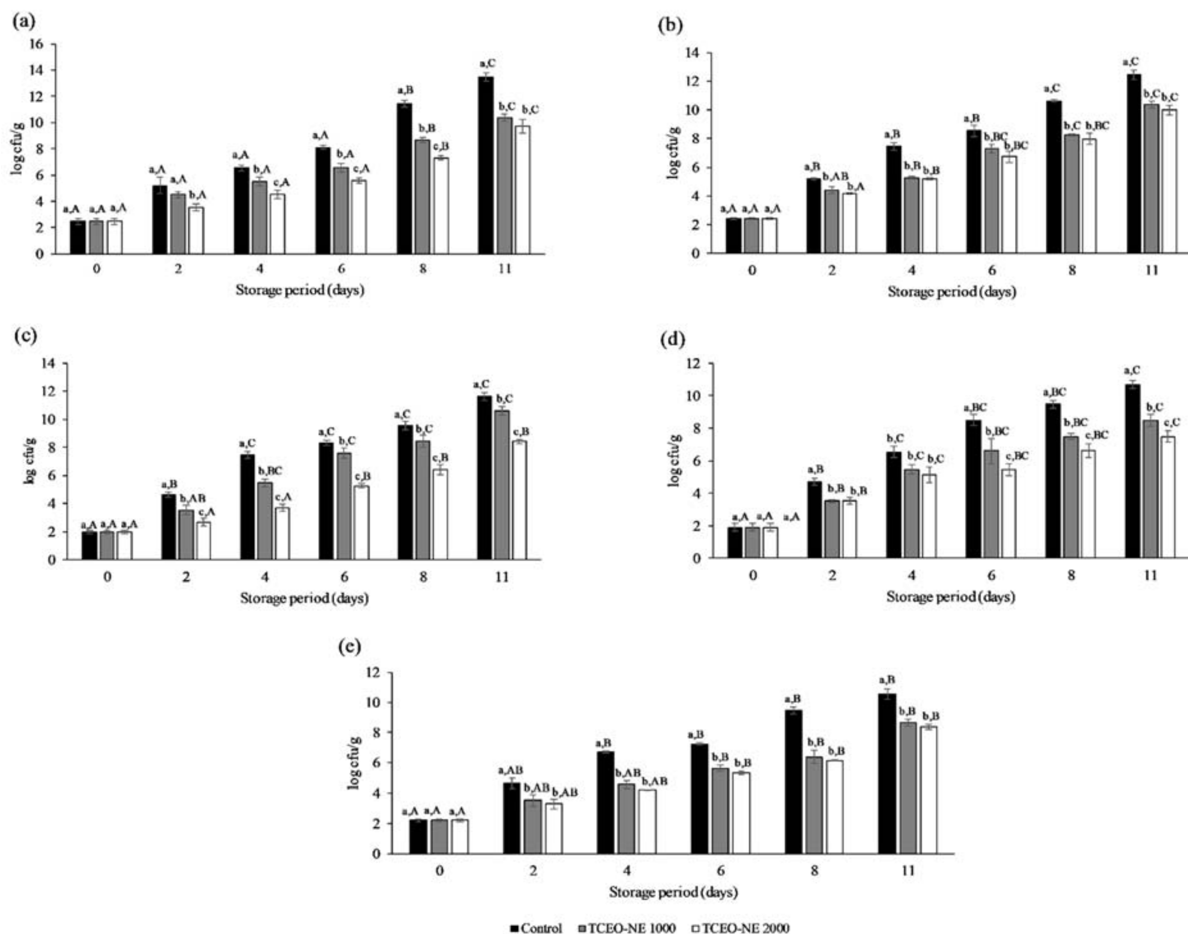


**Figure 4.** Microbial changes of *O. aguabonita* fillet coated with *T. copticum* EO nanoemulsion stored at 4 °C. (a): Total aerobic mesophilic count, (b): Psychrotrophs, (c): *Enterobacteriaceae*, (d): Lactic acid bacteria, (e): *S. aureus*

* Different lowercase letters show a significant difference at any time point between the three groups (P<0.05).

** Different uppercase letters show a significant difference in each group between seven time points (P<0.05).

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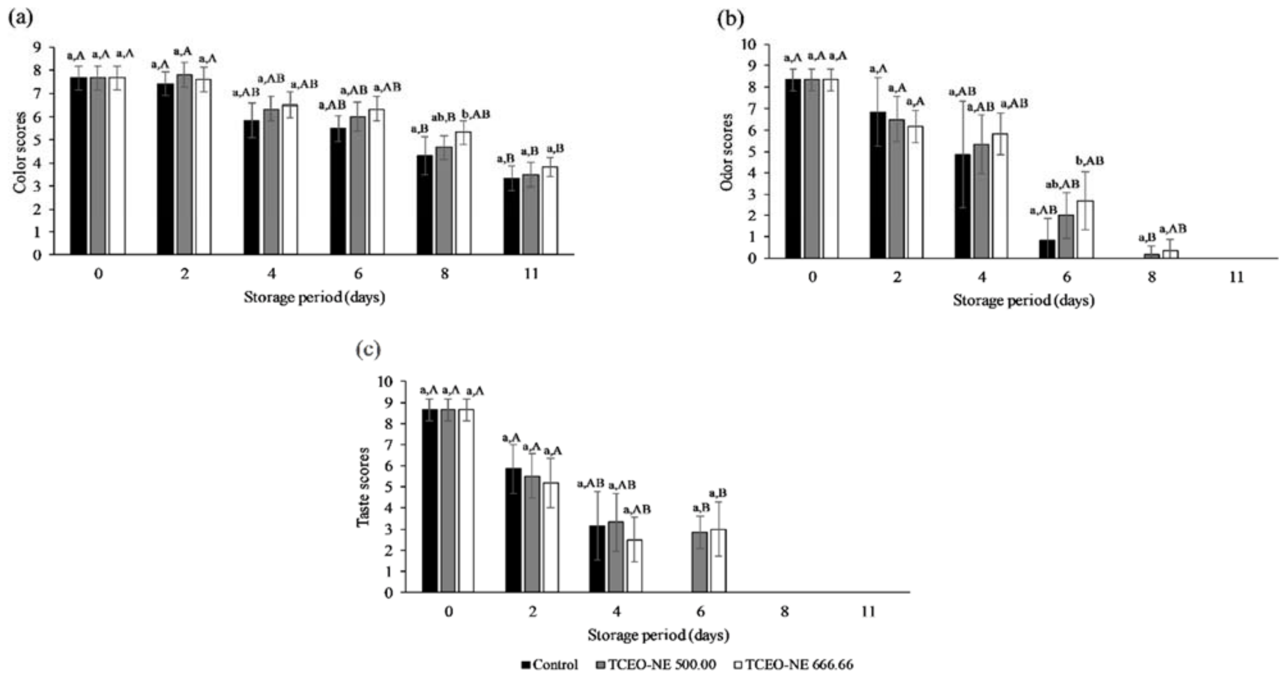


**Figure 5**. Sensory changes of *O. aguabonita* fillet coated with *T. copticum* EO nanoemulsion stored at 4 °C. (a): Color, (b): Odor, (c): Taste

* Different lowercase letters show a significant difference at any time point between the three groups (P<0.05).

** Different uppercase letters show a significant difference in each group between six time point (P<0.05).

Figure 5 shows the color changes of *O. aguabonita* fillet coated with TCEO-NE and stored at 4 °C. Color index had a decreasing trend during the study period which was more pronounced in TCEO-NE groups than in the control group (P>0.05). But the only significant difference was observed between the color of the control group and TCEO-NE 666.66 just on day 8 (P<0.05).

Figure 5 shows the odor changes in *O. aguabonita* fillet coated with TCEO-NE and stored at 4 °C. The odor index decreased during the storage period and this trend was more intense in the control group, so the control group did not receive any points on days 8 and 11 and TCEO-NE 500.00 and TCEO-NE 666.66 on day 11 (P<0.05).

Figure 5 shows the taste changes in *O. aguabonita* fillet coated with TCEO-NE and stored at 4 °C. In evaluating the taste of fish fillets, the groups containing TCEO-NE scored higher than the control group, so that the control group received no points on days 6, 8 and 11 and the other two groups on days 8 and 11. The taste index showed a significant difference between days 0 and 2 with days 6, 8 and 11 in all three groups (P<0.05).

## Discussion and Conclusion

In this study, the major constituent of *T. copticum* EO was thymol (53.17%). In the study of Jebelli Javan et al. (39) and Rabiei et al. (58), the major constituents of *T. copticum* EO were thymol, p-cymene and gamma-terpinene. Goudarzi et al. (30) also showed that the main constituent of *T. copticum* EO was thymol (36.7%). Differences between the constituents of *T. copticum* EO in different studies can be due to the genetic differences, geographical area of growth, plant age, part of the plant and the method used for essential oil extraction, and the type of solvent used in essential oil (11, 58).

According to our study, the droplet size of TCEO-NE was 127.6 nm, and PDI was 0.21. In other works, researchers focused on evaluating critical parameters for preparing stable essential oil nanoemulsions (69, 70). Their optimized formula was used for this study. Generally, oil in water nanoemulsions are very stable and can only be separated into two phases quickly using unique methods such as electric fields (34, 35, 69, 70). Naeim et al. (50) investigated the antifungal effect of *T. copticum* EO and its nanoemulsion against *Aspergillus niger* and its antioxidant properties in hamburgers and reported that the droplet size of the nanoemulsion was 146 nm. Sahraneshin Samani et al. (65) evaluated the effect of thyme and *T. copticum* EOs nanoemulsions in preventing the growth of *Byssochlamys fulva* in apple juice and showed that the droplet size of the nanoemulsion by ultrasound method was 15.13 nm and PDI was 0.253. Ozogul et al. (52) studied the effects of rosemary, laurel, thyme and sage EOs nanoemulsions on the sensory, chemical and microbial quality of rainbow trout fillets and reported the droplet size of the nanoemulsions prepared by the ultrasonic method as 112.82, 66.02, 63.02 and 59.48

nm, respectively. It seems that these differences in droplet size are due to the type of EOs, surfactants and methods of nanoemulsion preparation (19). The droplet size of nanoemulsions is as a criterion for determining their stability. Thus, the larger the droplet size of the nanoemulsion, the less stable the nanoemulsion (48).

We found that the moisture content of fillets was 73.66% on day zero. Askary Sary et al. (9) and Gokoglu et al. (29) reported the moisture content of rainbow trout carcasses as 77.9% and 73.38%, respectively. According to our study, the Ash content in fillets was 1.30% on day zero. Ash content in rainbow trout fillets was reported to be 1.57% and 1.35% (9, 29). In this study, the amount of protein in fillets was 17.50% on day zero. Askary Sary et al. (9) and Gokoglu et al. (29) reported the amount of protein in rainbow trout fillets as 19.46% and 19.80%, respectively. We found that the fat content of fillets was 1.36% on day zero. Askary Sary et al. (9) and Gokoglu et al. (29) reported the average fat content in rainbow trout fillets as 0.83% and 3.44%, respectively. Moisture, ash, protein and fat can vary based on fish species, size, diet, fishing time, spawning cycle, habitat and other environmental conditions (51).

In this study, the initial pH of the fillets was 7.00 on day zero and reached 7.78, 7.51, and 7.39 in the control group, TCEO-NE 500.00 and TCEO-NE 666.66, respectively on day 11. The increasing trend of pH value during the storage period is explained by the accumulation of nitrogen and alkali compounds such as ammonium and trimethylamines resulting from an increase in the number of spoilage bacteria and enzymatic activity (15). Durmus et al. (21) studied the effects of nanoemulsions of several edible oils (hazelnut, corn, canola, soybean, olive and sunflower) on chemical, sensory and microbial changes of vacuum-packed and refrigerated sea bass fillets. The lowest and the highest pH values at the end of the storage period (day 18) belonged to the group containing nanoemulsions of olive oil (6.88) and the control group (7.22), respectively. These differences were attributed to the inhibitory effect of the nanoemulsion on microbial growth. Ozogul et al. (51) reported that the initial pH of the control fish fillets was 6.89 on day zero and reached 7.21 on the final day of storage period (day 24). The lowest pH was reported in the group containing laurel nanoemulsion (7.07).

In the present study, TVB-N has reached the maximum acceptable level on day 4 in the control group and on day 6 in TCEO-NE 500.00 and TCEO-NE 666.66. TVB-N is an important indicator of fish quality and spoilage, which increases due to microbial spoilage and the activity of fish enzymes (61). The maximum acceptable level of TVB-N was 25 mg/100 g fish (28). Raeisi et al. (60) reported that rainbow trout flesh coated with 1.5 and 3% *T. copticum* extract, contained less than the permissible amount of TVB-N for up to days 9 and 15 of storage, respectively. This may be due to a rapid decrease in bacterial population or a decrease in the bacterial capacity for oxidative deamination of non-protein nitrogen compounds or a combination of these two mechanisms.

The maximum permissible level of peroxide in fish fillets for human consumption has been reported to be 10 meq/kg fish meat (57). In this study, the control group exceeded the acceptable limit on day 6 and TCEO-NE 500.00 and TCEO-NE 666.66 on day 8. Peroxide content is an indicator of fat oxidation that is used to measure hydroperoxides (24). Hydroperoxides are of the primary products of oxidation and tasteless compounds that consumers do not recognize. Still, secondary compounds such as aldehydes and ketones cause unpleasant taste and odor in food products (53). Raeisi et al. (60) investigated the antioxidant and antimicrobial effects of shallot (*Allium ascalonicum L.*) and ajwain (*T. ammi* (*L.*) Sprague) seed extract on semi-fried rainbow trout fillets. They found that the amount of peroxide in all treatments increased during the storage period, exceeding the allowable limit in the control group on day 6 and in the groups containing 1.5 and 3% of *T. copticum* seed extract on days 12 and 15, respectively.

TBARS index indicates the amount of secondary metabolites of fat oxidation, especially aldehydes (43). The maximum acceptable amount of TBARS in fish meat is 1-2 mg MDA/kg fish meat (52). We found that among the fillets stored in the refrigerator (4 °C), TCEO-NE 666.66 fillets exceeded the permissible limit on day 11, while the index in the other two groups exceeded the acceptable limit on day 8. Durmus (19) showed that none of the rainbow trout fillets containing citrus EO at the end of the storage period (day 16) exceeded the maximum TBARS level, but the control group exceeded this level on day 12 and reached 2.77 mg MDA/kg fish meat. The amount of TBARS in mandarin and grapefruit EOs reached 1.56 and 1.69 mg MDA/kg fish meat, respectively, which were the lowest amount of TBARS among all groups during the storage period.

In this study, the total aerobic mesophilic count on day zero was 2.47 log cfu/g indicating the fillets' good quality (66). The maximum allowable number of aerobic mesophilic count in fish is 7 log cfu/g (1). In the current study, aerobic mesophilic count in the control group exceeded the allowable level on day 6 and in TCEO-NE 500.00 and TCEO-NE 666.66, on day 8, increasing the shelf life by two days. A lower count of aerobic mesophilic in groups containing TCEO-NE may imply its antibacterial properties and its effective role in reducing the total microbial count. Similarly, Durmus (19) showed that aerobic mesophilic count in rainbow trout fillets was 2.43 log cfu/g on day zero. The lowest level of aerobic

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microorganisms at the end of the storage period was observed in the groups containing mandarin (7.43 lof cfu/g) and grapefruit nanoemulsions (7.63 log cfu/g). This amount exceeded the allowable level in the control group on the 10th day. Thus, the use of these nanoemulsions extended the shelf life from 4 to 6 days. Using virgin olive oil nanoemulsion extended the shelf life of lamb loins for 4 days (38). Shadman et al. (68) reported that aerobic mesophilic count in rainbow trout fillets in the control group was 2.31 log cfu/g which is similar to the present study.

One of the main causes of spoilage in fresh fish stored in the refrigerator is psychrotrophic bacteria, which produce compounds such as aldehydes and ketones that cause undesirable changes in taste, texture and odor, thereby reducing fish quality (20). The maximum allowable number of psychrotrophic bacteria is reported to be 7 log cfu/g (20, 66). We found that the number of psychrotrophic bacteria in the control group, TCEO-NE 500.00 and TCEO-NE 666.66 groups exceeded the allowable limit on days 4, 6 and 8, respectively, which may be due to the presence of compounds such as thymol, limonene, alpha-terpinene and other antibacterial compounds in *T. copticum* EO.

*Enterobacteriaceae* are a health indicator for fish meat and are part of the natural microbial flora of fresh trout (1). In the present study, the initial number of *Enterobacteriaceae* in fillets was 1.97 log cfu/g on day zero and reached 11.61, 10.58, and 8.41 in the control group, TCEO-NE 500.00 and TCEO-NE 666.66, respectively on day 11. Durmus (19) reported the initial number of *Enterobacteriaceae* in rainbow trout fillet to be between 1 to 1.35 log cfu/g and at the end of the storage period (day 16), *Enterobacteriaceae* count in the control group reached 9.67 log cfu/g.

Lactic acid bacteria, as part of the natural microbial flora of fish, are important causes of spoilage in meat products (1, 59). In this study, the number of lactic acid bacteria in the control group at the end of the storage period was approximately 9 log cfu/g, but in TCEO-NE 666.66, this number was approximately 5.5 log cfu/g. Pasbani and Amiri (56) showed that the number of lactic acid bacteria in beef in the control group at the end of the storage period increased by approximately 3 log cfu/g, while in the group containing thymol seed lipid nano-particles and aloe vera coating, this increase was approximately 1.4 log cfu/g.

We found that the initial number of *S. aureus* in fillets was 2.19 log cfu/g on day zero and reached 10.54, 8.66, and 8.35 in the control group, TCEO-NE 500.00 and TCEO-NE 666.66, respectively on day 11. Jebelli Javan et al. (39) concluded that *T. ammi* EO and ethanolic extract of propolis, especially when used in combination, reduce the growth rate of some foodborne pathogens such as *S. aureus* which can be caused by compounds such as thymol and carvacrol.

The color of fish is a remarkable criterion for consumers and can indicate freshness or spoilage. Changes in the color of fish tissue are largely due to reactions such as oxidation and Maillard, which are preventable by the presence of a strong antioxidant hindering discoloration by making oxygen unavailable (41). In the present study, the most desirable group during the storage period in terms of color was TCEO-NE 666.66. Khoshbouy Lahidjani et al. (41) reported that the color index of rainbow trout scored by panelists decreased over time, so that fish color in the control group reached an undesirable level after 10 days, but the groups containing the curcumin nanoemulsion coating had an acceptable color even after 15 days.

The odor index decreases with increasing TVB-N and the production of products such as dimethylamine, trimethylamine, ammonia and other volatile nitrogen compounds. Moreover, the increasing psychrotrophs count has a negative effect on the odor index (31). In this study, from the 4th day of storage, the control group received lower scores in the odor test as TVB-N increased and the number of psychrotrophs exceeded their allowable limit. Similarly, Khoshbouy Lahidjani et al. (41) reported that the rainbow trout fillets in the control group had an unpleasant odor from day 5 onwards, which was in line with increasing TVB-N and exceeding the allowable limit on day 5. Furthermore, the presence of nanoemulsion coating of curcumin during the storage period caused a significant difference with the control group so that the presence of curcumin at the level of 5% was able to prevent the formation of unpleasant odors during the storage period.

The results of the present study showed that the groups containing nanoemulsions received higher taste scores from evaluators than the control group, and the most desirable group during the maintenance period was TCEO-NE 666.66. Durmus (19) reported that the use of nanoemulsions had a positive effect on fish taste. The groups containing nanoemulsions received higher scores from the evaluators during the storage period than the control group. Among nanoemulsions, those of mandarin and grapefruit EOs received the highest scores.

Our results showed that the treatment groups and the control group exceeded TVB-N limit on day 6 and on day 4, respectively and the treatment groups and the control group exceeded peroxide limit on day 8 and on day 6 respectively. TBARS index in TCEO-NE 666.66 exceeded the allowable limit on day 11, while in the other two groups, it exceeded the acceptable level on day 8. Based on total aerobic mesophilic count, the use of TCEO-NE coating has extended the shelf life of fish fillets by two days. These effects can be attributed to thymol, limonene

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and alpha-terpinene in *T. copticum* EO. The color, odor and taste index had a negatively decreasing trend during the study period and was higher in the groups coated with TCEO-NE at all times than in the control group. The results showed that due to the properties of TCEO-NE and its positive effects on chemical, microbial and sensory changes of *O. aguabonita* fillets, this compound could be used as a natural food additive to increase shelf life.

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## Conflict of Interest

The authors declared that there is no conflict of interest.

## Author Contributions

RP designed the study and wrote the manuscript. MH and MHS carried out the experiments. All authors provided critical feedback and helped shape the research, analysis and manuscript.

## Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

## Ethical Statement

This study does not present any ethical concerns.

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# The presence of antibiotic resistance and molecular characterization of aminoglycoside and *PmrA* genes among food- and clinical-acquired *Acinetobacter* isolates

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## Abstract

The assessment of antibiotic resistance and related genes of foodborne *Acinetobacter* spp. and the analysis of whether they are genetically related to clinical infection-agent strains are crucial in terms of sustainability of food safety. The study at hand investigated antibiotic resistance, aminoglycoside-modifying enzyme (AME), and colistin resistance (*PmrA*) genes, clonal relationships while evaluating a possible correlation between antibiotic resistance and related genes between 27 foodborne and 50 clinical *Acinetobacter* spp. in Turkey. Antimicrobial susceptibilities, AME, *PmrA* genes, and clonal relatedness of the strains were performed by disc diffusion, PCR, and Pulsed Field gel Electrophoresis (PFGE) methods, respectively. The *aph-AI*, *aph-6*, *anth(3'')-I*, *aadA1*, *aadB,* and *PmrA* genes were found as 48%(n=24), 22%(n=11), 14%(n=7), 2%(n=1), 4%(n=2), and 92%(n=46) respectively, in clinical strains. This rate was found as 51.9%(n=14),59.3%(n=16), 70.4%(n=19), 7.4%(n=2), 0%(n=0), and 100%(n=27), respectively in foodborne isolates. A positive correlation existed between the number of *aph-AI* gene positivity and trimethoprim-sulfamethoxazole and gentamycin resistance; *anth (3'')-I* gene positivity, and colistin resistance; *PmrA* gene positivity and piperacillin-tazobactam, ceftazidime, meropenem, amikacin, and imipenem resistance in clinical strains ($P<0.05$). A positive correlation between trimethoprim-sulfamethoxazole resistance and *aadAI* gene positivity was found in foodborne strains ($P<0.05$). Clonal relations were absent between foodborne and clinical *A. baumanni* species. Finally, AME genes rise parallel to multidrug-resistance in the clinical isolates, and foods may be potential reservoirs for disseminating multi-AME and *PmrA* genes while being susceptible to several antibiotics.

## Introduction

*Acinetobacter baumannii* is a Gram-negative bacterium that can survive in harsh conditions both in nature, various food animals/poultry meat, dairy products, fruit/ vegetables, and in the human body. A widespread/ inappropriate application of broad-spectrum antibiotics in the medical field, agriculture, and veterinary area was conducted. As a result, multidrug-resistant *Acinetobacter* species that cause clinical nosocomial infections (bacteremia, pneumonia, meningitis, and urinary tract infections) have emerged because of fatal effects and economical losses around the world. Therefore, this bacterium is considered one of the six dangerous microorganisms by the Diseases Society of America. In the last decades, antibiotic resistance is an increasing problem worldwide, causing failures in the treatment of infections that affect not only hospital infections but also public health (19, 23, 30, 34). In *Acinetobacter* infections (humans and animals), beta-lactam group antibiotics are used for bactericidal effects during the lag period of the bacteria. In the resting period of the bacteria, aminoglycosides (gentamycin, tobramycin, and cephemycin) are used in combination with beta-lactam group drugs (imipenem and meropenem) due to their synergistic

effects. However, imipenem-resistant *A. baumannii* has become a worldwide problem due to the bacterial production of β-lactamase encoded by the *bla*$_{OXA-23}$ gene, which is also carried by foodborne *Acinetobacter* spp. as previously reported. However, there is less information on AME and colistin resistance genes carried by foodborne *Acinetobacter* spp. (17, 20). There are two main mechanisms of Gram-negative bacteria resistance to aminoglycosides. Firstly, aminoglycosides entering the bacteria are modified by aminoglycoside-modifying enzymes and become dysfunctional. Secondly, the methylation of target sites is induced by 16S rRNA methylases, resulting in decreased affinity of 16S rRNA for the antibiotic (7). With the inclusion of variant acetyltransferases [aac(3)-I, aac(3)-II, aac(3)-III, aac(6')-I, aac(6')-II, aac(6')-III], phosphotransferases [aph(3')-I, aph(3')-II, aph(3')-VI], and the nucleotidyltransferases [ant(3-)-I, ant(4')-I, and ant(2")-I], AMEs have been identified in *Acinetobacter* spp. Aminoglycoside resistance in *Acinetobacter* species is mostly due to the production of *aph-AI, aph-6, anth(3'')-I, aacC1, aadA1, aadB* genes responsible for AME enzymes by *Acinetobacter* spp. (4, 15, 19, 33).

Colistin is an antibiotic of last-line drug used in the treatment of *A. baumannii* infections. Colistin resistance is considered a serious problem, due to a lack of alternative antibiotics. The main mechanism of colistin resistance in *Acinetobacter baumannii* is the addition of a cationic group to the lipopolysaccharide layer of the bacteria; the complete loss of lipopolysaccharide production leads to resistance. The addition of a cationic group in *A. baumannii* in the *PmrAB*gene region depends on mutation (6, 27). The development of acquired antibiotic resistance in food-borne and clinical-*Acinetobacter* strains and the genes encoding AME enzymes and 16S rRNA methylase should be investigated, whether food-borne strains play a role in the spread of these genes and whether they are genetically related to clinical infection agent strains (5, 19, 21, 29). Several types of research have gone into the resistance mechanisms and genes in *Acinetobacter* spp. However, in comparison, there are so few studies on the spread of those genes due to their presence in the food chain (5, 17, 23).Investigation of phenotypic and genotypic resistance to antibiotics in *A. baumannii* strains may have global implications for the maintenance of antimicrobial chemotherapy (15). The high prevalence of AME and *PmrA* genes associated with phenotypic resistance makes it possible to choose the most accurate antibiotics in agriculture, veterinary and medical fields; it is so crucial in establishing the best policies to prevent the spread of genes encoding resistance (5, 15, 17, 23). However, there is a lack of studies about this issue in Middle East Countries (15) including Turkey. The relationship between mentioned encoding genes and phenotypic resistance analyses in our country/Middle East is a gap that this study aims to fill.

For this purpose; [1] five aminoglycoside-modifying enzymes (*aph-AI, aph-6, anth(3'')-I, aadA1, aadB*) and *PmrA* genes were evaluated in 27 foodborne *Acinetobacter* spp. and 50 *A. baumannii* strains of clinical originated. [2] A possible correlation between the aminoglycoside genes range and antibiotic resistance was statistically examined in all strains. [3] The analysis also answered the question as to whether genetically related *A. baumanni* species of food and clinical origin were investigated with the PFGE genetic comparison method, which is considered ''gold standard'' to discrimination of endemic strains. This method is accepted as a solid standard with high discrimination power in comparison of endemic species and other species. To the best of our knowledge, this is the first report of molecular characterization of antimicrobial-resistant *Acinetobacter* spp. from various foods and clinical samples in our country.

## Materials and Methods

***Place and sampling:*** In this study, a total of 250 samples were analyzed for the presence of *Acinetobacter* spp. A total of 102 food samples [n=25 cheese, n=39 fruit (banana, strawberries, tomatoes) and vegetables (lettuces, packed salads, cabbages), n=17 sucuk, n=8 veal, and n=13 chicken meat] were purchased from 12 markets in two provinces of southern Turkey. Clinical strains isolated from 148 clinical samples obtained from two hospitals in Adana and Mersin provinces were sent to Çukurova University, Clinical Microbiology Laboratory for a 4-year period (2018-2021). A total of 50 clinical strains (non-repetitive) were collected. Half of the strains were collected from female patients (25, 50%). The age ± standard deviation of the patients was 65.9± 15.1. Since the PFGE method can provide meaningful data for the analysis of short-term outbreaks, food isolates were collected in a similar period from the markets in the close location (campus) of the hospitals where the clinical isolates were collected.

***Isolation and identification of Acinetobacter spp.*:** The isolation of foodborne isolates was performed, as described previously (5, 23). The samples were cultured on Tryptone Soy Agar (TSA) plates containing 5% sheep blood (Oxoid) media at 37°C for 24 hours. The isolates were first evaluated by Gram staining, motility, catalase production, and the oxidase test to examine the morphology of colonies and biochemical test characteristics (7). To identify the presumptive colonies, a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF) (Bruker, Germany)

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was used (10). Columbia and Macconkey agars (Becton-Dickinson, Sparks, MD), were used to grow the clinical isolates. To obtain pure cultures, blood agar [containing 5% sheep blood (Oxoid)] was used and identified by (MALDI-TOF) system. All isolates were also confirmed by BBL Crystal E/NF test kit (Becton Dickinson, Australia) in the Microbiology Laboratory of Medical Faculty. The isolates were confirmed by the PCR method in terms of *bla*$_{OXA-51}$ gene carriage (35). Brain Hearth Broth medium (BHI) (including 10% of glycerol and10% of human blood) was used as a storage medium at -20ºC covering a genetic analysis of all identified species.

***Detection of the* AME *and PmrA genes:*** The genomic DNA was extracted with the boiling method as previously described (13). A spectrophotometer was used to measure the extracted DNA (100 ng/µL DNA for each sample) (UV-VIS Spectrophotometer CHIBIOS). The DNAs were stored at -20ºC before genotypic tests were performed. A multiplex PCR protocol was performed to screen specific *aph-AI, aph-A6, ant(''3)-I, aadB, aadAI,* and colistin resistance genes (*PmrA*) as described before (2, 16, 35, 37). The PCR conditions and the list of specific primers were presented in Table 1.The amplicons were run on 1.5% agarose gel [PegGOLD Universal Agarose, 91052 Erlangen Deutschland, 2%(w/v)], which was visualized on a UV transilluminator (Kodak, New York, USA).

***Antibiotic susceptibility test:*** The antimicrobial susceptibility test was performed through a disk diffusion method on Mueller-Hinton Agar (Merck KGaA, Germany)(7). The test was performed with nine antibiotic discs; ciprofloxacin (CIP, 5µg), piperacillin-tazobactam (TZP, 100/10µg), ceftazidime (CAZ, 30µg), imipenem (IPM, 10µg), meropenem (MEM, 10µg), amikacin (AK, 30 µg), trimethoprim/sulfamethoxazole (SXT,10 µg), gentamicin (CN,10 µg), and tetracycline (TE,30 µg), (all discs from Oxoid). The susceptibility was interpreted by Clinical and Laboratory Standards Institute (CLSI)) guidelines (7). A reference strain was obtained by using an index strain of *A. baumannii* (CI-74) as an infection agent. It was collected from the Medical Microbiology Laboratory. Colistin (CL) susceptibility and MIC value of the strains were determined by the agar dilution method according to EUCAST guidelines (7, 12).*Escherichia coli* ATCC 25922 was usedas a quality control (QC) strain for susceptibility tests. Isolates with a MIC ≤2 mg/L for colistin were accepted as susceptible (7, 12).

**Table 1.** The list of primers and amplification conditions used in this study.

| Genes | Primer sequences (5'-3') | Product size (bp) | Annealing (˚C) | Amplification conditions | Reference |
|---|---|---|---|---|---|
| *bla*$_{OXA-51}$ | GACCGAGTATGTACCTGCTTCGACC | 497 | 55 | The initial cycle of 94˚C for 4 min; 35 cycles of 94˚C for 30 s, 55˚C for 30 s, 72˚C for 1 min; 1 cycle of 72˚C for 7 min. | (35) |
| | GAGGCTGAACAACCCATCCAGTTAACC | | | | |
| *ant(3'')-I* | TGATTTGCTGGTTACGGTGAC | 284 | 55 | The initial cycle of 94ºC for 3 min.; 40 cycles of: 94ºC for 30 s, 55ºC for 40s,72ºC for 40 s;1 cycle: of 72ºC for 10 min. | (37) |
| | CGCTATGTTCTCTTGCTTTTG | | | | |
| *aph-AI* | ACAGAAGAGCTGCAGGAAATG | 623 | 55 | The initial cycle of 94ºC for 3 min.; 40 cycles of: 94ºC for 30 s, 55ºC for 40s,72ºC for 40 s;1 cycle: of 72ºC for 10 min. | (16) |
| | GACTGACGTCCAAGTTCCCAA | | | | |
| *aph-A6* | GCACGCTATTACCAACTATGA | 736 | 55 | | |
| | TAAGAAAGAACATCACCACGA | | | | |
| *aadAI* | AGATTTCATCTTTGATTCTTGG | 624 | 62 | The initial cycle of 94ºC for 3 min.; 40 cycles of: 94ºC for 30 s, 62ºC for 40s,72ºC for 40 s;1 cycle: of 72ºC for 10 min. | |
| | AATTGATTCTTAGCATCTGG | | | | |
| *aadB* | ACTCGGGGATTGATAGGC | 495 | 68 | The initial cycle of 94ºC for 3 min.; 40 cycles of: 94ºC for 30 s, 68ºC for 40s,72ºC for 40 s;1 cycle: of 72ºC for 10 min. | |
| | GCTGCTAAAGCTGCGCTT | | | | |
| *PmrA* | TCTGCAATAGAGATAGCCGC3 | 175 | 57 | The initial cycle of 94ºC for 3 min.; 40 cycles of: 94ºC for 30 s, 57ºC for 40s,72ºC for 40 s;1 cycle: of 72ºC for 10 min. | (2) |
| | GGAGTAGCTATCCCAGCATT | | | | |

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***PFGE analysis:*** Plug preparation, lysis, cell washing, restriction digestion, and electrophoresis were performed as previously described (8). PFGE was run in a CHEF-DR II apparatus (Bio-Rad, The USA), with pulses ranging from 5 to 30s at a voltage of 6 V/cm at 12°C for 20 h. Products were visualized after staining with ethidium bromide (50 µg/mL) and photographed. Gel images were exported to Gelcompar II software (version 3.0; Applied Maths, Sint Martens Latem, Belgium) for analysis. Comparisons were made by way of the band-based Dice coefficient. Dendrograms were generated by using the unweighted pair group method based on the arithmetic averaging method with a 1.5% position tolerance. Isolates were considered to be closely related if the Dice coefficient correlation was ≥80%.

***Statistical Analysis:*** The statistical program S-PLUS 20 (S-PLUS 20.00 for Windows, Professional Edition) was used for data analysis. The number and percentage of categorical measurements were summarized. The Chi-square test was used to compare AME/*PmrA* genes and antibiotic susceptibility. Fisher's exact test and Mann-Whitney U tests were used where appropriate. A P value of <0.05 was considered statistically significant.

## Results

A total of 27 foodborne isolates were identified as the following species; *A. baumannii* (n=14), *A. pittii* (n=5), *A. bereziniae* (n=2), *A. dijkshoorniae* (n=2), *A. calcoaceticus* (n=1), *A. baylyi* (n=1), *A. schindleri* (n=1), and *A. tandoii* (n=1). A total of 50 clinical isolates were identified as *A. baumannii*. The majority of the clinical isolates in a nosocomial environment were from intensive care internal medicine (*n*=17, 34%), nephrology/urology, (*n*=9, 18%), burn unit (*n*=6, 12%), and other clinics (general surgery anesthesia and brain surgery intensive care) (*n*=14, 28%), respectively. Clinical strains were isolated from aspiration fluid (*n*=14, 28%), wound (*n*=11, 22%), sputum (*n*=10, 20%), blood (*n*=8, 16%), and urine (*n*=7, 14%) samples, respectively. Distribution and antibiotic susceptibilities of the foodborne and clinical strains were presented in Table 2. The MIC value of clinical XDR (*n*=46, 92%), and MDR (*n*=4, 8%) strains were found between 64-128 µg/mL. All colistin-resistant strains' MIC value was ≥4 µg/mL. A summary of the incidence of the$bla_{\text{OXA-51}}$, AME, and *PmrA* genes among the *Acinetobacter* spp. and their origin was presented in Table 3. The gel image of $bla_{\text{OXA-51}}$ and AME genes of different sources was presented in Figure1. A summary of correlations between the AME, *PmrA* genes, and antibiotic resistance patterns of clinical and foodborne *Acinetobacter* spp. was presented in Tables 4 and 5.

PFGE results indicated a total of 34 *A. baumannii* isolates formed 24 different pulsotypes. The width of the clusters varied between 2–3 strains; a total of 6 groups were included 2 members such as"d, l, m, n, p, t". Two groups were included 3 members as "j, s". The "s" group has 100% similar clusters (s1). It was noted that there were 8 different PFGE groups. The clustering rate was calculated as 52.9. A total of 16 strains were unique, and 18 (52.9%) strains were collected in 8 closely related groups. There was no clonal association between foodborne and clinical strains. Foodborne and clinical isolates from different genetically unique/related groups among themselves (Figure 2).

**Table 2.** Antimicrobial susceptibility profile of all *Acinetobacter* spp. (*n*=77).

| | Resistance Patterns | | | No (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Isolates** | **A**/SXT | **B**/CIP/ | **C**/TZP | **D**/TE | **E**/AK | **F**/MEM | CL | **G**/CAZ | **E**/CN | **F**/IMP |
| Foodborne strains (27) | | | | | | | | | | |
| *A. baumannii*(14) | 2(14.3)* | | | 2(14.3) | | | | | | |
| *A. calcoaceticus*(1) | | | | | | | | | | |
| *A.bereziniae*(2) | | | | | | 1(50)* | | 1(50)* | | |
| *A. baylyi*(1) | | | | | | | | | | |
| *A.dijkshoorniae*(2) | 1(50) | | | 1(50) | | 1(50) | | | | 1(50) |
| *A.pittii*(5) | 1(20) | | | | | | | 1(20)* | | |
| *A.tandoi*(1) | | | | | | | | | | |
| *A. schindleri*(1) | | | | | | | | | | |
| Total | 4(14.8) | | | 3(11.1) | | 2(7.4) | | 2(7.4)* | | 1(3.7) |
| Clinical Strains(50) | | | | | | | | | | |
| *A. baumannii* (50) | 44(88) | 45(90) | 47(94) | | **46(92)** | 46(92) | **7(14)** | 47(94) | **43(86)** | 47(94) |
| Total | 44(88) | 45(90) | 47(94) | | **46(92)** | 46(92) | **7(14)** | 47(94 | **43(86)** | 47(94) |

*: Intermediately sensitive; CIP, Ciprofloxacin; SXT, Trimethoprim-Sulfamethoxazole; TE, Tetracycline; CL, Colistin; TZP, Piperacillin-Tazobactam; CAZ, Ceftazidime; IPM, Imipenem; CN. Gentamycin; AK, Amikacin; MEM, Meropenem. **A:** Folate pathway inhibitors, **B:** Fluoroquinolones, **C:** β-lactam/β-lactamase inhibitor combinations, **D:** Tetracyclines, **E: Aminoglycosides**, **F:** Carbapenems, **G:** Cephems.

**Table 3.** Distribution of $bla_{OXA-51}$, aminoglycoside, and *PmrA* resistances genes of *Acinetobacter* spp. in the present work.

| | Resistance genes | | | No (%) | | | |
|---|---|---|---|---|---|---|---|
| **Isolates** | $bla_{OXA-51}$ | ***aph-AI*** | ***aph-6*** | ***anth(3'')-I*** | ***aadA1*** | ***aadB*** | ***PmrA*** |
| Foodborne strains (**27**) | | | | | | | |
| *A. baumannii*(14) | 14(100) | 9(64.3) | 9(64.3) | 9(64.3) | 2(14.3) | 0 | 14(100) |
| *A. baylyi*(1) | 0 | 0 | 0 | 0 | 0 | 0 | 1(100) |
| *A. bereziniae* (2) | 0 | 1(50) | 1(50) | 2(100) | 0 | 0 | 2(100) |
| *A. calcoaceticus*(1) | 0 | 1(100) | 0 | 1(100) | 0 | 0 | 1(100) |
| *A. dijkshoorniae*(2) | 0 | 1(50) | 1(50) | 1(50) | 0 | 0 | 2(100) |
| *A. pittii*(5) | 0 | 2(40) | 5(100) | 4(80) | 0 | 0 | 5(100) |
| *A. schindleri*(1) | 0 | 0 | 0 | 1(100) | 0 | 0 | 1(100) |
| *A. tandoi*(1) | 0 | 0 | 0 | 1(100 | 0 | 0 | 1(100) |
| Total | 14(51.9) | 14(51.9) | 16(59.3) | 19(70.4)) | 2(7.4) | 0 | 27(100) |
| Clinical Strains (**50**) | | | | | | | |
| *A. baumannii* (**50**) | 50(100) | 24(48) | 11(22) | 7(14) | 1(2) | 2(4) | 46(92) |
| Total | 50(100) | 24(48) | 11(22) | 7(14) | 1(2) | 2(4) | 46(92) |

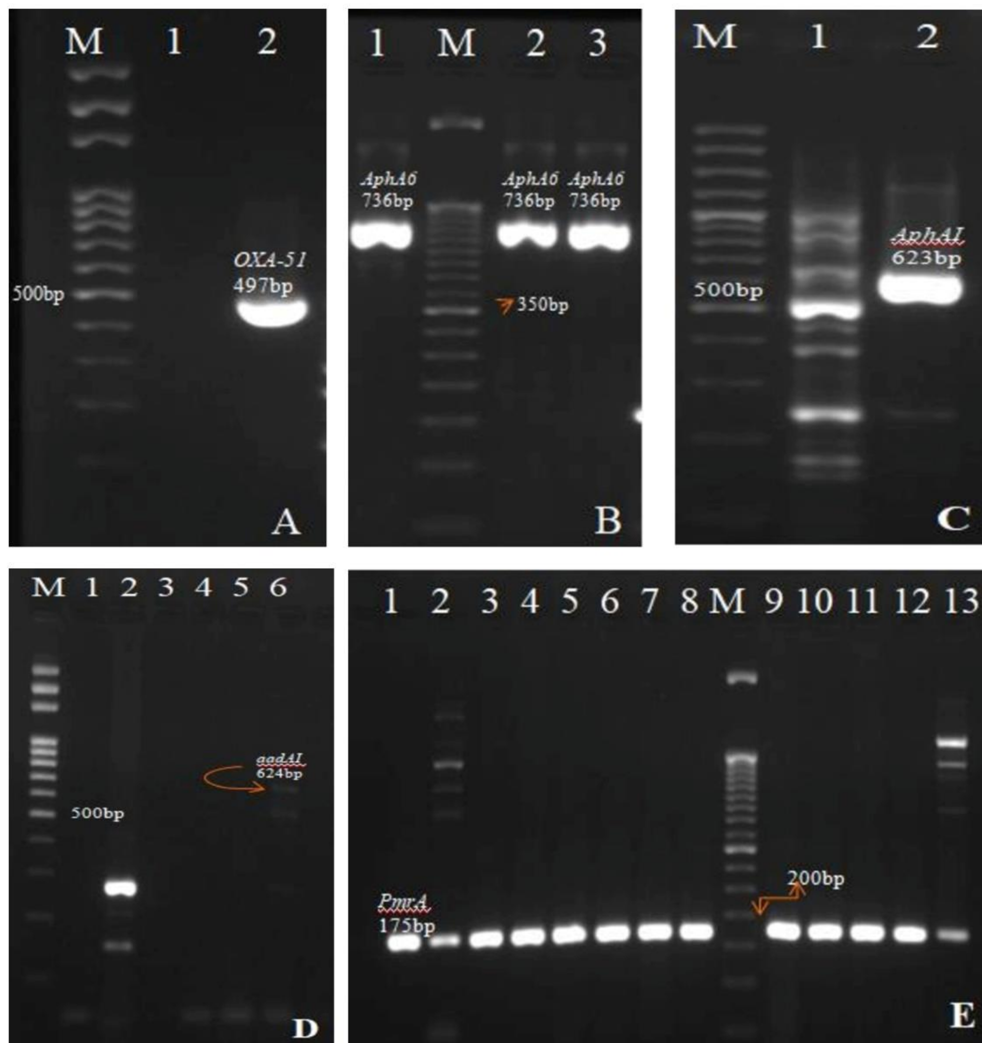


**Figure 1.** Agarose gel electrophoresis of PCR-amplified products of *Acinetobacter* spp.
(**A**)-($bla_{OXA-51}$); M: Marker (100bp), 1: Negative control, 2: *A. baumannii*(lettuce). (**B**)-(*aph-A6*);1: *A. baumannii*(veal), M: Marker(50bp), 2: *A. baumannii*(sucuk), 3: *A. schindleri*(cheese). (**C**)-(*aph-AI*);M: Marker (100bp), 1: *A. baumannii*(purple cabbage), 2: *A. baumannii*(cheese). (**D**)-(*aadAI*); M: Marker (100bp), 1: *A. pitti* (cheese), 2: *A. pittii* (packet salad), 3: *A. bereziniae*(veal), 4: *A. dijkshoorniae*(packet salad), 5: *A. baumannii*(packet salad), 6: *A. baumannii*(lettuce). (**E**)-(*PmrA*); 1: *A. pittii*(cheese), 2: *A. bereziniae*(veal), 3: *A. dijkshoorniae*(packet salad), 4: *A.pittii* (packet salad), 5: *A. baumannii*(packet salad), 6: *A. baumannii*(lettuce), 7: *A. baumannii*(lettuce) 8: *A. baumannii*(spinach), M: Marker (50bp), 9: *A. baumannii*(purple cabbage), 10: Control strain (*A. baumannii*), 11: *A. pittii* (packet salad), 12: *A. baumannii*(cheese), 13: *A. baumannii*(traditional cheese).

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**Table 4.** Antibiotic susceptibility and resistant genes relations in clinical *Acinetobacter* spp. strains ($n$=50) in the present study.

| Tested Antibiotics | | Resistance genes No (%) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *aph-AI* | | *P* | *aph-6* | | *P* | *anth(3'')-I* | | *P* | *aadA1* | | *P* | *aadB* | | *P* | *PmrA* | | *P* |
| | | *n=26/0*$^a$ | *n=24/1*$^b$ | | *n=39/0*$^a$ | *n=11/1*$^b$ | | *n=43/0*$^a$ | *n=7/1*$^b$ | | *n=49/0*$^a$ | *n=1/1*$^b$ | | *n=48/0*$^a$ | *n=2/1*$^b$ | | *n=4/0*$^a$ | *n=46/1*$^b$ | |
| | | No (%) | | | No (%) | | | No (%) | | | No (%) | | | No (%) | | | No (%) | | |
| **TZP** | **R** | 23(88.5) | 24(100) | | 36(92.3) | 11(100) | | 40(93) | 7(100) | | 46(93.9) | 1(100) | | 45(93.8) | 2(100) | | 2(50) | 45(97.8) | |
| | **S** | 3(11.5) | 0 | 0.236$^c$ | 3(7.7) | 0 | 1000$^c$ | 3(7) | 0 | 1000$^c$ | 3(6.1) | 0 | 1000$^c$ | 3(6.3) | 0 | 1000$^c$ | 2(50) | 1(2.2) | 0.014$^c$ |
| **CAZ** | **R** | 23(88.5) | 24(100) | | 36(92.3) | 11(100) | | 40(93) | 7(100) | | 46(93.9) | 1(100) | | 45(93.8) | 2(100) | | 2(50) | 45(97.8) | |
| | **S** | 3(11.5) | 0 | 0.236$^c$ | 3(7.7) | 0 | 1000$^c$ | 3(7) | 0 | 1000$^c$ | 3(6.1) | 0 | 1000$^c$ | 3(6.3) | 0 | 1000$^c$ | 2(50) | 1(2.2) | 0.014$^c$ |
| **CIP** | **R** | 21(80.8) | 24(100) | | 36(92.3) | 9(81.8) | | 38(88.4) | 7(100) | | 44(89.8) | 1(100) | | 43(89.6) | 2(100) | | 2(50) | 43(93.5) | |
| | **S** | 5(19.2) | 0 | 0.051$^c$ | 3(7.7) | 2(18.2) | 0.301$^c$ | 5(11.6) | 0 | 1000$^c$ | 5(10.2) | 0 | 1000$^c$ | 5(10.4) | 0 | 1000$^c$ | 2(50) | 3(6.5) | 0.045$^c$ |
| **SXT** | **R** | 20(76.9) | 24(100) | | 33(84.6) | 11(100) | | 38(88.4) | 6(85.7) | | 43(87.8) | 1(100) | | 42(87.5) | 2(100) | | 2(50) | 42(91.3) | |
| | **S** | 6(23.1) | 0 | 0.023$^c$ | 6(15.4) | 0 | 0.317$^c$ | 5(11.6) | 1(14.3) | 1000$^c$ | 6(12.2) | 0 | 1000$^c$ | 6(12.5) | 0 | 1000$^c$ | 2(50) | 4(8.7) | 0.066$^c$ |
| **CN** | **R** | 19(73.1) | 24(100) | | 33(84.6) | 10(90.9) | | 37(86) | 6(85.7) | | 42(85.7) | 1(100) | | 41(85.4) | 2(100) | | 2(50) | 41(89.1) | |
| | **S** | 7(26.9) | 0 | 0.010$^c$ | 6(15.4) | 1(9.1) | 1000$^c$ | 6(14) | 1(14.3) | 1000$^c$ | 7(14.3) | 0 | 1000$^c$ | 7(14.6) | 0 | 1000$^c$ | 2(50) | 5(10.9) | 0.089$^c$ |
| **MEM** | **R** | 22(84.6) | 24(100) | | 35(89.7) | 11(100) | | 39(90.7) | 7(100) | | 45(91.8) | 1(100) | | 44(91.7) | 2(100) | | 2(50) | 44(95.7) | |
| | **S** | 4(15.4) | 0 | 0.111$^c$ | 4(10.3) | 0 | 0.563$^c$ | 4(9.3) | 0 | 1000$^c$ | 4(8.2) | 0 | 1000$^c$ | 4(8.3) | 0 | 1000$^c$ | 2(50) | 2(4.3) | 0.028$^c$ |
| **AK** | **R** | 22(84.6) | 24(100) | | 35(89.7) | 11(100) | | 39(90.7) | 7(100) | | 45(91.8) | 1(100) | | 44(91.7) | 2(100) | | 2(50) | 44(95.7) | |
| | **S** | 4(15.4) | 0 | 0.111$^c$ | 4(10.3) | 0 | 0.563$^c$ | 4(9.3) | 0 | 1000$^c$ | 4(8.2) | 0 | 1000$^c$ | 4(8.3) | 0 | 1000$^c$ | 2(50) | 2(4.3) | 0.028$^c$ |
| **IMP** | **R** | 23(88.5) | 24(100) | | 36(92.3) | 11(100) | | 40(93) | 7(100) | | 46(93.9) | 1(100) | | 45(93.8) | 2(100) | | 2(50) | 45(97.8) | |
| | **S** | 3(11.5) | 0 | 0.236$^c$ | 3(7.7) | 0 | 1000$^c$ | 3(7) | 0 | 1000$^c$ | 3(6.1) | 0 | 1000$^c$ | 3(6.3) | 0 | 1000$^c$ | 2(50) | 1(2.2) | 0.014$^c$ |
| **CL** | **R** | 2(7.7) | 5(20.4) | | 5(12.8) | 2(18.2) | | 4(9.3) | 3(42.9) | | 7(14.3) | 0 | | 6(12.5) | 1(50) | | 0 | 7(15.2) | |
| | **S** | 24(92.3) | 19(79.2) | 0.239$^c$ | 34(87.2) | 9(81.8) | 0.641$^c$ | 39(90.7) | 4(57.1) | 0.048$^c$ | 42(85.7) | 1(100) | 1000$^c$ | 42(87.5) | 1(50) | 0.263$^c$ | 4(100) | 39(84.8) | 0.536$^c$ |

*0*$^a$: No gene, *1*$^b$: Gene present, ***c*:** fisher`s exact test, R: Resistant, S: Sensitive and intermediate sensitive; TZP, Piperacillin-Tazobactam; CAZ, Ceftazidime; CIP, Ciprofloxacin; SXT, Trimethoprim-Sulfamethoxazole; CN, Gentamycin; MEM, Meropenem; AK, Amikacin; IPM, Imipenem; CL, Colistin. The significant correlations were indicated in ***bold P*** value.

**Table 5.** Antibiotic susceptibility and resistant genes relations in foodborne *Acinetobacter* spp. ($n$=27) in this study.

| Tested Antibiotics | | Resistance genes No (%) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *aph-AI* | | *P* | *aph-6* | | *P* | *anth(3'')-I* | | *P* | *aadA1* | | *P* | *aadB* | | *P* | *PmrA* | | *P* |
| | | *n=13/0*[a] | *n=14/1*[b] | | *n=11/0*[a] | *n=16/1*[b] | | *n=8/0*[a] | *n=19/1*[b] | | *n=25/0*[a] | *n=2/1*[b] | | *n=27/0*[a] | *n=0/1*[b] | | *n=0/0*[a] | *n=27/1*[b] | |
| | | No (%) | | | No (%) | | | No (%) | | | No (%) | | | No (%) | | | No (%) | | |
| **TZP** | **R** | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N |
| | **S** | 13(48.1) | 14(51.9) | | 11(100) | 16(100) | | 8(100) | 19(100) | | 25(100) | 2(100) | | 27(100) | 0 | | 0 | 27(100) | |
| **CAZ** | **R** | 1(7.7) | 1(7.1) | 1000[c] | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N |
| | **S** | 12(92.3) | 13(92.9) | | 11(100) | 16(100) | | 8(100) | 19(100) | | 25(100) | 2(100) | | 27(100) | 0 | | 0 | 27(100) | |
| **CIP** | **R** | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N |
| | **S** | 13(48.1) | 14(51.9) | | 11(100) | 16(100) | | 8(100) | 19(100) | | 25(100) | 2(100) | | 27(100) | 0 | | 0 | 27(100) | |
| **SXT** | **R** | 2(15.4) | 2(14.3) | 0.249[c] | 1(9.1) | 3(18.8) | 0.761[c] | 0 | 4(21.1) | 0.191[c] | 3(12) | 1(50) | **0.030**[c] | 4(14.8) | 0 | N | 0 | 4(14.8) | N |
| | **S** | 11(84.6) | 12(85.7) | | 10(90.9) | 13(81.2) | | 8(100) | 15(78.9) | | 22(88) | 1(50) | | 23(85.2) | 0 | | 0 | 23(85.2) | |
| **CN** | **R** | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N |
| | **S** | 13(48.1) | 14(51.9) | | 11(100) | 16(100) | | 8(100) | 19(100) | | 25(100) | 2(100) | | 27(100) | 0 | | 0 | 27(100) | |
| **MEM** | **R** | 1(7.7) | 0 | 0.481[c] | 1(9.1) | 0 | 0.407[c] | 0 | 1(5.3) | 1000[c] | 1(4) | 0 | 1000[c] | 1(3.7) | 0 | N | 0 | 1(3.7) | N |
| | **S** | 12(92.3) | 14(100) | | 10(90.9) | 16(100) | | 8(100) | 18(94.7) | | 24(96) | 2(100) | | 26(96.3) | 0 | | 0 | 26(96.3) | |
| **AK** | **R** | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N |
| | **S** | 13(48.1) | 14(51.9) | | 11(100) | 16(100) | | 8(100) | 19(100) | | 25(100) | 2(100) | | 27(100) | 0 | | 0 | 27(100) | |
| **IMP** | **R** | 1(7.7) | 0 | 0.481[c] | 1(9.1) | 0 | 0.407[c] | 0 | 1(5.3) | 1000[c] | 1(4) | 0 | 1000[c] | 1(3.7) | 0 | N | 0 | 1(3.7) | N |
| | **S** | 12(92.3) | 14(100) | | 10(90.9) | 16(100) | | 8(100) | 18(94.7) | | 24(96) | 2(100) | | 26(96.3) | 0 | | 0 | 26(96.3) | |
| **CL** | **R** | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N | 0 | 0 | N |
| | **S** | 13(48.1) | 14(51.9) | | 11(100) | 16(100) | | 8(100) | 19(100) | | 25(100) | 2(100) | | 27(100) | 0 | | 0 | 27(100) | |

*0*[a]: No gene, *1*[b]: Gene present, ***c*:** fisher`s exact test, R: Resistant, S: Sensitive and intermediate sensitive, N: No applicable; TZP, Piperacillin-Tazobactam; CAZ, Ceftazidime; CIP, Ciprofloxacin; SXT, Trimethoprim-Sulfamethoxazole; CN, Gentamycin; MEM, Meropenem; AK, Amikacin; IPM, Imipenem; CL, Colistin**.** The significant correlations were indicated in ***bold P*** value.

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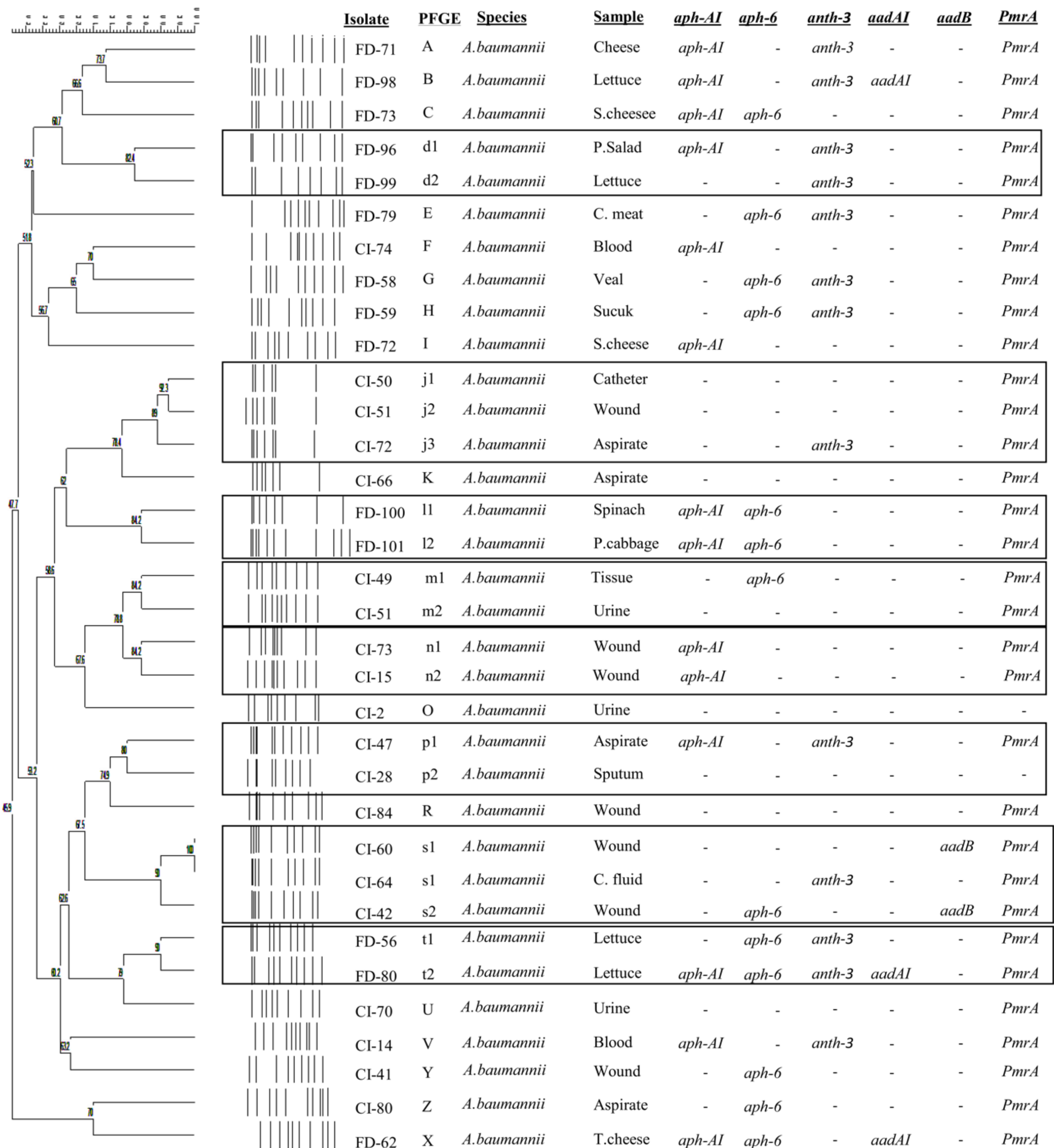


**Figure 2.** PFGE dendrograms among the 34 strains of *A. baumannii* from human infection agents (20) and foods (14) are included. The closely-related clusters (≥80% similarity) are presented boxed.

FD: Food isolate, CI: Clinical Isolate, S. Cheese: String Cheese, P. salad: Packed Salad, C. Meat: Chicken Meat, P. Cabbage: Purple Cabbage, C. Fluid: Cerebrospinal Fluid, T. Cheese: Traditional Cheese.

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## Discussion and Conclusion

Due to its gaining of multidrug resistance pattern, *A. baumanni* became an important foodborne and nosocomial opportunistic pathogen (15, 21). The most abundant species of our foodborne (n=14, 51.9%) and, clinical strains were *A. baumanni* (n=50, 100%), *A. pitti* (n=5, 18.5%). These species and *A. calcoaceticus* (n=1, 3.7%) were also considered the *A. baumannii* group most constantly associated with nosocomial infections worldwide (26, 38, 39). In the current study, the rate of this group was 90.9% (70/77). Fewer phenotypic- resistant strains in food samples were found in our study. Only one *A. dijkshoorniae* strain was found MDR and this result (3.7%, MDR) was lower than previous reports in Portugal (29.8%, MDR in fruits and vegetables) and Iran (50%, MDR in chicken meat), respectively (5, 3). Overall, 92% of clinical isolates were resistant to at least five classes of antibiotics, hence meeting the criteria for extensive drug resistance (XDR resistance) (16). The frequency rate of antimicrobial resistance of *A. baumannii* recovered from clinical specimens was found between 88% and 94% for all tested antibiotics (Table 2). Similarly, in China, Pakistan, and Iran profiles with higher resistance (100%) were reported (15, 31, 40).

Screening for genes encoding AMEs demonstrated that 92% of the clinical isolates that are amikacin resistant contained the phosphotransferase gene *aphA6* with the rate of 22% (11/50). Other genes encoding AMEs included the adenylyltransferase genes *aadA1* 2% (1/50) and, *aadB* 4% (2/50) genes were found in this study (Table 3). Our results were lower than Mortazavi et al. (25)'s study in Iran. They reported as *aphA6*, *aadA1*, and *aadB* genes with the rate of 22.5%, 11.25%, and 30% respectively. They also found a positive correlation between *aadB* and *aphA6* genes positivity with high resistance against gentamicin and amikacin inconsistent with our research (Table 4). A similar study conducted in Germany by Wareth et al. (36) reported that 19% of strains were found resistant to amikacin and the new subclass of intrinsic aminoglycoside nucleotidyltransferase, *ant(3")-IIa*, was widely distributed in humans, animals, and milk powder samples. Subsequently, the intrinsic aminoglycoside nucleotidyltransferases (*aadA* and *aadAI* genes) were detected in 9 percent of the isolates. By contrast in our study, the amikacin resistance of our clinical strains was higher than their result (92%), and the *aph-AI* gene was the most prevalent in foodborne and clinical strains (38, 54.3%). Besides, the *anth(3'')-I* gene was widely seen in foodborne strains (19, 70%) and *aadA1* gene prevalence was less in our study (Table 3). The *aph(3'')-I* gene (70%) rate in clinical strains were higher than Moniri et al. (24)'s results (41.7%) in Iran, but lower than Wen et al. (37)'s results (85%) in China. Tested AME genes were more frequent among foodborne isolates than clinical strains and carried more multi- AME genes (P<0.001) (Table 3). Many of these genes are widespread in *Pseudomonas aeruginosa* and *A. baumannii* and mirror those described in a collection of MDR *A. baumannii* isolates from Europe (clone types I, II, and III) (16). In *Acinetobacter* spp.; some important AME genes can be located in plasmids [*ant(3")-Ia, aadA1, aph(3')-VIa, aph(6)-Id*)], integron [*(ant(3")-Ia, aadA1*], transposon [*ant(3")-Ia, aadA1, aph(3')*], integrative conjugative element [*aph(6)-Id, aph(3")-Ib*], chromosome [*aph(3")*], and chromosomal genomic island [*aph(6)-Id*]. AME genes can be transferred by means of mobilizable or conjugative plasmids, natural transformation, or transduction (14, 22, 28). Thus, the findings of AME genes in our foodborne isolates mean that tested AME genes can be transferred by other pathogenic bacteria in food production processes (the use of contaminated/sewage water in the agricultural sector, unhygienic practices in slaughter and milk production processes (improper heating/pasteurization or contamination by food workers, etc.) or some of them carried in their chromosome/chromosomal genomic island.

In this study, 14% of clinical *A. baumannii* isolates were found colistin-resistant. This rate is higher than the resistance ratio (2.9%) reported from Southwestern Iran by Khoshnood et al. (18), and higher than previous works in Saudi Arabia and Pakistan, no colistin-resistant *A. baumannii* isolates were found in clinical samples (18, 30). These varying rates of resistance may arise from differences in the epidemiology and the infection treatment regulatory policies of respective countries, management patterns, and antibiotic use. The *pmrA* gene rate in clinical colistin-resistant and sensitive *A. baumannii* isolates was found (46, 92%) which were higher than Sepahvand et al. (30)'s study in Iran. In their study, they detected the *PmrA* gene at a rate of 70 percent and the *PmrB* gene at a rate of 30 percent. They reported that among the *A. baumanni* isolates carrying these genes, there are also colistin-resistant and sensitive ones. In our study, similar to the results of Sepahvand et al. (30)'s, colistin-resistant and susceptible *Acinetobacter* spp. strains carried the *PmrA* gene. The increased expression of the *PmrAB* system is necessary for *A. baumannii* resistance to colistin and the expression rate of *pmrA/pmrB* genes should be compared to colistin-sensitive strains (1). All foodborne strains carried *PmrA* genes but their sensitivity to colistin may be due to the chromosomes carried in this gene and also we did not measure the expression rate of the *PmrA*/*PmrB* genes (Figure 1).

The *aadA1* gene confers streptomycin and spectinomycin resistance, *aadB* gene confers tobramycin, gentamicin, and kanamycin resistance, *aphA6* gene confers amikacin, gentamicin, kanamycin, and neomycin

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resistance (13). In clinical *A. baumannii* strains; there was a significant association between the harboring of *aph-AI* gene positivity and trimethoprim-sulfamethoxazole and gentamycin resistance (P=0.023; P=0.010); and *anth(3'')-I* gene positivity and colistin resistance (P=0.048); *PmrA* gene positivity and piperacillin-tazobactam, ceftazidime, meropenem, amikacin, and imipenem resistances (P=0.014; P=0.014; P=0.028, P=0.028; and P=0.014) were found respectively (Table 4). In the foodborne *Acinetobacter* spp. (Table 5) section of the results, a positive correlation was found between *aadAI* gene positivity and trimethoprim-sulfamethoxazole resistance (P=0.030). By this correlation, we can assume that in foodborne strains, trimethoprim-sulfamethoxazole resistance acquisition may be the result of the effect of the *aadAI* gene. The finding is in agreement with the fact that streptomycin and spectinomycin are the usual substrates for *aadAI* gene, but the correlation between trimethoprim-sulfamethoxazole and *aadAI* gene in foodborne strains means that a combination with streptomycin and spectinomycin usage (in agriculture/animal husbandry) may have entailed this result. A similar result was seen in our clinical strains (*aph-AI* gene positivity and trimethoprim-sulfamethoxazole and gentamycin resistance). However, other correlations were the unexpected substrates related to the genes; [*anth(3'')-I* and *PmrA* genes] which were in agreement with Sheikhalizadeh et al. (32)'s report in Iran [*ant(2')-Ia,aac(3')-IIa* encoding genes and related antibiotic non-susceptibility], and South Africa on clinical SAK strain of *A. baumannii* (9). These results may vary according to antibiotic combination usage in clinical settings. To this very day, we have not encountered any specialist text investigating the relationship between antibiotic resistance and aminoglycoside (AME) and colistin (*PmrA*) resistance genes in foodborne *Acinetobacter* spp. strains. Therefore, we were not able to make a comparison with a relevant field study.

The clonal relationships were not found between foodborne and clinical strains by PFGE. This difference in results may be due to various effectors including time, place, and methodology. For instance; we collected the samples during the Covid-19 pandemic. Therefore, we couldn't collect colonization strains from healthy patients. If we could have collected these isolates, they would have enabled us to reach wider data.

Interestingly, some closely related clusters have the same AME and *PmrA* genes; in the group of ''d'' and ''m''. Except for *aph-AI* and *aph-6*, all tested genes are shown in the same cluster. Similarly, in the group of ''j'', except for *anth(3'')-I*, all tested genes are shown in the same cluster; in the groups of ''l'' and ''n'' all tested genes carriage are shown in the same cluster (Figure 2). Thus, the same clones dissemination occured in foodborne and clinical strains. Our results are consistent with the literature; the cluster analysis showed that AME genes and *16S rRNA* methylase genes are often associated with genetic markers of moveable genetic elements (i.e., these genes are removable rear-mediated genetic elements). This acquired resistance mechanism facilitates horizontal spread (20).

Finally, *Acinetobacter* spp. from food can carry AME and *PmrA* genes and may rarely be resistant to aminoglycoside and carbapenems. However, the *A. baumanni* and *A. pitti* bacteria isolated from foods (especially from fruit and vegetables, chicken, turkey, and veal meat) have AME resistance genes at various rates and are more common than the clinical isolates. Foodborne *A. baumannii*, *A. pittii*, and *A. dijkshoorniae* strains may have been potential sources of the dissemination of AME and, *PmrA* genes confer to aminoglycoside and colistin resistance. The positive correlation between the positivity of *aph-AI*, *anth(3'')-I,* and *PmrA* genes in clinical *A. baumannii* isolates and resistance to various antibiotics are crucial findings. The spread of AME genes/resistance formation due to the positive relationship between *aadAI* gene positivity and trimethoprim-sulfamethoxazole resistance in foodborne isolates points to the food chain being a factor in the spread/formation of AME genes/ resistance. Foodborne *A. baumanni* isolates are not genetically related to clinical strains which suggests that foodborne strains don't play a role in infection development. Our study has some limitations; low numbers of *Acinetobacter* spp. are analyzed in the study at hand. The cause of limitation is due to the difficulty of isolation of food and the difficulties encountered in the identification. Further studies including more *Acinetobacter* spp. isolates of foodborne and clinical with more antibiotics resistance, AME, *16SrRNA* methylase genes, as well as efflux pumps genes in wider geographical areas/countries are needed to ensure food safety in the food industry and lend to clinical microbiology.

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## Conflict of Interest

The authors declared that there is no conflict of interest.

## Author Contributions

MT, ZE, and FK conceived and planned the experiments. MT carried out the experiments. MT, ZE, and FK planned and carried out the simulations. MT, ZE, and FK contributed to sample preparation. MT, ZE, and FK contributed to the interpretation of the results. MT took the

lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

## Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

## Ethical Statement

This study was carried out after the clinical samples were approved by Çukurova University Local Ethics Committee (Decision number: 14.06.2019-89).

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# Meta-analysis and meta-regression of subclinical mastitis prevalences in dairy cattle in Türkiye

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## Abstract

In this study, it was aimed to determine the prevalence of subclinical mastitis obtained from 38 studies conducted in Türkiye between 1988 and 2019 by meta-analysis method and to calculate common prevalence. The estimated prevalence of subclinical mastitis in the studies were evaluated separately as cow-based (11182 cows in 37 studies) and udder quarter-based (48990 udder quarters in 33 studies). According to the results, the common prevalence of subclinical mastitis was calculated as 0.48 (95% CI: 0.41-0.56) in cow-based studies and 0.32 (95% CI: 0.26-0.37) in quarter based-based studies. As a result of this study, the results of individual studies on the prevalence of subclinical mastitis in dairy cows in Türkiye were combined with the meta-analysis method and a more precise estimate of the prevalence was obtained.

## Introduction

Mastitis is an infection that occurs in the mammary gland as a result of infectious agents such as bacteria, yeast, fungi, and viruses entering the body through the teat canal. The form of mastitis in which milk or mammary gland abnormalities or secondary clinical signs can be observed is defined as clinical mastitis; and the form that can be diagnosed by using different test methods to identify the inflammatory cells formed by the glandular tissue but cannot otherwise be detected is defined as subclinical mastitis (6, 9, 18).

Subclinical mastitis is a type of mastitis that has a high risk of contamination in the herd and can turn into clinical mastitis, if not treated, causing deterioration in milk structure and decrease in milk yield (9). It has been reported that subclinical mastitis causes more economic loss than clinical mastitis in the dairy industry due to the difficulty of its detection. The economic loss due to subclinical mastitis in Türkiye has been estimated to be approximately 8 trillion dollars per year, whereas the amount allocated for maintaining mammary health has been determined to be 33 dollars per animal (6, 9, 14).

Sublinical mastitis and its economic losses can be avoided by following a versatile mastitis control plan in the herds (6, 18). Subclinical mastitis is one of the most important problems of the dairy industry and is still up to date. Since many studies have been conducted both in the world and in Türkiye to estimate the prevalence of subclinical mastitis and to determine the factors affecting it, systematic review or meta-analysis studies are needed on this subject.

Due to variations in the achieved results, the results should be statistically interprited according to breed, herd size and geographic properties (1, 13).

In this study, it was aimed to evaluate the prevalence of subclinical mastitis in dairy cows in Türkiye by meta-analysis and to calculate the common prevalence as well as to determine the effects of breed, geographical region,

the years of the studies, and herd size on subclinical mastitis by meta-regression analysis.

## Materials and Methods

Thirty-8 studies, which were about the prevalence of subclinical mastitis in Türkiye and published between 1988 and 2019 were evaluated in this study. In these studies, cows and udder quarters were used as materials to determine the prevalence of subclinical mastitis. For this reason, meta-analyses in the study were conducted separately as cow-based and udder quarter-based. The characteristics of these studies were given in Table 1.

**Table 1.** Characteristics of studies included in the meta-analysis of subclinical mastitis prevalences in Türkiye.

| Rank No | Authors | Year | Province | Breed | Base on * |
|---|---|---|---|---|---|
| 1 | **Alaçam E, Tekeli T, Erganiş O, et al** (1989): *The Diagnosis, Isolation of Etiological Agents and Antibiotic Susceptibility Test Results in Cows and Buffalos Suffering from Subclinical Mastitis*. Eurasian J Vet Sci **5**, 91–101. | 1989 | Afyonkarahisar | Brown Swiss and Crossbreed | 1 |
| 2 | **Nizamlıoğlu M, Kalaycıoğlu L, Dinç DA, et al** (1992): *Determination of N-acetyl B-D glucosaminidase enzyme activity in bovine milk for the early diagnosis of subclinical mastitis*. S Ü Vet Fak Derg **8**, 60–63. | 1992 | Konya | Holstein and Crossbreed | 1 |
| 3 | **Gürtürk K, Boynukara B, Ekin İH, et al** (1998*): A study on the etiology of subclinical mastitis in dairy cows in and around Van*. Van Vet J **9**: 1–4. | 1998 | Van | Unspecified Breeds | 2 |
| 4 | **Vural MR, Esendal Ö, İzgür H, et al** (1999): *Subclinical mastitis cases in primiparous holstein cows part 2. Intramammary infections during first lactation*. Veterinary Journal of Ankara University **46**, 287 - 298. | 1999 | Ankara | Holstein and Crossbreed | 1, 2 |
| 5 | **Yüksel H** (1999): *Pathologic investigations on mastitis in cows slaughtered at the Elazığ Elet abattoir*. PhD thesis, Fırat University Institute of Health Sciences, Elazığ. | 1999 | Elazığ | Indigenous Breeds and Crossbreed | 2 |
| 6 | **Ak S** (2000): *Bacterial agents cause contagious and environmental bovine mastitis in Trakya district and their susceptibility to antibiotics*. J Fac Vet Med Univ Istanbul **26**, 353–365. | 2000 | - | Unspecified Breeds | 1, 2 |
| 7 | **Şeker İ, Rişvanlı A, Kul S, et al** (2000): *Relationships Between CMT Scores and Udder Traits and Milk Yield in Brown-Swiss Cows*. Lalahan Hay Araşt Enst Derg **40**, 29–38. | 2000 | Malatya | Brown Swiss and Crossbreed | 2 |
| 8 | **Uzmay C, Kaya A, Akba Y** (2001): *Studies on Prevalence of Mastitis and Factors Affecting Prevalence in Herds of Izmir Holstein Breeders Association. 2. Relationships Between Managerial Practices and Subclinical Mastitis*. Ege Üniv. Ziraat Fak. Derg **38**, 71-78. | 2001 | İzmir | Unspecified Breeds | 1, 2 |
| 9 | **Rişvanlı A** (2001): *Clinic and subclinic incidence and isolation, antibiotic sensitivity of the microorganisms caused mastitis in dairy cows in Elazığ district*. PhD thesis, Fırat University Institute of Health Sciences, Elazığ. | 2001 | Elazığ | Holstein and Crossbreed, Brown Swiss and Crossbreed, Indigenous Breeds and Crossbreed, Other Breeds | 1, 2 |
| 10 | **Beytut E, Aydın F, Özcan K, et al** (2002): *Pathological and Bacteriological Investigations on Bovine Mastitis in Kars Region and Its Surround*. Kafkas Üniv Vet Fak Derg **8**, 111–122. | 2002 | Kars | Unspecified Breeds | 1 |
| 11 | **Kireçci E, Çolak A** (2002*): Methicillin Resistance in Staphylococci Strains Isolated from Dairy Cows with Subclinical Mastitis the Onset of the Dry Period*. Kafkas Üniv Vet Fak Derg 8, 98–100. | 2002 | Erzurum | Other Breeds | 1, 2 |
| 12 | **Uzmay C, Kaya I, Akbaş Y, et al** (2003): *Effects of Udder and Teat Morphology, Parity and Lactation Stage on Subclinical Mastitis in Holstein Cows*. Turkish J Vet Anim Sci **27**, 695-701. | 2003 | İzmir | Unspecified Breeds | 1 |
| 13 | **Sabuncuoğlu N, Çolak A, Akbulut Ö, et al** (2003): *Relationships Between CMT Scores and Some Milk Yield Traits in Holstein Friesian and Brown Swiss Cows.* Atatürk Univ. J. of Agricultural Faculty **34**, 139–143. | 2003 | Erzurum | Brown Swiss and Crossbreed, Holstein and Crossbreed | 2 |
| 14 | **Ergün Y, Aslantaş Ö, Doğruer G, et al** (2004): *Epidemiology of Subclinical Mastit is in Family Size Dairy Farms in Hatay Region*. Vet Bil Derg **20**, 25–28. | 2004 | Hatay | Holstein and Crossbreed | 1 |
| 15 | **Gülcü HB, Ertaş HB** (2004): *Bacteriological Investigation of Udder Lobes of Cows with Mastitis Slaughtered in the Elazığ Region*. Turk J Vet Anim Sci **28**, 91–94. | 2004 | Elazığ | Indigenous Breeds and Crossbreed | 1, 2 |
| 16 | **Abay M, Bekyürek T** (2006): *The Compare of Efficiency of Cefquinome and Amoxycillin+Clavulonic Acide Treatment on Subclinical Staphylococcus Aureus Mastitis in Lactating Dairy Cows*. JHSM **15**, 189–193. | 2006 | Sivas | Holstein and Crossbreed | 1 |
| 17 | **Musal B, İzgür İH** (2006): *The efficacy of intramammary, systemic and combined antibiotics administered during dry off in cows with subclinical mastitis*. Ankara Univ Vet Fak Derg **53**, 175–178. | 2006 | Eskişehir | Brown Swiss and Crossbreed | 1, 2 |

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| Rank No | Authors | Year | Province | Breed | Base on * |
|---|---|---|---|---|---|
| 18 | **Çoban Ö, Tüzemen N** (2007): *Risk Factors For Subclinical Mastitis In Holstein Friesian and Brown Swiss Cows*. Uludag Univ J Fac Vet Med **26**, 27–31. | 2007 | Erzurum | Other Breeds | 2 |
| 19 | **Baştan A, Kaçar C, Acar DB, et al** (2008): *Investigation of the incidence and diagnosis of subclinical mastitis in early lactation period cows*. Turk J Vet Anim Sci **32**, 119–121. | 2008 | - | Unspecified Breeds | 2 |
| 20 | **Tel OY, Keskin O, Zonturlu AK, et al** (2009): *Subclinical Mastitis Prevalance and Determination of The Antibiotics Susceptibility in Sanliurfa Region*. F.U. Vet.J.Health.Sci **23**, 101–106. | 2009 | Şanlıurfa | Unspecified Breeds | 1, 2 |
| 21 | **Türkyılmaz S, Yıldız Ö, Oryaşın E, et al** (2010): *Molecular identification of bacteria isolated from dairy herds with mastitis*. Kafkas Üniv Vet Fak Derg **16**, 1025–1032. | 2010 | - | Unspecified Breeds | 1 |
| 22 | **Macun HC, Pir Yağcı İ, Ünal N, et al** (2011): *Agent Isolation and Antibiotic Resistance in Dairy Cows with Subclinical Mastitis in Kırıkkale*. J Fac Vet Med Univ Erciyes **8**, 83–89. | 2011 | Kırıkkale | Unspecified Breeds | 1 |
| 23 | **Bardakcioglu HE, Sekkin S, Oral Toplu HD** (2011): *Relationship between some teat and body measurements of Holstein cows and sub-clinical mastitis and milk yield*. J Anim Vet Adv **10**, 1735–1737. | 2011 | Aydın | Holstein and Crossbreed | 1, 2 |
| 24 | **Yeşilmen S, Özyurtlu N, Bademkıran S** (2012): *The Isolation of Subclinical Mastitis Agents and Determination of the Sensitive Antibiotics in Dairy Cows in Diyarbakır Province*. Dicle Üniv Vet Fak Derg **1**, 24–29. | 2012 | Diyarbakır | Unspecified Breeds | 1 |
| 25 | **Acar G, Yılmaz E, Solmaz H, et al** (2012): *Isolation of Streptococcal Agents from Cattle with Subclinical Mastitis in Hatay Region and Detection of their Susceptibilities against some Antibiotics*. AVKAE Derg **2**, 1-5 | 2012 | Hatay | Unspecified Breeds | 1, 2 |
| 26 | **Çokal Y, Konuş R** (2012): *Isolation of Aerobic Bacteria From Cow Milks With Subclinical Mastitis*. BAUN Health Sci J **1**, 65–69. | 2012 | Balıkesir | Unspecified Breeds | 1, 2 |
| 27 | **Koçyiğit R** (2012): *The determination of subclinical mastitis incidence in dairy cows in Bolu Mudurnu region*. MSc thesis, Afyon Kocatepe University Institute of Health Sciences, Afyonkarahisar. | 2012 | Bolu | Other Breeds | 1, 2 |
| 28 | **Ayanoğlu K** (2012): *Investigation of the Effect of Pregnancy Rate of Mastitis That Formed in Cows During Early Pregnancy*. PhD thesis, Selçuk University | 2012 | Isparta | Holstein and Crossbreed | 1, 2 |
| 29 | **Kaygısız A, Karnak İ** (2012): *Evaluation of Somatic Cell Count in Raw Milk Samples Collected from Dairy Farms in Kahramanmaras Province for EU Norms and Subclinical Mastitis*. KSU J Nat Sci **15**, 9-15. | 2012 | Kahramanmaraş | Holstein and Crossbreed | 2 |
| 30 | **Ikiz S, Başaran B, Bingöl EB, et al** (2013): *Presence and antibiotic susceptibility patterns of contagious mastitis agents (staphylococcus aureus and streptococcus agalactiae) isolated from milks of dairy cows with subclinical mastitis*. Turk J Vet Anim Sci **37**, 569–574. | 2013 | - | Unspecified Breeds | 1 |
| 31 | **Özdemir S, Kaymaz M** (2013): *Comparison of Diagnostic Methods and Incidence of Subclinical Mastitis on Local Breeds*. Atatürk University J Vet Sci **8**, 71–79. | 2013 | Sivas | Unspecified Breeds | 2 |
| 32 | **Büyükcangaz E, Mat B, Ahmed MKAA** (2012): *Microbiological Analysis and Antimicrobial Resistance Pattern of the Isolates Derived From Dairy Cattle With Subclinical Mastitis*. Uludag Univ J Fac Vet Med **31**, 35–44. | 2013 | Bursa | Holstein and Crossbreed | 2 |
| 33 | **Baştan A, Salar S, Cengiz M, et al** (2015): *The prediction of the prevalence and risk factors for subclinical heifer mastitis in Turkish dairy farms*. Turk J Vet Anim Sci **39**, 682–687. | 2015 | - | Unspecified Breeds | 1 |
| 34 | **Gezgen C** (2015): *Investigation of methicillin resistance and Panton-Valentine leukocidin in Staphylococci isolated from bovine mastitis*. MSc thesis, Afyon Kocatepe University Institute of Health Sciences, Afyonkarahisar. | 2015 | İzmir | Unspecified Breeds | 1, 2 |
| 35 | **Dalgıç D, Sarıbay MK** (2015): *Distribution of Lesions Occurred in Teat Skin and Their Effects on Mastitis in Cows*. F.U. Vet.J.Health.Sci **29**, 111–117. | 2015 | Hatay | Holstein and Crossbreed | 2 |
| 36 | **Akdağ F, Gürler H, Teke B, et al** (2017): *The Effect of the Scores and Various Assessments of the Scores for CMT on Milk Yield, Milk Composition and the Diagnosis of Subclinical Mastitis in Jersey Cows*. J Fac Vet Med Istanbul Univ **43**, 44–51. | 2016 | Samsun | Other Breeds | 1 |
| 37 | **Tepeli SÖ, Zorba NN** (2017): *Some Properties of Raw Milk Produced in Çanakkale (Yenice) City and The Incidence of Subclinical (Hidden) Mastitis*. TUJNS **18**, 41–47. | 2017 | Çanakkale | Unspecified Breeds | 1 |
| 38 | **Saydan M, Kalkan C** (2017): *Prevalence of Subclinical Mastitis in Dairy Cattle in Malatya Arguvan District*. F.U. Vet.J.Health.Sci **31**, 193–200. | 2017 | Malatya | Holstein and Crossbreed, Brown Swiss and Crossbreed, Other Breeds | 1, 2 |

*: 1=Cow-based Prevalence, 2=Quarter-based Prevalence.

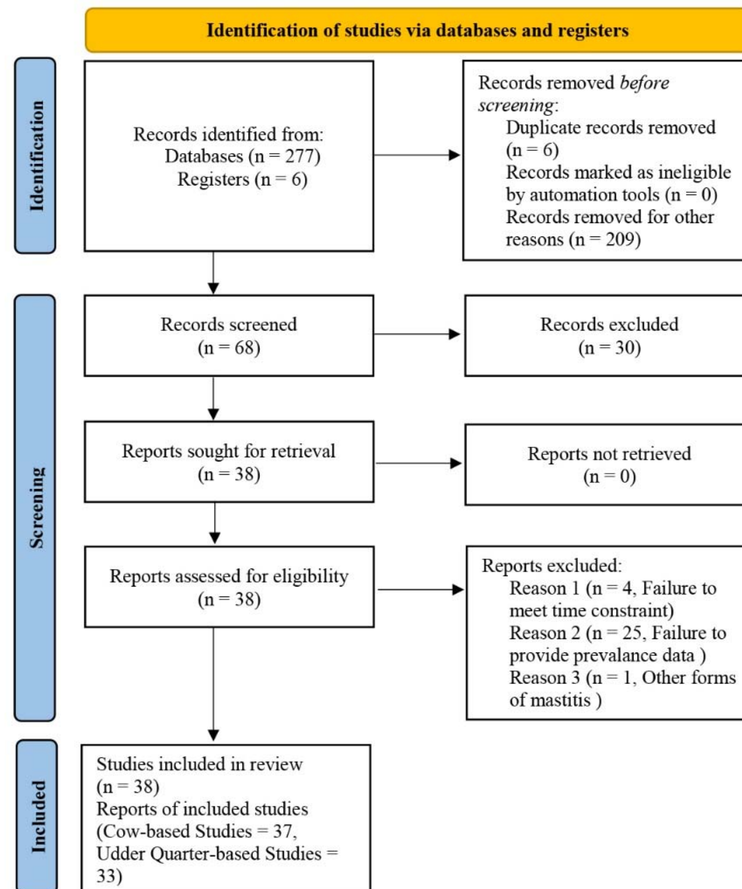


**Figure 1.** Flow diagram of prevalences of subclinical mastitis in dairy cows (16).

The literature search for the studies to be included in the meta-analysis was carried out between 06.07.2017 and 03.05.2020. The keywords were chosen as 'Subclinical mastitis', 'Dairy cattle', and 'Prevalence', and the literature search was made using Google Scholar, PubMed, ScienceDirect, and Scopus electronic databases. The inclusion criteria for the studies used in the meta-analysis were determined as “have been done in Türkiye and to have the calculated or calculable prevalence of subclinical mastitis in dairy cow breeds”. Studies to be included in meta-analyses were determined according to the PRISMA 2020 checklist and the flowchart was given in Figure 1 (17). Meeting the inclusion criteria as a result of literature search, subclinical mastitis prevalence data from 11182 dairy cows in 37 cow-based studies and 48990 udder quarters from 33 udder-quarters-based studies were used in the meta-analyses. In the study samples, Begg and Mazumdar rank correlation test and Egger's linear regression test were performed to determine publication biases, and the funnel plots were drawn. One Study Removed method was used to determine the effect of individual studies on the common prevalence.

Der Simonian-Laird method was used to determine the heterogeneity between studies. In order to determine the sources of heterogeneity, subgroups were formed according to the categories of region, breed, year groups, and herd size covariates, and meta-regression analyses of subclinical mastitis prevalence were performed for each group. Subgroups with fewer than three studies were not included in the analysis.

For the subgroup analyses conducted separately for cow and udder quarter-based studies, the subgroups were formed as 5 geographical regions in Türkiye (Mediterranean Region, Eastern Anatolia Region, Aegean Region, Central Anatolia Region, and Marmara Region), cow breeds (Holstein and its crossbreeds, Swiss Brown and its crossbreeds, the local breed and its crossbreeds, other breeds, and unspecified breeds), year groups (1988-1999, 2000-2009, 2010-2019) and herd sizes (small (n<100), medium (100≤n<300), large (300≤n)).

In the applied meta-regression analyzes, method of moments was preferred for the calculation of the model coefficients. Comprehensive Meta-Analysis (CMA) and R 4.1.0 (www.r-project.org) softwares were used in the application of meta-analyses. In R software, "meta", "metaphor" and "tidyverse" packages were used. In order to test the hypothesis that the prevalence was not different from 0.5 in the analysis, the significance level was determined as P<0.05, while the significance level for the significance controls of Cochran's Q heterogeneity statistics was taken as P<0.10.

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## Results

Cow and udder quarter-based meta-analyses were conducted regarding the prevalence of subclinical mastitis throughout Türkiye, and according to the results of Der Simonian-Laird method used to determine the heterogeneity among the studies, high heterogeneity was detected between both cow-based and udder quarter-based studies (Cochrane's Q=4918.44, df=36, $I^2$=99.27, P<0.001; Cochrane's Q=11907.87, df=32, $I^2$=99.73, respectively) (Table 2). Thus, the random effect model was used to calculate the effect sizes in the analyses. According to the results of the random effects model, the common prevalence of subclinical mastitis in Türkiye was calculated as 0.48 (95% CI: 0.41-0.56) in cow-based studies and 0.32 (95% CI: 0.26-0.37) in udder quarter-based studies (Table 2).

According to the results of One Study Removed method, which was used to determine the effects of individual studies on the prevalence, it was observed that the pooled prevalence estimation did not change when the studies were removed one by one from the analysis. The forest plots of the studies were given in Figure 2.

According to Begg and Mazumdar rank correlation test and Egger's linear regression test which was used to detect publication bias in the study samples, there was no publication bias in the study samples (P>0.05) (Table 3). In addition, no asymmetry was observed in the funnel plots (Figure 3).

For cow and udder quarter-based studies, separate subgroup analyses were made with the variables of 5 geographical regions in Türkiye, cow breeds, year groups, and herd sizes. This subgroup analyses were made according to geographical regions in Türkiye. The Southeastern Anatolia Region and the Black Sea Region, where there were not enough studies to form a group, were excluded from the analysis, and the remaining 5 regions were compared. It was determined that the prevalence of subclinical mastitis varies according to regions in both cow and udder quarter-based studies (P=0.071, P=0.008, respectively). In the cow-based studies, the highest prevalence of subclinical mastitis was calculated in the Marmara Region (0.68), and the lowest prevalence was calculated in the Aegean Region (0.33); in the udder quarter-based studies the highest prevalence was calculated in the Central Anatolian Region (0.50), and the lowest prevalence was calculated in the Marmara Region (0.14) (Table 4).

It was determined that the prevalence of subclinical mastitis did not show a statistically significant variation in the subgroup analyses according to cow breeds (P=0.687) (Table 5).

In the subgroup analyses performed according to the years of the studies, it was determined that the prevalence of subclinical mastitis showed a statistically significant variation (P=0.053). Accordingly, the highest prevalence values in both the cow-based studies and the udder quarter-based studies were calculated between 2010 and 2019 (0.54 and 0.32, respectively), and the lowest prevalence values were calculated between 1988 and 1999 (0.17 and 0.12, respectively) (Table 6).

In the subgroup analyses performed according to the herd's size used in the studies, the prevalence of subclinical mastitis did not show a statistically significant variation in the cow-based studies (P=0.207), while it showed a significant variation in udder quarter-based studies (P<0.001). Accordingly, the highest prevalence value in udder quarter-based studies was observed in small-scale herds (0.42) (Table 7).

In this study, meta-regression analysis was performed in order to theoretically calculate the sources of heterogeneity among the studies included in the meta-analysis. For this purpose, multivariate meta-regression models were created for both the cow and the udder quarter-based studies.

The model with the highest $R^2$ analog value in the multivariate meta-regression analysis created for cow-based and udder quarter-based studies is given in Table 8.

According to the model test for the cow-based studies, there was a significant difference in disease prevalence between the subgroups of the year group and herd size variables (Q=9.42, df=4, P=0.052). This model explains 28% of the variance in the true effects ($R^2$ analog=0.28). In this model, the effects of year groups and herd size were found to be significant. When 1988-1999 subgroup was taken as the reference, the prevalence of subclinical mastitis was calculated to be 1.22 times higher in 2000-2009; and 1.63 times higher in 2010-2019 (P<0.05). When the large-scale herds were taken the reference, the prevalence of subclinical mastitis was calculated to be 0.40 times higher in medium-sized herds and 0.68 times higher in small-scale herds (P>0.05). For udder quarter-based studies, there was a significant difference in disease prevalence among all subgroups of the region, year group, and herd size variables according to the model test (Q=33.16, df=8, P<0.001). This model explains 30% of the variance in the true effects ($R^2$ analog=0.30). According to the model, when the Marmara Region was taken as the reference, the prevalence of subclinical mastitis was 1.57 times higher in the Aegean Region and 1.73 times higher in the Central Anatolia Region (P<0.05).

When 1988-1999 subgroup was taken as the reference, the prevalence of subclinical mastitis was calculated to be 1.08 times higher in 2000-2009, and 1.38 times higher in 2010-2019 (P<0.05). When large-scale herds are taken as the reference, the prevalence of subclinical mastitis was calculated to be 0.50 times higher in medium-sized herds and 0.97 times higher in small-scale herds (P>0.05, P<0.05, respectively).

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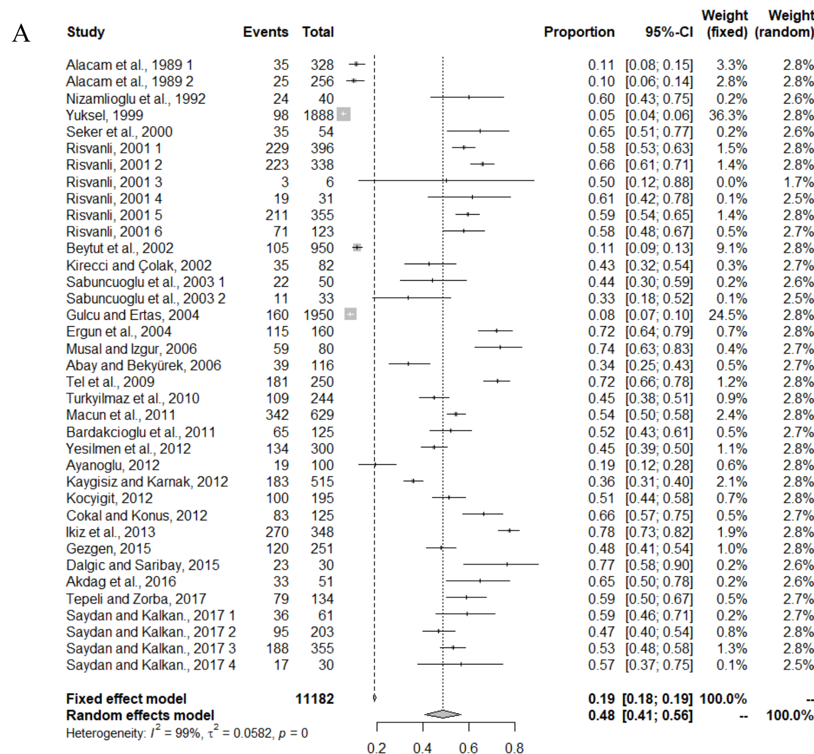
**Table 2.** Cow-based and udder quarter-based pooled subclinical mastitis prevalences across Türkiye.

| Statistics | Cow-based (n=37) | Quarter-based (n=33) |
|---|---|---|
| **Total Numbers** | 11182 | 48990 |
| **No of Subclinical Mastitis (+)** | 3596 | 10310 |
| **Simple Ratio** | 0.322 | 0.210 |
| **Pooled Prevalence and 95% C.I.** | **0.48 (0.41-0.56)** | **0.32 (0.26-0.37)** |
| **P Value** | <0.001 | <0.001 |
| **Heterogeneity Test** | | |
| **Cochran's Q** | 4918.44 | 11907.87 |
| **df (Cochran's Q)** | 36 | 32 |
| **$\tau^2$** | 0.058 | 0.025 |
| **$I^2$ (%)** | 99.27 | 99.73 |
| **P Value (Cochran's Q)** | **<0.001** | **<0.001** |



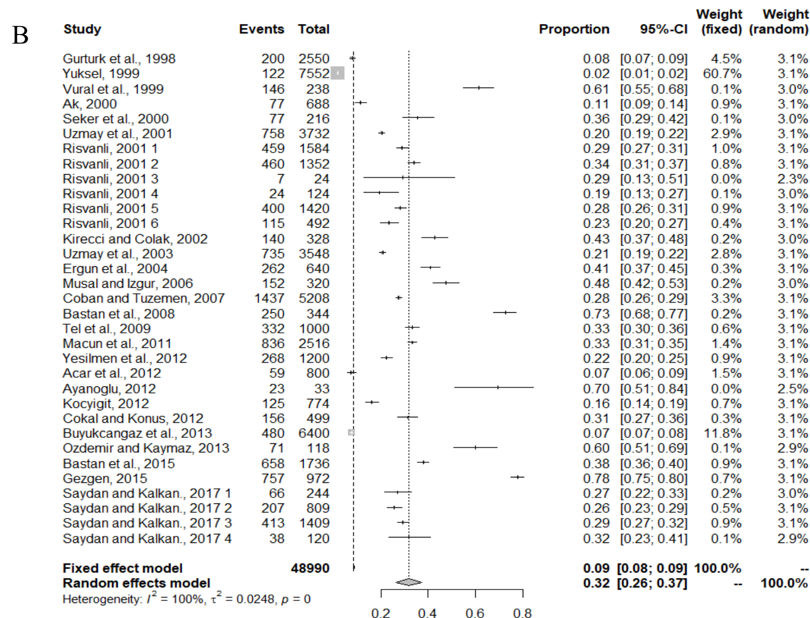




**Figure 2. A)** Forest plot of cow-based meta-analysis **B)** Forest plot of udder quarter-based meta-analysis.

**Table 3.** Publication bias tests of cow-based and udder quarter-based study samples.

| | Begg and Mazumdar Rank Correlation Test | | Egger's Regression Test | |
|---|---|---|---|---|
| | **Cow-Based** | **Quarter-Based** | **Cow-Based** | **Quarter-Based** |
| **Intercept** | -0.068 | <0.001 | 5.064 | 2.318 |
| **t statistic** | - | - | 1.610 | 0.501 |
| **z statistic** | 0.586 | <0.001 | - | - |
| **P Value** | 0.558 | 1.000 | 0.117 | 0.621 |

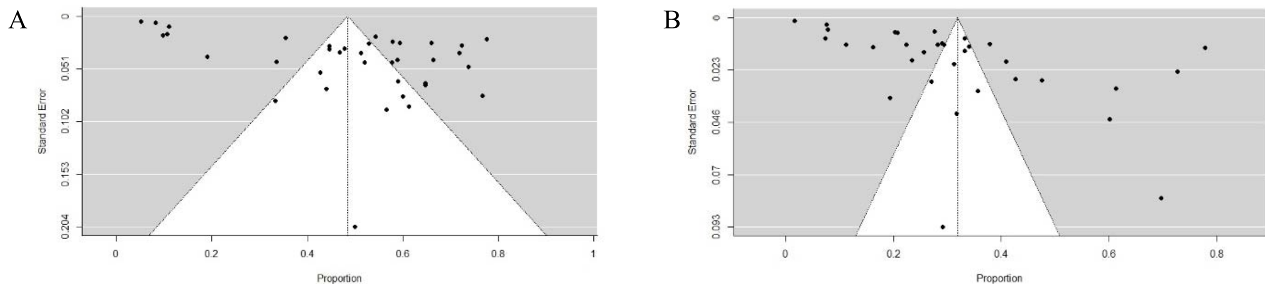



**Figure 3. A)** Cow-based funnel plot of the study sample on subclinical mastitis prevalences **B)** Udder quarter-based funnel plot of the study sample on subclinical mastitis prevalences across Türkiye.

**Table 4.** Results of subgroup analysis of cow-based and quarter-based subclinical mastitis prevalences in Türkiye by region.

| | Regions | | | | |
|---|---|---|---|---|---|
| | **The Mediterranean region (n=4)** | **The Eastern Anatolia Region (n=18)** | **The Aegean Region (n=3)** | **The Central Anatolia Region (n=4)** | **The Marmara Region (n=3)** |
| **Cow-Based (n=32)** | | | | | |
| **Number of Cows** | 805 | 7205 | 704 | 865 | 607 |
| **Number of Cows with Subclinical Mastitis (+)** | 340 | 1692 | 202 | 464 | 432 |
| **Simple Proportion** | 0.42 | 0.24 | 0.29 | 0.54 | 0.71 |
| **Pooled Prevalence and 95% CI** | 0.50 (0.26-0.74) | 0.42 (0.28-0.58) | 0.33 (0.12-0.64) | 0.55 (0.40-0.69) | 0.68 (0.56-0.79) |
| **P Value** | 0.988 | 0.338 | 0.286 | 0.491 | **0.006** |
| **Heterogeneuity Test** | | | | | |
| **Cochran's Q** | 92.05 | 1584.61 | 103.31 | 30.64 | 17.72 |
| **df (Cochran's Q)** | 3 | 17 | 2 | 3 | 2 |
| $\tau^2$ | 1.085 | 1.886 | 1.296 | 0.327 | 0.208 |
| $I^2$ | 96.741 | 98.927 | 98.064 | 90.208 | 88.710 |
| **P Value (Cochran's Q)** | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Between Regions** | | | | | |
| **Cochran's Q =8.635, df=4, p= 0.071** | | | | | |
| **Udder Quarter-Based (n=29)** | **The Mediterranean region (n=3)** | **The Eastern Anatolia Region (n=16)** | **The Aegean Region (n=3)** | **The Central Anatolia Region (n=4)** | **The Marmara Region (n=3)** |
| **Number of Udder Quarter** | 1473 | 24632 | 8252 | 3192 | 7587 |
| **Number of Udder Quarter with Subclinical Mastitis (+)** | 344 | 4433 | 2250 | 1205 | 713 |
| **Simple Proportion** | 0.23 | 0.18 | 0.27 | 0.38 | 0.09 |
| **Pooled Prevalence and 95% CI** | 0.33 (0.08-0.73) | 0.23 (0.17-0.30) | 0.38 (0.15-0.68) | 0.50 (0.35-0.65) | 0.14 (0.05-0.33) |
| **P Value** | 0.417 | **<0.001** | 0.449 | 0.982 | **0.001** |
| **Heterogeneity Test** | | | | | |
| **Cochran's Q** | 210.103 | 1615.283 | 1002.477 | 110.385 | 257.516 |
| **df (Cochran's Q)** | 2 | 15 | 2 | 3 | 2 |
| $\tau^2$ | 2.270 | 0.584 | 1.230 | 0.387 | 0.928 |
| $I^2$ | 99.048 | 99.071 | 99.800 | 97.282 | 99.223 |
| **P Value (Cochran's Q)** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| **Between Regions** | | | | | |
| **Cochran's Q =13.802, df=4, P= 0.008** | | | | | |

df= degree of freedom, C.I.: Confidence Interval, SE: Standard Error, $I^2$: Ratio of variance in observed effects to variance in true effects rather than sampling error, $\tau^2$: Variance in true effect sizes.

**Table 5.** Subgroup analysis results of cow-based and quarter-based subclinical mastitis prevalences in Türkiye by breed.

| | Breeds | | | | |
|---|---|---|---|---|---|
| | **Holstein and Crossbreed (n=10)** | **Brown Swiss and Crossbreed (n=6)** | **Indigenous and Crossbreed (n=4)** | **Other Breeds (n=5)** | **Unspecified Breeds (n=7)** |
| **Cow-Based (n=32)** | | | | | |
| **Number of Cows** | 1.518 | 1111 | 4316 | 504 | 2737 |
| **Number of Cows with Subclinical Mastitis (+)** | 738 | 475 | 540 | 262 | 1133 |
| **Simple Proportion** | 0.49 | 0.43 | 0.13 | 0.52 | 0.41 |
| **Pooled Prevalence and 95% CI** | 0.50 (0.38-0.62) | 0.48 (0.28-0.68) | 0.24 (0.06-0.62) | 0.52 (0.47-0.57) | 0.50 (0.31-0.70) |
| **P Value** | 0.967 | 0.821 | 0.169 | 0.467 | 0.987 |
| **Heterogeneity Test** | | | | | |
| **Cochran's Q** | 161.986 | 171.267 | 701.100 | 4.279 | 511.783 |
| **df (Cochran's Q)** | 9 | 5 | 3 | 4 | 6 |
| $\tau^2$ | 0.572 | 1.058 | 2.823 | 0.005 | 1.226 |
| $I^2$ | 94.444 | 97.081 | 99.572 | 6.518 | 98.828 |
| **P Value (Cochran's Q)** | **<0.001** | **<0.001** | **<0.001** | 0.370 | **<0.001** |
| **Between Breeds** | | | | | |
| **Cochran's Q = 2.263, df=4, P = 0.687** | | | | | |
| | | | | | |
| **Udder Quarter-Based (n=29)** | | | | | |
| | **Holstein and Crossbreed (n=6)** | **Brown Swiss and Crossbreed (n=4)** | **Indigenous and Crossbreed (n=3)** | **Other Breeds (n=6)** | **Unspecified Breeds (n=10)** |
| **Number of Udder Quarter** | 8907 | 2929 | 9464 | 7213 | 16623 |
| **Number of Udder Quarter with Subclinical Mastitis (+)** | 1437 | 895 | 637 | 2059 | 3917 |
| **Simple Proportion** | 0.16 | 0.31 | 0.07 | 0.29 | 0.24 |
| **Pooled Prevalence and 95% CI** | 0.36 (0.17-0.61) | 0.34 (0.26-0.43) | 0.11 (0.02-0.48) | 0.30 (0.26-0.35) | 0.25 (0.16-0.37) |
| **P Value** | 0.282 | <0.001 | 0.042 | <0.001 | <0.001 |
| **Heterogeneity Test** | | | | | |
| **Cochran's Q** | 1117.273 | 55.256 | 886.331 | 39.425 | 1697.403 |
| **df (Cochran's Q)** | 5 | 3 | 2 | 5 | 9 |
| $\tau^2$ | 1.590 | 0.137 | 3.113 | 0.052 | 0.797 |
| $I^2$ | 99.552 | 94.571 | 99.774 | 87.318 | 99.470 |
| **P Value (Cochran's Q)** | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Between Breeds** | | | | | |
| **Cochran's Q =3.252, df=4, P=0.517** | | | | | |

df= degree of freedom, C.I.: Confidence Interval, SE: Standard Error, $I^2$: Ratio of variance in observed effects to variance in true effects rather than sampling error, $\tau^2$: Variance in true effect sizes.

**Table 6.** Subgroup analysis results of cow-based and quarter-based subclinical mastitis prevalences in Türkiye by years.

| | Years | | |
|---|---|---|---|
| | **1988-1999 (n=3)** | **2000-2009 (n=14)** | **2010-2019 (n=15)** |
| **Cow-Based (n=32)** | | | |
| **Number of Cows** | 2256 | 4564 | 3366 |
| **Number of Cows with Subclinical Mastitis (+)** | 157 | 1222 | 1769 |
| **Simple Proportion** | 0.07 | 0.27 | 0.53 |
| **Pooled Prevalence and 95% CI** | 0.17 (0.05-0.47) | 0.45 (0.28-0.63) | 0.54 (0.47-0.62) |
| **P Value** | <0.001 | <0.001 | 0.012 |
| **Heterogeneity Test** | | | |
| **Cochran's Q** | 100.652 | 1066.515 | 227.944 |
| **df (Cochran's Q)** | 2 | 13 | 14 |
| $\tau^2$ | 1.611 | 1.941 | 0.309 |
| $I^2$ | 98.013 | 98.781 | 93.858 |
| **P Value (Cochran's Q)** | <0.001 | <0.001 | <0.001 |
| **Between Years** | | | |
| **Cochran's Q =5.890, df=2, P=0.053** | | | |

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| Quarter-Based (n=29) | | | |
|---|---|---|---|
| | **1988-1999 (n=3)** | **2000-2009 (n=12)** | **2010-2019 (n=14)** |
| **Number of Udder Quarters** | 10340 | 13828 | 20968 |
| **Number of Udder Quarters with Subclinical Mastitis (+)** | 468 | 3404 | 5073 |
| **Simple Proportion** | 0.05 | 0.25 | 0.24 |
| **Pooled Prevalence and 95% CI** | 0.12 (0.02-0.53) | 0.27 (0.23-0.33) | 0.32 (0.23-0.43) |
| **P Value** | 0.066 | <0.001 | 0.002 |
| **Heterogeneity Test** | | | |
| **Cochran's Q** | 805.132 | 368.279 | 2215.519 |
| **df (Cochran's Q)** | 2 | 11 | 13 |
| **$\tau^2$** | 3.687 | 0.171 | 0.804 |
| **$I^2$** | 99.752 | 97.013 | 99.413 |
| **P Value (Cochran's Q)** | <0.001 | <0.001 | <0.001 |
| **Between Years** | | | |
| **Cochran's Q = 1.666, df=2, P= 0.435** | | | |

df= degree of freedom, C.I.: Confidence Interval, SE: Standard Error, $I^2$: Ratio of variance in observed effects to variance in true effects rather than sampling error, $\tau^2$: Variance in true effect sizes.

**Table 7.** Subgroup analysis results of cow-based and quarter-based subclinical mastitis prevalences in Türkiye according to herd size.

| | Herd Sizes | | |
|---|---|---|---|
| | **Small (n=12)** | **Medium (n=9)** | **Large (n=11)** |
| **Cow-Based (n=32)** | | | |
| **Number of Cows** | 597 | 1537 | 8052 |
| **Number of Cows with Subclinical Mastitis (+)** | 303 | 801 | 2044 |
| **Simple Proportion** | 0.51 | 0.52 | 0.25 |
| **Pooled Prevalence and 95% CI** | 0.53 (0.42-0.64) | 0.53 (0.46-0.61) | 0.34 (0.18-0.55) |
| **P Value** | 0.58 | 0.37 | 0.12 |
| **Heterogeneity Test** | | | |
| **Cochran's Q** | 72.154 | 63.414 | 1875.242 |
| **df (Cochran's Q)** | 11 | 8 | 10 |
| **$\tau^2$** | 0.528 | 0.173 | 1.988 |
| **$I^2$** | 84.755 | 87.385 | 99.467 |
| **P Value (Cochran's Q)** | <0.001 | <0.001 | <0.001 |
| **Between Herd Sizes** | | | |
| **Cochran's Q =3.155, df=2, P=0.207** | | | |
| **Udder Quart -Based (n=29)** | | | |
| | **Small (n=10)** | **Medium (n=8)** | **Large (n=11)** |
| **Number of Udder Quarter** | 1765 | 6100 | 37271 |
| **Number of Udder Quarter with Subclinical Mastitis (+)** | 744 | 1901 | 6300 |
| **Simple Proportion** | 0.42 | 0.32 | 0.17 |
| **Pooled Prevalence and 95% CI** | 0.42 (0.33-0.51) | 0.27 (0.15-0.45) | 0.18 (0.12-0.25) |
| **P Value** | 0.078 | **0.012** | **<0.001** |
| **Heterogeneity Test** | | | |
| **Cochran's Q** | 118.494 | 1074.647 | 2437.061 |
| **df(Cochran's Q)** | 9 | 7 | 10 |
| **$\tau^2$** | 0.319 | 1.246 | 0.582 |
| **$I^2$** | 92.405 | 99.349 | 99.590 |
| **P Value (Cochran's Q)** | **<0.001** | **<0.001** | **<0.001** |
| **Between Herd Sizes** | | | |
| **Cochran's Q =15.922, df=2, P<0.001** | | | |

df= degree of freedom, C.I.: Confidence Interval, SE: Standard Error, $I^2$: Ratio of variance in observed effects to variance in true effects rather than sampling error, $\tau^2$: Variance in true effect sizes.

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**Table 8.** Multivariate meta-regression model of the prevalence of cow-based and quarter-based subclinical mastitis in Türkiye.

| **Cow-Based**<br>**Covariate**<br>**Years** | **Intercept** | **SE** | **95% CI** | | **Z-Statistic** | **P-Value** |
|---|---|---|---|---|---|---|
| **1988-1999 (Referance)** | 0.00 | - | - | - | - | - |
| **2000-2009** | 1.22 | 0.69 | -0.13 | 2.57 | 1.78 | 0.076 |
| **2010-2019** | 1.63 | 0.71 | 0.25 | 3.02 | 2.31 | 0.021 |
| **Herd Sizes** | | | | | | |
| **Large (Referance)** | 0.00 | - | - | - | - | - |
| **Medium** | 0.40 | 0.51 | -0.59 | 1.40 | 0.80 | 0.425 |
| **Small** | 0.68 | 0.45 | -0.20 | 1.57 | 1.51 | 0.131 |
| **Constant** | -1.81 | 0.63 | -3.04 | -0.58 | -2.87 | 0.004 |
| **Model Test** | | | | | | |
| Q = 9.42, sd = 4, P = 0.052 | | | | | | |
| **Goodness of Fit Test** | | | | | | |
| $Tau^2$ = 1.077, Tau = 1.038, $I^2$ = 97.97%, Q = 1326.86, df = 27, P<0.001 | | | | | | |
| **Total Between-Study Variance (Constant Only)** | | | | | | |
| $Tau^2$ = 1.496, Tau = 1.223, $I^2$ = 98.60%, Q = 2210.22, df = 31, P<0.001 | | | | | | |
| **Ratio of Explained Variance** | | | | | | |
| $R^2$ analog = 0.28 | | | | | | |

| **Quarter-Based**<br>**Covariate**<br>**Region** | **Intercept** | **SE** | **95% CI** | | **Z-Statistic** | **P-Value** |
|---|---|---|---|---|---|---|
| **The Marmara Region (Referance)** | 0.00 | - | - | - | - | - |
| **The Mediterranean region** | 0.60 | 0.62 | -0.61 | 1.82 | 0.97 | 0.331 |
| **The Eastern Anatolia Region** | 0.69 | 0.49 | -0.27 | 1.65 | 1.40 | 0.160 |
| **The Aegean Region** | 1.57 | 0.61 | 0.38 | 2.76 | 2.59 | 0.010 |
| **The Central Anatolia Region** | 1.73 | 0.64 | 0.48 | 2.98 | 2.72 | 0.007 |
| **Years** | | | | | | |
| **1988-1999 (Referance)** | 0.00 | - | - | - | - | - |
| **2000-2009** | 1.08 | 0.50 | 0.11 | 2.05 | 2.18 | 0.029 |
| **2010-2019** | 1.38 | 0.50 | 0.41 | 2.35 | 2.78 | 0.005 |
| **Herd Size** | | | | | | |
| **Large (Referance)** | 0.00 | - | - | - | - | - |
| **Medium** | 0.50 | 0.38 | -0.25 | 1.25 | 1.31 | 0.191 |
| **Small** | 0.97 | 0.36 | 0.27 | 1.67 | 2.72 | 0.007 |
| **Constant** | -3.40 | 0.64 | -4.67 | -2.14 | -5.29 | <0.001 |
| **Model Test** | | | | | | |
| Q = 33.16, df= 8, P < 0.001 | | | | | | |
| **Goodness of Fit Test** | | | | | | |
| $Tau^2$ = 0.512, Tau = 0.715, $I^2$ = 98.83%, Q = 1711.85, df = 20, P < 0.001 | | | | | | |
| **Total Between-Study Variance (Constant Only)** | | | | | | |
| $Tau^2$ = 0.731, Tau = 0.855, $I^2$ = 99.33%, Q = 4175.77, df = 28, P < 0.001 | | | | | | |
| **Ratio of Explained Variance** | | | | | | |
| $R^2$ analog = 0.30 | | | | | | |

df= degree of freedom, C.I.: Confidence Interval, SE: Standard Error, $I^2$: Ratio of variance in observed effects to variance in true effects rather than sampling error, $Tau^2$: Variance in true effect sizes.

## Discussion and Conclusion

The prevalence values calculated in studies conducted in Türkiye on subclinical mastitis showed a wide range between 5% and 78% in cow-based studies, and between 2% and 78% in udder quarter-based studies. With this study, a stronger and more precise estimation was provided by eliminating the inconsistencies in the individual studies regarding the effect size of the subclinical mastitis prevalence in the population. As a result of meta-analysis, the prevalence of subclinical mastitis was calculated as 48% in cow-based studies, and as 32% in udder quarter-based studies. In a meta-analysis study, the prevalence of subclinical mastitis was calculated as 41% in India (11). In a study performed by Bangar et al. (3), the prevalences of cow-based and udder quarter-based subclinical mastitis in India were calculated as 46% and 23%, respectively Getaneh and Gebramedhin (7), reported the prevalence of subclinical mastitis as 37% in Ethiopia in a meta-analysis study. In this study, the prevalence of subclinical mastitis in Türkiye was found to be similar to or even higher than in the aforementioned countries where the studies were conducted for the same purpose.

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Findings obtained in subgroup analyses based on geographical regions in Türkiye, are in line with the view of Philpot and Nickerson who conducted a study on the geographical evaluation of the prevalence of subclinical mastitis and suggested that latitude may have an effect on the prevalence of the disease. In the same study, higher tendency in somatic cell count was reported in South America where the temperature and humidity are high (18). In a meta-analysis study comparing five regions in India in terms of the prevalence of subclinical mastitis, the effect of latitude was investigated and it was reported that the Eastern (47%) and Southern (50%) regions have a higher prevalence than the Western (37%) and Northern (39%) regions (11).

In the udder quarter-based analysis, the reason for the Central Anatolia Region having the highest prevalence can be explained as administrative factors in dairy enterprises in the Central Anatolia Region and by the infection in more than one udder quarter of a cow with mastitis. In addition, since the exclusion of the Southeastern Anatolia Region and the Black Sea Region from the evaluation due to the lack of sufficient number of studies for creating a subgroup, a new subgroup analysis for region comparisons became necessary.

Bangar et al. (3) calculated the disease prevalence in local breeds (24.2%) to be lower than that of crossbreed (31.4%). This result can be interpreted as local breeds having more adaptability and more resistant than culture breeds as well as lower milk yield. Tuke et al. (19), calculated the prevalence of mastitis for local breeds (17.64%) to be lower than exotic breeds (61.51%) and explained the reason for this as certain psychological and anatomical differences between breeds. It has been determined that the highest prevalence is in Holstein cows and crossbreeds, and it has been suggested that this is due to their drooping udders, high milk yield and relatively more open teat canals (19). In contrast, Hoque et al. (10) reported in their study that this difference between breeds may be due to different environmental conditions and administrative factors. Biffa et al. (4), also reported that the prevalence of subclinical mastitis in Zebu x Holstein crossbreeds and Jersey cows was lower than the prevalence in local breeds. In this study, the prevalence of subclinical mastitis in cow-based studies in Türkiye was calculated to be higher in Holsteins and their crossbreeds compared to other breeds, which is similar to what other studies also reported. This result can be explained by the fact that local breeds have more adaptability and disease resistance than culture breeds, and that their milk yield is lower (12).

In both cow-based and the udder quarter-based studies the prevalence of subclinical mastitis tends to increase over the years. Krishnamoorthy et al. (11), showed that the prevalence of subclinical mastitis tends to increase by performing subgroup analysis compared to the years 2005-2010 and 2011-2016 (29% and 45%, respectively). The increase in the prevalence of mastitis in recent years can be explained by the extensive breeding of culture-breed cows in the world and in Türkiye, and the low disease resistance of these cows despite their high milk yield. However, Philpot and Nickerson reported in their study that the prevalence of mastitis has decreased in many countries in recent years (18).

When the prevalence of mastitis is evaluated according to herd size in Türkiye, it has been observed that large scale herds have a lower rate than medium and small-scale herds in both cow and udder quarter-based studies. This result can be explained by the fact that small-scale herds do not have the quality of management available to large-scale herds and that milk production is higher in large-scale herds (18). Additionally, this result was supported by the studies reporting that the mean somatic cell counts in the herd decrease with the increasing herd size (2, 15, 16).

$R^2$ analog is the ratio of the variance explained by the independent variables to the total variance in a meta-regression model. In this study, the models with the highest $R^2$ analog value were used in the multivariate meta-regression models created to calculate the heterogeneity between studies.

According to the multivariate meta-regression model created to determine the factors affecting the prevalence of cow-based subclinical mastitis in Türkiye, only the variables of years and herd sizes were found to be effective on the prevalence of subclinical mastitis, and it was determined that the variance observed in the created model reflected 28% of the true variance. In the udder quarter-based studies, this rate was calculated as 30% and the variables of regions, years and herd sizes were included in the model. These results showed that models created with regions, years and herd sizes are not sufficient to explain the prevalence of subclinical mastitis.

In this study, the prevalence of subclinical mastitis in dairy cows in Türkiye was evaluated by meta-analysis and the common prevalences were calculated. In this way, an opportunity was obtained to make strong and precise predictions with a large sample. It was determined that the prevalence of subclinical mastitis in Türkiye increased in the years 2010-2019 compared to the years 1988-1999 and 2000-2009. In contrast, the prevalence of subclinical mastitis in Finland has decreased from 22.3% (1991) and 20.1% (2001) to 19% (2010) in the last 20 years (9). In addition, it was clearly shown that the prevalence of subclinical mastitis in Türkiye [0.48 (95% CI: 0.41- 0.56)] is significantly higher than in India [0.41 (95% CI: 0.33-0.49)] and in Ethiopia [0.37 (95% CI: 0.33-0.41)] (7, 11).

Considering the importance of a fully integrative large farm structure in the control of diseases in dairy

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cattle breeding, examining the management mechanisms of these enterprises and modeling similar mechanisms by small enterprises will be effective in reducing subclinical mastitis. Due to the multifactorial nature of subclinical mastitis, it is important to keep a holistic approach in the combat against this disease and develop a multi-faceted control mechanism. In addition, it is necessary to conduct meta-analyses of studies examining different factors and to calculate the effects of these factors on the prevalence of subclinical mastitis.

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## Conflict of Interest

The authors declared that there is no conflict of interest.

## Author Contributions

AA supervised, conceptualizated, administrated project. EÇG conducted formal analysis, writed original draft, investigated and visualizated. EÇG ve AA contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis and manuscript.

## Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

## Ethical Statement

This study does not present any ethical concerns.

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# Superficial pyoderma in cats and dogs: A retrospective clinical study

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## ABSTRACT

Superficial pyoderma is a common complication of a range of feline and canine skin diseases. The objective of the present study is to retrospectively evaluate bacterial and fungal skin culture samples in superficial pyoderma cases of cats and dogs and to reveal in detail the pathogens and their susceptibility and/or resistance to antimicrobials, as well as demographic data and clinical symptoms of the patients. Medical records of 28 cats and 35 dogs meeting the criteria for inclusion were reviewed. *Staphylococcus spp.* and *Trichophyton* species were found to be the most common cultured microorganisms in both cats and dogs. Antimicrobial resistance was determined both in cat and dog samples. As a result, it has been demonstrated that skin culture is particularly important for the management of diagnosis and treatment processes and the regulation of treatment protocols in pyoderma in terms of preventing the increasing antibiotic resistance in recent years and thus protecting both human and animal health.

## Introduction

While pyoderma typically refers to pyogenic changes occurring in the epidermis and/or follicular epithelium, superficial pyoderma is confined to the superficial portion of the hair follicle (1). Although mostly used to describe bacterial infections, fungi may also cause pyoderma lesions (21, 23). For the formation of superficial pyoderma, microorganisms must be able to adhere to the skin surface and colonize. In most cases, pyoderma develops secondary to various causes such as trauma, hormonal changes, impaired immune system, parasitic infestations, exposure to allergens, and follicular dysplasia (17, 28).

In pyoderma, a chronic and recurrent inflammatory process may easily occur. Antibiotics are often used empirically for the treatment of infectious bacterial diseases, leading to multidrug resistance in many cases. The threat posed by increasing antimicrobial resistance adds a new dimension to the public health implications of the management of cat and dog pyoderma and creates the need to develop new strategies for patient management in clinics (12). Therefore, deeper examinations including skin cultures in dermatological lesions become a great need, especially considering the decrease in the ability to effectively treat pyoderma.

The aim of the present study is to reveal the microorganisms and their susceptibility and/or resistance to antimicrobials in superficial pyoderma cases of cats and dogs referred to our clinics and to evaluate the possible effects of breed, age, gender, sterilization status, and seasonal factors on the disease.

## Materials and Methods

The records of feline and canine superficial pyoderma cases brought to Ankara University Faculty of Veterinary Medicine Small Animal Teaching Hospital between June 2018 and June 2022 were reviewed retrospectively from the hospital software database. The keywords ‘feline’ and 'canine' for animal species and ‘pyoderma’ for diagnosis were used to identify the cases to be included in the study. Superficial pyoderma was defined as animals presenting with dermatological complaints with the presence of neutrophils and/or intracellular bacteria on skin surface cytology (31). Among those, only the animals with the results of skin bacterial and fungal cultures and antibiogram analysis were included in the study. Of animals with multiple records, the data from the first visit were considered to avoid duplication. Demographic data, clinical signs, the season of presentation, age of onset, and duration of dermatological problems were evaluated. The localization and type of cutaneous lesions, the manifestation of the disease, as well as skin surface cytology and skin culture results were reviewed. All the examinations and procedures were performed after obtaining a written consent form from the owners (Ankara University Animal Experiments Local Ethics Committee Decision Number: 2022-14-132 & 2022-16-151).

***Bacterial Culture of the Skin:*** All skin samples are taken by following a routine method in our clinic. The skin swabs were taken very carefully to avoid possible contamination. Surface skin lesions were harvested by rotating a swab across them for 5 seconds and placed in a sterile container (8). The samples were sent to the laboratory immediately and, cultured within one hour. Inoculation was done on 5% Sheep Blood Agar and MacConkey Agar for bacterial growth from the swabs. After the incubation, the Petri dishes were incubated at 37°C for 24-48 hours. After the colonies formed, Gram staining was performed and, bacteria were classified as Gram-positive and Gram-negative. *Staphylococcus spp.* were separated from other bacteria by performing oxidation-fermentation tests and catalase tests. Afterward, coagulase, maltose, and mannitol tests were performed on *Staphylococcus spp* (29).

***Fungal Culture of the Skin:*** The swabs were inoculated on Sabouraud Dextrose Agar (SDA) for fungal growth and then the Petri dishes were incubated at 25 °C for 4 weeks. During the incubation, the formed colonies were identified according to their macroscopic and microscopic features including size, duration, structure, and pigmentation. Microscopic examination involved using Lactophenol Cotton Blue solution to assess dermatophytes at the genus level, focusing on features like hyphae structures, macroconidia, and spore formations within The swabs were inoculated on Sabouraud Dextrose Agar (SDA) for fungal growth and then the Petri dishes were incubated at 25 °C for 4 weeks. During the incubation, the formed colonies were identified according to their macroscopic and microscopic features including size, duration, structure, and pigmentation. Microscopic examination involved using Lactophenol Cotton Blue solution to assess dermatophytes at the genus level, focusing on features like hyphae structures, macroconidia, and spore formations within the fungal colonies (24).

***Antimicrobial Susceptibility Test:*** The antimicrobial susceptibility of bacterial isolates was tested on Mueller-Hinton agar (Merck, USA) using the disk diffusion method according to CLSI guidelines from 2013 (2). The following panel of antimicrobials was used: amoxicillin (10 µg/disk), amoxicillin/ clavulanic acid (30 µg/disk), ampicillin (10 µg/disk), Clindamycin (10 µg/disk),ciprofloxacin (5 µg/disk), enrofloxacin (5 µg/disk), danofloxacin (5 µg/disk), erythromycin (15 µg/disk), gentamicin (10 µg/disk), imipenem (10 µg/disk), lincomycin (15 µg/disk), meropenem (10 µg/disk), mupirocin (200 µg/disk), novobiocin (30 µg/disk), oxytetracycline (30 µg/disk), penicillin (10 units/disk), streptomycin (10 µg/disk), tetracycline (30 µg/disk). Staphylococcus aureus reference strain ATCC 25923 was used for quality control in the study.

## Results

***Prevalence, Signalment, and Seasonality:*** A total of 88 cats and 59 dogs with the diagnosis of superficial pyoderma were reviewed in the study. Of these, 28 cats and 35 dogs met the criteria for inclusion. This account for 31.8% and 59.3% of all suspected cases of cats and dogs, respectively. The age, breed, gender, and sterilization status were shown in Table 1.

Age at the time of diagnosis ranged from 9 months to 11.5 years in cats and 10 months to 18 years in dogs. The median age was 4.3 years in cats and 6.8 years in dogs. Upon presentation, the duration of skin lesions ranged from less than 1 month to more than 2 years, with a median duration of 5 months in cats and from 1 month to more than 4 years with a median of 11 months in dogs.

When the distribution of the patients in terms of seasons was evaluated, it was determined that the presentation of pyoderma in cats was higher in winter (from December to February; 10/28, 35.7%) followed by summer (from June to August; 7/28, 25%), spring (from March to May; 5/28, 17.8%) and autumn (from September to November; 6/28, 21.4%). In dogs, the incidence of the diagnosis was higher in winter (15/35, 42.8%), followed by summer (9/35, 25.7%), spring (7/35, 20%), and autumn (4/35, 11.4%).

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**Table 1**. Breed, age, gender and, sterilization status of cats and dogs included in the study.

| Cats | Dogs |
|---|---|
| **Breed** | |
| Domestic short hair (n=18); Scottish Fold (n=5); British Shorthair (n=3); Norwegian Forest (n=1); Bombay (n=1) | Mix-Breed (n=6); Golden Retriever (n=6); Terrier (n=4); Turkish Kangal Shepherd (n=3); Pug (n=3); German Shepherd (n=2); Labrador Retriever (n=2); Akita (n=1); American Cocker (n=1); Belgian Shephard (n=1); French Bulldog (n=1); Hungarian Hound (n=1); King Charles Spaniel (n=1); Poddle (n=1); Shar-Pei (n=1); Siberian Husky (n=1) |
| **Age (mean± std)** | |
| 4.34±2.22 | 6.80±4.09 |
| **Gender and Sterilization Status** | |
| Female active / spayed 7/6<br>Male active / castrated 14/1 | Female active / spayed 11/1<br>Male active / castrated 21/1 |

***Clinical Signs:*** In cats, the most common complaints and findings at the presentation were pruritus (n=22/28), multifocal alopecia (n=22/28), crusting (n=20/28), pustules (n=19/28), effusive lesions (n=13/28), and hyperpigmentation (n=12/28) while crusty and effusive lesions (n=32/35), scratching (including licking and biting if the area is accessible; n=28/35), multifocal alopecia areas (n=25/35) and hyperpigmentation (n=22/35) were the most common complaints in dogs.

In cats, cutaneous lesions were mostly multifocal (n=11). Apart from these, regions, where specific localization was identified were; head/neck (n=7), inguinal region (n=6), limbs and axillar region (n=4), and ventral abdomen (n=1). In dogs, the specific lesions were mostly located on the inguinal region (n=11), limbs and axillar region (n=9), head/neck (n=4), ventral abdomen (n=3), and tail (n=1). The number of dogs with multifocal lesions was noted as 7.

***Bacterial and Fungal Culture Findings:*** Bacterial growth was observed in 17 of 28 cats. In two of these cats, concomitant colonizing bacteria were grown, while a single bacterial type was determined in the remaining cats. The most frequently recovered bacterial genus was *Staphylococcus spp.* (n=15) while *Pseudomonas spp. (n=4)* and *Corynebacterium spp. (n=2)* growth was also observed. Among the studied samples, *Staphylococcus aureus* (11/17, 64.7%) represented the most frequently recovered bacterial isolates. The other staphylococci recovered from the studied animals was *Staphylococcus pseudointermedius (S. pseudointermedius)* isolated from 4 samples (23.5%).

Fungal species were recovered from 16 of the studied cats (57.1%) and more than one fungal microorganism grew in 5 of these cats. *Trichophyton* species were found to be the most common fungal microorganism in this study (6/16 isolates, 37.5%). The other microorganisms grown were found to be *Aspergillus* (5/16, 31.25%) and *Penicillium* (4/16, 25%) followed by overgrowth of *Microsporum, Alternaria, Cladosporium*, *Candida* and, *Rhizopus* species each in one sample (1/16, 6.25%), Yeast overgrowth was also seen in one cat. The list of isolated bacteria and fungi from feline patients are presented in Table 2.

In dogs, bacterial growth was observed in 29 out of 35. In 3 dogs concomitant colonizing bacteria growth was seen. The most detected bacterial genus was *Staphylococcus* (26/29, 89.65 %) followed by *Pseudomonas* (3/29; 10.34%) and *Streptococcus* and *Proteus* were detected in one dog each (1/29; 3.44%). Among those, *S. pseudointermedius* represented the majority of the isolates (13/29; 44.82%).

Fungal pathogens were grown in 17 dogs and more than one microorganism growth was identified in 8. *Trichophyton* species (8/17; 47.05%) were found to be the most common fungal microorganism as it is in cats. This was followed by *Penicillium* (5/17; 29.41%), which was observed as a mixed infection with *Microsporum gypseum* growth in one dog, *Alternaria* (5/17; 29.41%), *Aspergillus* (3/17; 17.64%), *Mucor* (2/17; 11.76%), *Rhizopus* (2/17; 11.76%), *Candida* (1/17; 5.88%) species and yeast (n=2) growth. Bacterial and fungal culture results of the dogs are presented in Table 3.

***Antimicrobial susceptibility test:*** The antibiotics to which staphylococci are most susceptible in cats were oxytetracycline (12/17; 70.58%), enrofloxacin (11/17; 64.7%), and danofloxacin (7/17; 41.17%). All staphylococcal isolates were susceptible to at least 2 antimicrobial agents. A total of 10 isolates were resistant to at least one agent. Antimicrobial resistance was most determined against ampicillin (50%), followed by amoxicillin (30%), lincomycin (30%), oxytetracycline (30%), and enrofloxacin (30%). Six of the resistant samples of staphylococci were determined to be resistant to three or more antimicrobials which are defined as multi-resistance in this presented study.

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**Table 2.** Bacteria and fungi isolated from skin samples from cats.

| Cat | Bacterial Culture | Fungal Culture |
|---|---|---|
| **1** | *Staphylococcus aureus* | - |
| **2** | *Staphylococcus aureus* | - |
| **3** | *Staphylococcus aureus* | - |
| **4** | - | *Aspergillus fumigatus Trichophyton mentagrophytes* |
| **5** | *Staphylococcus aureus* | - |
| **6** | *Staphylococcus pseudointermedius* | - |
| **7** | *Staphylococcus aureus* | - |
| **8** | *Staphylococcus aureus* | - |
| **9** | *Pseudomonas spp.* | *Trichophyton mentagrophytes* |
| **10** | - | *Alternaria spp.* |
| **11** | - | *Penicillium spp.* |
| **12** | *Staphylococcus pseudointermedius* | *Rhizopus spp.* |
| **13** | *Staphylococcus pseudointermedius* | - |
| **14** | - | *Penicillium spp.* |
| **15** | *Staphylococcus pseudointermedius* | - |
| **16** | - | *Trichophyton rubrum* |
| **17** | - | *Aspergillus niger* |
| **18** | *Staphylococcus aureus Corynebacterium spp.* | - |
| **19** | *Staphylococcus aureus* | *Trichophyton mentagrophytes Aspergillus fumigatus Alternaria spp.* |
| **20** | - | *Penicillium spp.* |
| **21** | - | *Trichophyton mentagrophytes* |
| **22** | - | *Penicillium spp.* |
| **23** | *Staphylococcus aureus* | *Candida spp. Cladosporium spp.* |
| **24** | - | *Microsporum ferrugineum* |
| **25** | - | *Aspergillus niger Penicillium spp.* |
| **26** | *Staphylococcus aureus* | *Aspergillus niger* |
| **27** | *Staphylococcus aureus* | - |
| **28** | *Staphylococcus aureus* | - |

**Table 3.** Bacteria and fungi isolated from skin samples from dogs.

| Dog | Bacterial Culture | Fungal Culture |
|---|---|---|
| **1** | *Staphylococcus intermedius* | *Penicillium spp.* |
| **2** | *Staphylococcus epidermidis* | *Candida albicans* |
| **3** | - | *Aspergillus fumigatus* |
| **4** | *Pseudomonas aeruginosa* | - |
| **5** | *Staphylococcus intermedius* | *Penicillium spp. Microsporum gypseum* |
| **6** | *Staphylococcus epidermidis* | - |
| **7** | *Staphylococcus aureus Streptococcus canis* | - |
| **8** | *Staphylococcus pseudointermedius* | *Trichophyton mentagrophytes Aspergillus niger Alternaria spp.* |
| **9** | *Staphylococcus intermedius* | *Aspergillus fumigatus* |
| **10** | - | - |
| **11** | *Staphylococcus intermedius* | *Trichophyton mentagrophytes Alternaria spp.* |
| **12** | - | *Trichophyton mentagrophytes Alternaria spp.* |
| **13** | *Pseudomonas aeruginosa* | - |
| **14** | *Proteus mirabilis* | - |
| **15** | *Staphylococcus pseudointermedius* | - |
| **16** | *Staphylococcus aureus* | *Penicillium spp.* |
| **17** | *Staphylococcus aureus* | - |
| **18** | *Staphylococcus epidermidis* | *Penicillium spp.* |
| **19** | *Staphylococcus aureus* | - |
| **20** | *Staphylococcus pseudointermedius* | *Rhizopus spp.* |
| **21** | *Staphylococcus epidermidis* | - |
| **22** | *Staphylococcus epidermidis* | *Rhodotorula glutinis* |
| **23** | *Staphylococcus intermedius* | - |
| **24** | *Staphylococcus intermedius* | - |
| **25** | *Staphylococcus aureus* | *Trichophyton rubrum Penicillium spp.* |
| **26** | *Staphylococcus epidermidis* | - |
| **27** | *Staphylococcus pseudointermedius Pseudomonas aeruginosa* | |
| **28** | - | *Aspergillus niger* |
| **29** | *Staphylococcus epidermidis* | - |
| **30** | - | *Aspergillus fumigatus Trichophyton mentagrophytes* |
| **31** | *Staphylococcus intermedius* | *Rhizopus spp.* |
| **32** | - | *Alternaria spp. Trichophyton rubrum* |
| **33** | *Staphylococcus intermedius* | *Alternaria spp. Trichophyton mentagrophytes* |
| **34** | *Staphylococcus intermedius* | *Trichophyton mentagrophytes* |
| **35** | *Staphylococcus aureus* | - |

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All staphylococcal strains cultured from dog samples were susceptible to at least 2 antimicrobials listed enrofloxacin (20/29; 68.96%), oxytetracycline (12/29; 41.3%) and ciprofloxacin (7/29; 24.13%). Antimicrobial resistance against staphylococci was most prevalent against amoxicillin (34.48%), followed by meropenem (13.79%) and metronidazole (10.34%). Twelve samples showed multidrug resistance in dogs. All *Pseudomonas spp.* cultured samples were susceptible to ciprofloxacin while resistant to ampicillin and/or amoxicillin. While *Proteus* and *Streptoccous* isolates were susceptible to enrofloxacin, they were resistant to amoxicillin and ampicillin, respectively.

## Discussion and Conclusion

According to the results of the presented study, bacterial and/or fungal growth was observed in approximately 30% of cats and 60% of dogs who presented to our clinics with dermatological complaints with a preliminary diagnosis of pyoderma. These rates are consistent with the previous findings reported from different regions of the world (17, 20, 31).

The vast majority of the cats in this study (about 65%) were domestic shorthairs. The dominance of this breed was found to be consistent with previously presented reports of feline pyoderma (30, 31). However, it seems plausible that this is a proportional excess because of the regional adoption of this breed rather than a breed predisposition. The same assessment may also be valid for canine pyoderma cases, which were more intensely detected in mix-breed and Golden retriever dogs in this report.

One of the surprising findings in our study was the predominance of the male sex in both cats and dogs. Previous literature on pet animals do not show a clear gender predominance in the development of dermatological diseases in cats (31) or dogs (23). However, a study including 30 cats determined that pyoderma was more common in male cats than females (25). A similar finding was reported in a pyoderma study in which 60 dogs were examined and, according to this study, male dogs represent 60% of the cases (20). These findings may be explained by the fact that superficial traumas are more common in males than females, leading to the formation of pyoderma. Nevertheless, this finding is an outcome that should be considered carefully, especially in clinical practice, considering studies showing that multidrug resistance in pyoderma cases is more commonly determined in samples taken from male dogs, as well (10).

Our results showed that pyoderma can be detected at an earlier age in cats than in dogs. This trend is mostly considered to be associated with early-onset hypersensitivity in most feline patients (31). Many researchers stated the likelihood of the formation of pyoderma in dogs decreases after 5 years of age (9, 22). Although there were patients in whom the first manifestation of clinical signs begins from the age of 10 months among the dogs included in our study, the higher average age (6.8 y mean) may be associated with an increased risk of developing pyoderma as a result of the immune system declining with advancing age in the included dogs.

The incidence of dermatological problems may develop depending on the season and climate. The incidence of pyoderma cases in winter months was found to be higher in cats and dogs than in other months in this study. Contrary to our findings, previous studies show a higher frequency of pyoderma cases in warmer seasons (11, 31). This may be related to the fact that the number of patients presented to our hospital in the winter period is higher than in the summer leading the proportional differences between the presented and previously reported studies. However, it should be considered that this finding may also be related to the fact that anxiety experienced by cats and dogs due to the decrease in sunlight and sub-zero temperatures causes cold stress during winter, regardless of whether it is an indoor or outdoor pet (18).

The most common clinical signs were pruritus and multifocal lesions in cats while dogs presented crusted lesions especially localized in the inguinal and axillar region, in line with the previous reports (26, 31). This distribution corresponds to that of feline hypersensitivities and its nature of generalized localization. However, lesions are distinctly localized in bacterial pyoderma in dogs. Lesions often start in the limb, groin, and axilla, which may be due to the fact that these areas are more humid and offer a suitable environment for the proliferation of bacterial microorganisms (6). Although it is assumed that microorganisms can adhere to corneocytes in these regions more easily than other anatomical regions, it has been shown that there is no significant difference in adhesion to these regions compared to other regions, and adhesion is probably not an important factor in the susceptibility of canine bacterial pyoderma to affect specific localizations (5).

In the presented study, staphylococci are the most cultured microorganism in both cats and dogs. While *S. aureus* was the most isolated bacteria from cats, *S. pseudointermedius* was isolated in the majority of the dogs. Given the recent understanding of staphylococcal infections may be effective in the formation of atopic dermatitis in dogs, the same suggestions may be valid for cats (3). Staphylococci, if there is a predisposition to atopy, can worsen the patient's clinical condition by producing *Staphylococcus* -specific IgE and staphylococcal protein A, which can bind nonspecifically to IgE molecules on mast cells (15). Controlled prospective clinical trials are needed to further

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characterize *Staphylococcus-induced* pyoderma in both cats and dogs to advance the understanding of pyoderma lesions and related diseases such as atopic dermatitis.

Although it is known that zoonotic and multi-drug resistant strains have increased in veterinary medicine in recent years, no significant progress has been made in the control mechanisms of drug use in the control of infections in clinical practice (7, 19). However, there is a growing need to encourage the prudent and judicious use of antibiotics in pet medicine, as the recent much greater incidence of multidrug-resistant bacteria in dogs and cats highlights a significant threat (10, 19). We have revealed an increased risk of multi-drug resistance in antimicrobial susceptibility tests against *Staphylococcus spp*. Exchange and acquisition of new strains of Staphylococci may occur between pets with normal daily contact. Animals receiving antibiotic therapy may be particularly at risk for acquiring resistant organisms, as this may promote the transfer of organisms through antibiotic-induced reduction of the normal resident *Staphylococcus* population (14). This finding has an additional severe consequence as these microorganisms are mostly of zoonotic importance, in addition to the prolongation of the treatment period of patients admitted to veterinary clinics and the rapid deterioration in animal health and welfare.

In the presented study, while the bacterial isolates were mostly susceptible to enrofloxacin and oxytetracycline, the resistance was determined against ampicillin and amoxicillin in cats and dogs, respectively. Penicillin groups are antimicrobials that have been widely used in human and animal medicine for many years in the treatment of various diseases, including pyoderma (13). Although, similar to our findings, resistance to ampicillin and amoxicillin, has been reported in feline canine staphylococci infections previously (4), a study among primary care practitioners showed that in most dogs diagnosed with pyoderma, the amoxicillin group was the most commonly prescribed antibiotic for empirical therapy (27). Inappropriate use of antibiotics in pyoderma isolates increases the risk of developing multidrug resistance. Therefore, evidence of methicillin resistance in staphylococci, although still rare in veterinary medicine, should be carefully evaluated in animals, considering the risks to human health (16).

In the presented study, besides bacterial agents, fungal agents were also evaluated. *Trichophyton* was the most frequently detected dermatophyte in cats and dogs. The high isolation rates obtained in this study showed that dermatophytes still pose a problem in cats and dogs. Since the majority of fungal agents isolated from domestic animals also cause disease in humans, these cases should be followed carefully, and the effectiveness of fungal infections should be taken into account when designing the treatment protocol in pyoderma cases.

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## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

NKY and BB planned the study, reviewed the patient files and contributed to the interpretation of the results. NKY took the lead in writing the manuscript. NKY and BB provided critical feedback and helped shape the research, analysis and manuscript.

## Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

## Ethical Statement

This study was reviewed by the Local Animal Ethics Committee of Ankara University (Decision number: 2022-14-132 & 2022-16-151).

## Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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# Tie-cross acrylic external skeletal fixator: Technique and 13 cases

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## Abstract

This study aims to describe a novel design of an acrylic external skeletal fixator (ESF) system for stabilizing epiphyseal/metaphyseal fractures with or without articular involvement and evaluate its efficiency in 13 cases. Client-owned five cats and eight dogs with epiphyseal/metaphyseal fractures were included in this study. Cross pins with or without a transcortical pin were included in "J" shaped acrylic ESF, and this novel technique was called the Tie-cross ESF. The functional use of the extremity was evaluated, and the joint range of motion was assessed and compared with the contralateral side. Radiographs were evaluated for bone healing and potential complications. The first use of the extremities changed from the day of surgery to the 3rd day. Fracture healing occurred in 35-69 days, and ESFs were removed. No persistent lameness was observed, and total functional recovery was provided in all cases. Fixation of epiphyseal and metaphyseal fractures can be challenging, especially when it involves the articular surface. Including cross-pins in an acrylic ESF (Tie-cross ESF) is useful and can be considered an alternative technique for stabilizing these fractures. This technique encourages the patient to use the extremity during the fracture healing and enables joint functions; therefore, additional physical therapy will not be necessary.

## Introduction

Physeal, epiphyseal and metaphyseal fractures are common; some may include articular surfaces (8, 31). All fractures need complete anatomical reduction and stable fixation. However, it is necessary to secure the articular surface in complete anatomical position and normal axial alignment for intra-articular fractures to restore early joint mobility. Additionally, an immediate joint motion is necessary to prevent joint stiffness and ensure articular healing and functional recovery (2, 7, 25, 28, 36). Many techniques can be used to perform this goal (14, 18, 21, 23, 32).

Rush-pin or Cross-pin technique, if necessary, combined with a transcondylar screw or pin, is one of these methods frequently used for the stabilization of epiphyseal fractures with or without articular involvement (4, 6, 18, 30, 38). Cross-pining is sufficient with simple Salter-Harris fractures without needing an additional fixation method or a bandage. It encourages the patient to use its extremity step by step in the early healing period and enables joint functions. Complications of this technique include pin loosening, displacement of the pins, fixation failure, skin perforation, and soft tissue or/and bone infection (2, 3, 7, 8, 14).

Due to the possible complications mentioned above, we decided to include cross pins in an acrylic external skeletal fixator to prevent pin displacement. This novel technique was called "tie-cross" acrylic ESF. ESF is a non-/minimal-invasive technique frequently used in fractures to restore limb functions in the early period. The tie-in ESF is commonly preferred for comminuted fractures to collegiate bone fragments and segments. Therefore, we thought the tie-cross technique would also be a successful fixation method for epiphyseal and metaphyseal fractures with or without articular involvement.

Following the successful results in the first patient, this technique was also used on some patients, and fracture healing, joint range of motion, and functional recovery were evaluated clinically and radiographically.

## Materials and Methods

***Animals and case selection:*** The surgical protocol was approved by the Near East University animal care ethics committee (No: 27.11.2020/121). Before the procedures, patient owners were informed, and their signed consent was requested.

Eight dogs and five cats with an intra-articular or extra-articular distal or proximal part of femoral, tibial, or humeral fractures were included in this study. None of the patients had any life-threatening condition at the clinical presentation. Still, three cases (cases #7, 9, and 10) also had contralateral side fractures. Clinical and radiographical evaluations were performed as routine. Information about cases is indicated in Table 1.

***Acrylic ESF Preparation:*** Commercially available self-curing, dental cold acrylic was used as an external frame. Liquid and powder were mixed with a ratio of 1:2, respectively. Because the mixture was liquid, to restrict the liquid acrylic before curing, it was first applied into a sterile endotracheal tube. The reason for choosing the endotracheal tube was that the tube was strong and flexible, and also sterile. Before this procedure, the tube had already been used as an external mold in the sterile surgical procedure as described below; pins were first passed through the endotracheal tube and the skin to the bone or drilled from the fracture line and then sent out of the skin and passed from the tube.

***Surgical procedure:*** Before the surgical procedure, medetomidine (for cats: 100 mcg/kg IM, for dogs: 25 mcg/kg IM) and butorphanol (0.1 mg/kg SC) were administered for premedication, propofol (4 mg/kg IV) was used for induction, then patients were intubated, and anesthesia was maintained with sevoflurane in oxygen. Cefazolin (30 mg/kg IV) was used as a single shot before anesthetic induction. The surgical site was clipped and prepared for aseptic surgery as routine.

Tie-cross Acrylic ESF was performed in all cases with an open surgical technique (17). Following exposure of the fracture site, intra-articular (intercondylar or interfragmentary) fracture fixation was aimed first. For this, a threaded pin (Kirschner or Schanz) was first passed through the endotracheal tube and the skin (percutaneous). Then the pin was applied transcondylary to stabilize the two fracture fragments; before this, a pointed bone holding forceps was temporarily used to stabilize both condyles. During this application, utmost care was taken to bring every bone fragment to its exact anatomic place to avoid potential adverse effects on joint mobility. Then, with or without intercondylar fractures, the condylar segment's stabilization to the bone's main body was performed by cross-pinning. Although it varies according to cases, the cross pins were sent from the distal fragment to the proximal main segment (such as an anterograde pin application), as in the routine cross pin application, angled upwards from the lateral and medial sides of the condyles (Figure 1). It is also possible to apply the cross-pins in a retrograde fashion in femur and tibia fractures. Before being applied, pins were passed through the tube used as an acrylic mold.

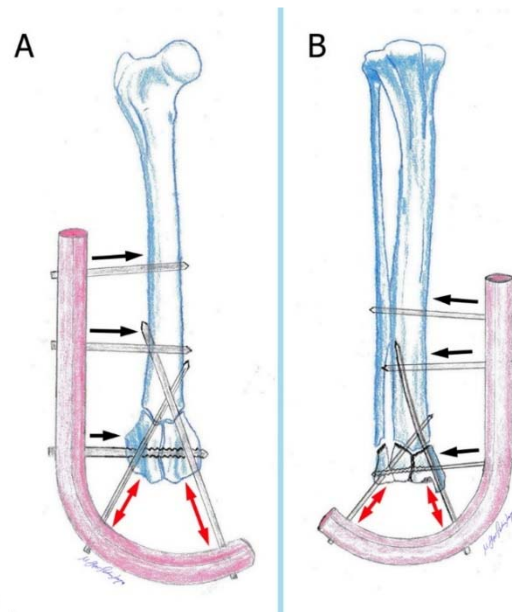


**Figure 1.** Cross-pins can be applied in both anterograde and retrograde fashion (red arrows) for femoral (A) and tibial fractures (B). If there is an intercondylar fracture, a transcondylar threaded pin should be applied before cross pins. Additional pins are sent above the fracture line from the tube to the bone (black arrow) to achieve a "J" shaped tie-cross ESF.

For humeral fractures, cross-pins were advanced from the fracture lines of each condyle (like retrograde pin application) and directed into the medullary canal of lateral and medial epicondylar crests. Then drilled out of the bone and the skin, passed through the plastic tube, and driven back from opposite ends until the inside ends aligned with the fracture line. Pins were advanced upward following fracture reduction to stabilize the condyles to the main body (Figure 2). Cross-pin ends were passed through the opposite cortex or driven into the medulla.

The plastic tube was bent in a "J" fashion for all patients, and additional pins were sent from the tube to the bone above the fracture line (Figure 3). Care was taken to keep an appropriate distance between the tube and the skin. Following the operation, the surgical site was sutured routinely, and cold-curing acrylic was prepared and injected inside the plastic tube. The acrylic body and pins were cooled with sterile saline to prevent possible thermal injury during the polymerization process of the acrylic.

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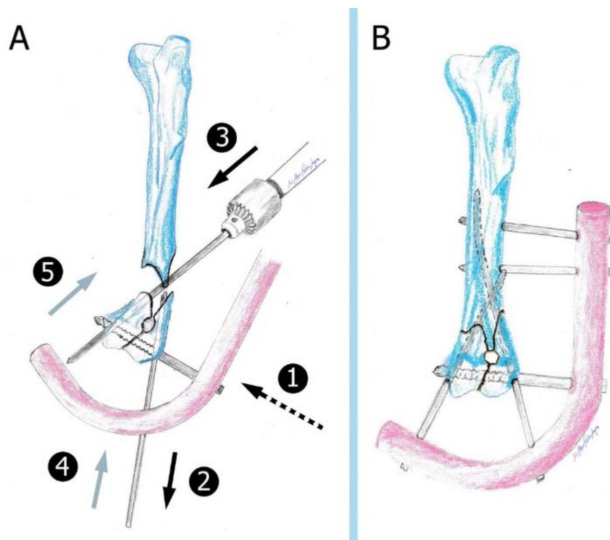


**Figure 2.** A. For humerus fractures following transcondylar pin application (dotted black arrow; no.1), two pins (black arrows; no. 2 and 3) are inserted into the lateral and medial epicondylar canals from fracture lines and drilled outside of the skin. Following reduction, these pins are driven into the main bone (grey arrows; no. 4 and 5) to achieve cross-pin fixation. B. After cross-pinning, additional pins are sent from the tube to the bone above the fracture line.

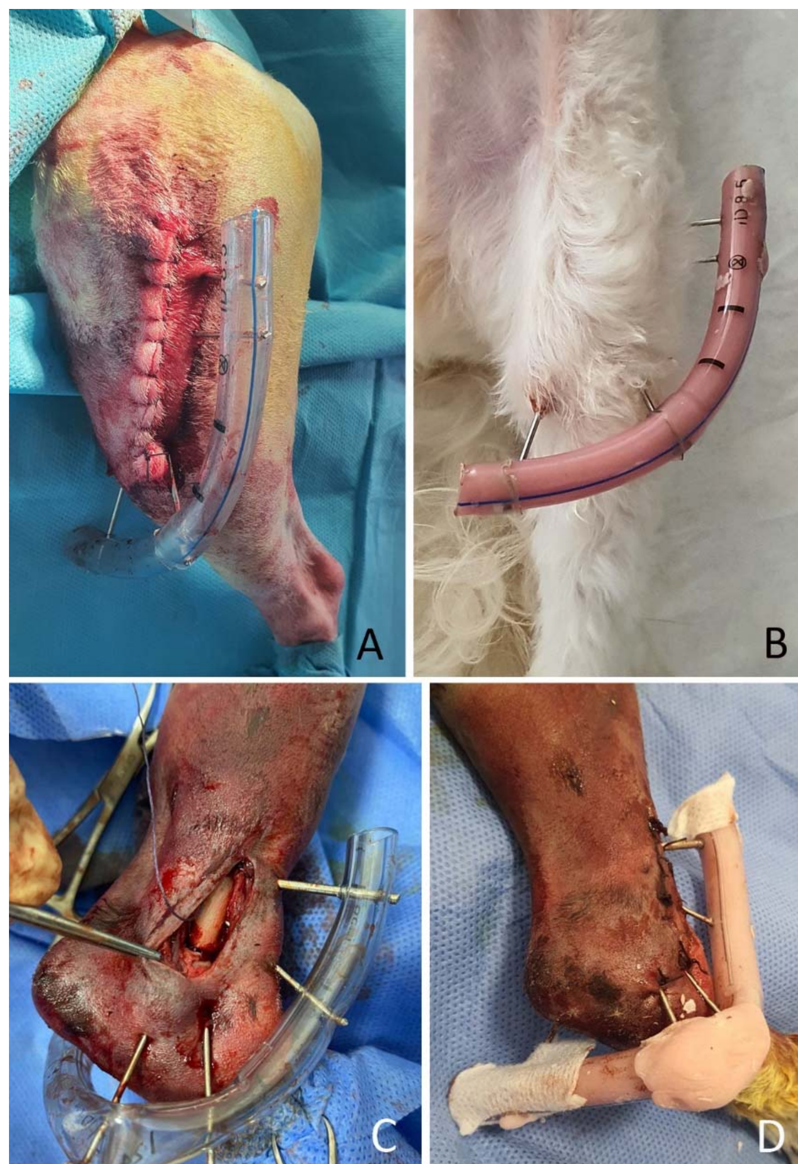


**Figure 3.** Sterile endotracheal tube was bent in a "J" fashion, and additional pins were sent from the tube to the bone above the fracture line (Case #13).
**(A)**. In the same case before acrylic frame removal **(B)**. Intraoperative **(C)** and immediately after the surgery **(D)** views of Case #8; a sterile endotracheal tube was used to keep the liquid acrylic mixture in a mold.

***Postoperative Care:*** Following surgical procedures, postoperative radiographs were taken to evaluate the fixation further. Patients were hospitalized for ten days. Carprofen (3 mg/kg/day SC) for dogs and Meloxicam (0.2 mg/kg/day SC) for cats were preferred for pain management for 3-4 days. To keep the pin–skin interface clean, 10% povidone-iodine solution and a local antibiotic spray were applied daily. An Elizabeth collar was used for all cases to prevent any damage to the fixator and self-contamination.

At the end of the hospital care, patient owners were strictly informed about the daily care of the pin–skin interface until the removal of the fixator. During the treatment period, animals were not allowed to do any active exercise except for short leash walks, and they also were confined in a small place/room for six weeks.

***Postoperative Evaluations:*** Postoperative clinical and radiographical evaluations were performed not in particular periods due to owners' and doctors' availability. Clinical assessments include the joint range of motion, functional use of the limb, fixator stability, and possible complications, such as any discharge in the pin-skin interface. Radiographical evaluations include bone and pins condition, fracture stability, and fracture healing.

Limb functionality and any lameness were evaluated and graded subjectively by two surgeons as described before (10, 26); excellent (no lameness, clinically normal), good (slight lameness after extensive exercise), fair (slight to moderate intermittent lameness but consistent weight-bearing), and poor (non-weight-bearing lameness). The joint range of motion was assessed by the method previously used by the authors (15, 24) with a plastic goniometer at different times; following surgery under general anesthesia, on the 10th day of hospitalization without sedation, and on the day of ESF removal under general anesthesia. The tie-cross acrylic ESFs were removed under general anesthesia after radiographically determined evidence of fracture union.

## Results

Dogs' ages ranged from 7 months to 2 years, and cats' ages ranged from 3.5 months to 2 years. The cause of traumas was falling from high in cats and vehicular accidents in dogs. Weights ranged from 1.2 to 3 kg in cats and 4 to 29 kg in dogs.

Humerus fracture was determined in 8 cases (cases #1, 2, 3, 4, 5, 6, 9, 12), three of which were intraarticular. These were Salter-Harris type II (case #1), distal metaphyseal transversal (case #2), proximal metaphyseal oblique (case #3), condylar "Y" fracture (case #4), distal metaphyseal oblique fracture (case #5 and 6), Salter-Harris type IV (case #9 and 12) (Figure 4). A comminuted Salter-Harris type IV fracture of the distal tibia in case #7 and a distal metaphyseal short oblique fracture of the tibia and fibula in case #8 were determined (Figure 5). Femoral fractures were detected in 3 cases (cases #10, 11, and 13); all had Salter-Harris type I fracture (Figure 6). Three cases also had additional fractures of the contralateral side, including the pelvis and tibial fractures in case #7, a lateral condylar fracture of the right humerus in case #9, and a right side intraacetabular fracture in case #10 (Table 1).

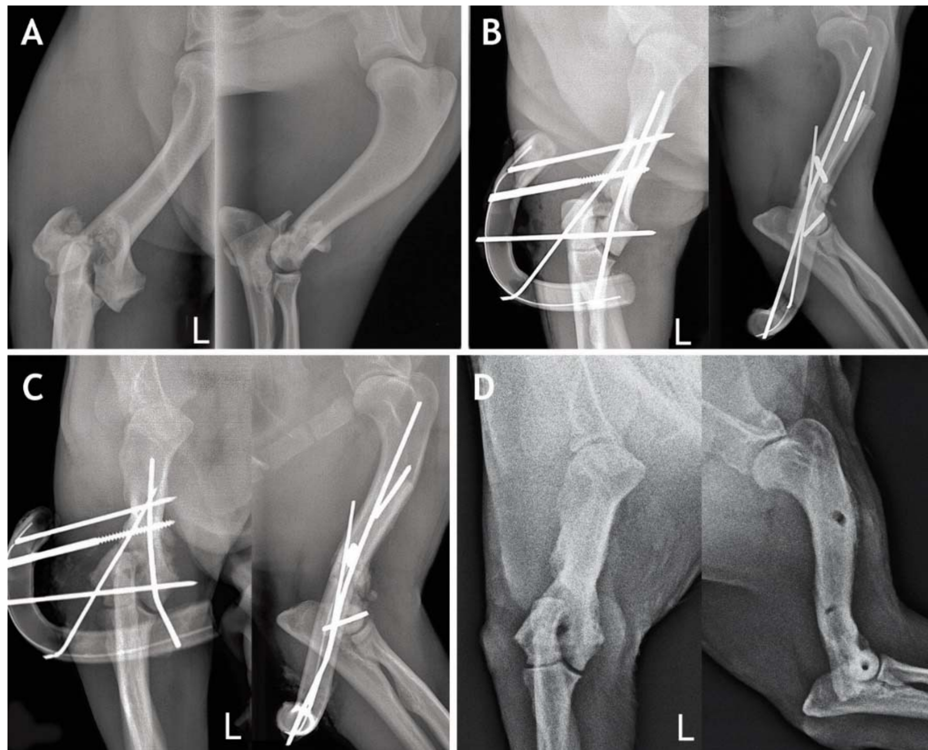


**Figure 4.** Case #4; left humerus condylar "Y" fracture with tiny bone fragments.
A. Preoperative, B. immediately after surgery, C. before ESF removal, and D. after ESF removal radiographs.

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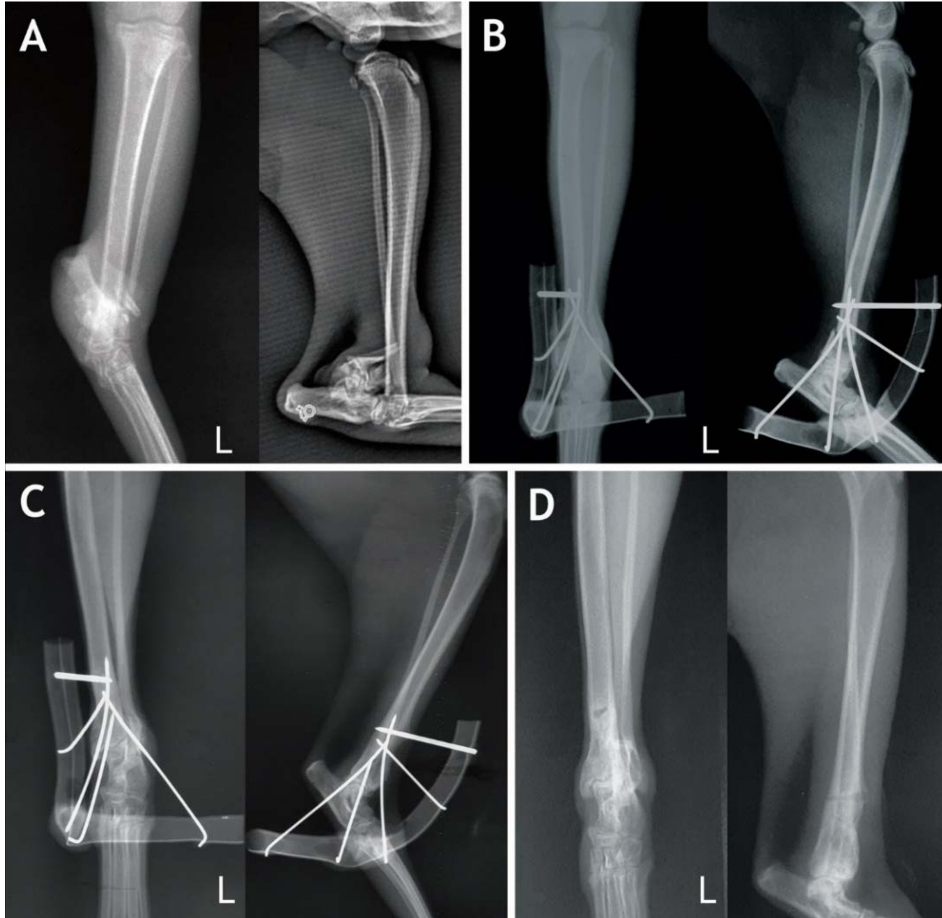


**Figure 5.** Case #8; left tibia fibula distal metaphyseal short oblique fracture.
A. Preoperative, B. immediately after surgery, C. before ESF removal, and D. after ESF removal radiographs.

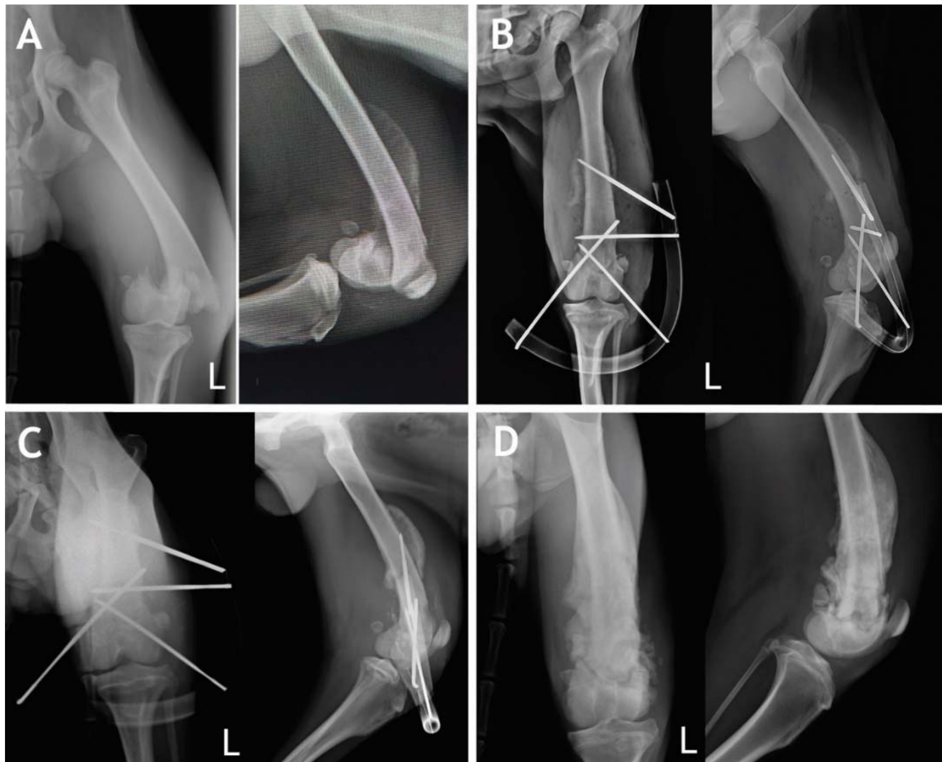


**Figure 6.** Case #10; left femur Salter-Harris Type I fracture.
A. Preoperative, B. immediately after surgery, C. before ESF removal, and D. after ESF removal radiographs.

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**Table 1.** Summary of information about cases.

| Case | Signalment | History | Fracture localization and type | K-wire / pin size | Other fractures | Complications | First time to use the limb/fixation removal (day) | PROM / Limb functionality |
|---|---|---|---|---|---|---|---|---|
| 1 | Dog / Mix/ 23kg / 9 m. ♂ | VA | Right **humerus** condylar Salter-Harris Type II | 1.5, 2, and 2.5 mm | None | None | 1/55 | Elbow Flex 32° - Ext 164° / Excellent |
| 2 | Dog / Mix/ 27kg / 1.5 y. ♀ | VA | Right **humerus** distal metaphyseal transversal (2nd surgery Tie-cross) | 2.5 mm | None | None | 1/49 | Elbow Flex 34° - Ext 162° / Excellent |
| 3 | Dog / Mix/ 24kg / 7m. ♂ | VA | Left **humerus** proximal metaphyseal short oblique | 2 mm, and threaded 3 mm | None | None | 0/35 | Shoulder Flex 55° - Ext 166° / Excellent |
| 4 | Dog / French bulldog / 14 kg / 2 y. ♂ | VA | Left **humerus** condylar “Y” fracture | 2, 2.5 mm, and threaded 3 mm | None | None | 0/63 | Elbow Flex 37° - Ext 165° / Excellent |
| 5 | Cat / Mix / 3kg / 2 y. ♂ | Fall | Left **humerus** distal metaphyseal supracondylar long oblique | 0.8, 2 mm | None | None | 1/45 | Elbow Flex 24° - Ext 163° / Excellent |
| 6 | Dog / Mix/ 29kg / 8 m. ♂ | VA | Right **humerus** distal metaphyseal supracondylar short oblique | 1.5, 2, and 2.5 mm | None | Minor pin tract discharge, mild periosteal reaction, severe soft tissue reaction | 2/41 | Elbow Flex 39° - Ext 161° / Excellent |
| 7 | Cat / Mix / 3 kg / 8 m. ♂ | Fall | Right **tibia** Salter-Harris Type IV, distal epiphyseal comminuted | 0.8, 1.5, 2 mm, and threaded 1,2 mm | Right tuber ischii apophyseal fracture, left corpus ischii fissure, left tibia short oblique mid-diaphyseal fracture | None | 1/69 | Tarsal Flex 25° - Ext 165° / Excellent |
| 8 | Cat / Mix/ 3kg / 9 m. ♀ | Fall | Left **tibia fibula** distal metaphyseal short oblique | 0.8 mm, and threaded 1,2 mm | None | Frame-caused contact dermatitis | 1/45 | Tarsal Flex 22° - Ext 168° / Excellent |
| 9 | Cat / Mix/ 2kg / 4.5 m. ♀ | Fall | Left **humerus** Salter-Harris Type IV, comminuted condylar “Y” fracture | 0.8 mm | Right humerus lateral condylar | None | 0/42 | Elbow Flex 24° - Ext 164° / Excellent |
| 10 | Dog / Mix/ 26kg / 7 m. ♂ | VA | Left **femur** Salter-Harris Type I | 2 mm, and threaded 2,5 mm | Right intraacetabular | Minor pin tract discharge, severe periosteal reaction | 2/50 | Stifle Flex 45° - Ext 164° / Excellent |
| 11 | Dog / Mix/ 26kg / 9 m. ♂ | VA | Left **femur** Salter-Harris Type I | 2 mm, and threaded 3 mm | None | None | 2/45 | Stifle Flex 45° - Ext 165° / Excellent |
| 12 | Cat / Mix/ 1.2kg / 3.5 m. ♀ | Fall | Right **humerus** Salter-Harris Type IV with segmented lateral supracondylar crest | 0.8 mm, and threaded 1.2 mm | None | Minor pin tract discharge, mild periosteal reaction | 1/52 | Elbow Flex 22° - Ext 162° / Excellent |
| 13 | Dog / Terrier / 4kg / 9 m. ♂ | VA | Left **femur** Salter-Harris Type I | 2 mm | None | Minor pin tract discharge, mild periosteal reaction | 3/40 | Stifle Flex 40° - Ext 167° / Excellent |

m. month, y. Year, VA vehicular accident, ♂ male, ♀female.

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Immediately after surgical procedures, postoperative examinations revealed no crepitus during joint movements but a slight decrease in range of motion because of tightness in periarticular muscles and postoperative edema. Additionally, the acrylic frame and construction of the tie-cross ESF were strong enough to be weight-bearing and allowed limb functions. After surgeries, radiographs revealed a good fracture reconstruction, even in intra-articular fractures, and no abnormalities such as articular involvement of a pin, and a fissure. Patients that recovered from anesthetic effects were allowed to leash walk on the same day. Three cases (#3, 4, and 9) started using their extremity at this time; two had an intraarticular fracture. Besides, all patients were willing to walk at varying degrees, including in three cases (#7, 9, and 10) with contralateral extremity fractures.

During the 10-day hospitalization period, limb functions and joint range of motion were evaluated (on the 10$^{th}$ day) and compared with the healthy side. Because of the post-surgical mild edema, the joint range of motion gradually increased from the day after surgery; however, animals did not allow evaluating the goniometric measurements while applying full extension and flexion of the affected side. Because of this fact, measurements were not consistent on the 10$^{th}$ day. Willing to walk and functions also gradually increased during this period. In addition to 3 cases (#3, 4, and 9) used their extremities on the same day of surgery, the remaining cases also started using their extremities on different days (Table 1); on the day after surgery (cases #1, 2, 5, 7, 8, and 12), on the second day after surgery (cases #6, 10, and 11), and on the third day after surgery (case #13). At the end of the hospitalization period, the patients were sent home, and their owners provided care.

Frame-caused contact dermatitis was observed in case #8 and eliminated by trimming the contacting side with an electrical bur and reinforcing the other side of the frame with the additional acrylic application.

During follow-up examinations, a mild pin tract discharge and soft tissue infection were observed in four cases (#6, 10, 12, and 13). These were probably due to the negligence of the patient owners, and/or these cases broke their hygiene. This complication was resolved by using oral antibiotics, cleaning the pin-skin interface, and covering the frame with a soft cloth. Mild to severe periosteal reactions were also encountered in mild pin tract discharge cases. This complication resolved gradually after the acrylic frame and pin removal.

On radiographic evaluations, fracture unions were good enough, and acrylic ESF frame and pin removal time ranged from 35 to 69 days. Following the removal of ESF under general anesthesia, passive joint range of motion (PROM) in maximal flexion and extension was evaluated with a standard plastic goniometer and compared with the opposite side. One shoulder, seven elbows, three stifles, and two tarsal joints were measured and compared to the opposite side (Table 1);

In case #1, PROM of the elbow joint for the affected side was Flexion 32° - Extension 164°, and the normal side was Flexion 32° - Extension 166°; *a slight difference in extension*.

In case #2, PROM of the elbow joint for the affected side was Flexion 34° - Extension 162°, and the normal side was Flexion 34° - Extension 165°; *a slight difference in extension*.

In case #3, PROM of the shoulder joint for the affected side was Flexion 55° - Extension 166°, and the normal side was Flexion 56° - Extension 165°; *a slight difference in flexion and extension*.

In case #4, PROM of the elbow joint for the affected side was Flexion 37° - Extension 165°, and the normal side was Flexion 36° - Extension 165°; *a slight difference in flexion*.

In case #5, PROM of the elbow joint for the affected side was Flexion 24° - Extension 163°, and the normal side was Flexion 22° - Extension 163°; *a slight difference in flexion*.

In case #6, PROM of the elbow joint for the affected side was Flexion 39° - Extension 161°, and the normal side was Flexion 36° - Extension 165°; *a slight difference in flexion and extension*.

In case #7, PROM of the tarsal joint for the affected side was Flexion 25° - Extension 165°, and the normal side was Flexion 21° - Extension 166°; *a slight difference in flexion and extension*.

In case #8, PROM of the tarsal joint for the affected side was Flexion 22° - Extension 168°, and the normal side was Flexion 24° - Extension 168°; *a slight difference in flexion*.

In case #9 with bilateral distal intra-articular humerus fracture, PROM of the left elbow (Tie-cross fixation) was Flexion 24° - Extension 164°, and for the right elbow (transcondylar and IM K-wire fixation) was Flexion 25° - Extension 163°; *a slight difference in flexion and extension*.

In case #10, PROM of the stifle joint for the affected side was Flexion 45° - Extension 164°, and the normal side was Flexion 42° - Extension 165°; *a slight difference in flexion and extension*.

In case #11, PROM of the stifle joint for the affected side was Flexion 45° - Extension 165°, and the normal side was Flexion 42° - Extension 165°; *a slight difference in flexion*.

In case #12, PROM of the elbow joint for the affected side was Flexion 22° - Extension 162°, and the normal side was Flexion 22° - Extension 164°; *a slight difference in extension*.

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In case #13, PROM of the stifle joint for the affected side was Flexion 40° - Extension 167°, and the normal side was Flexion 35° - Extension 170°; *a slight difference in flexion and extension*.

## Discussion and Conclusion

Reconstruction and stabilization of physeal, epiphyseal and metaphyseal fractures of bones are more challenging, especially if this fracture involves the joint. However, orthopedic surgeons can tackle this by using many techniques or combinations (2, 3, 7, 8, 14). Cross-pin, cross-pin with a transcondylar pin or screw, bone plate, bone plate with a transcondylar pin or screw, and external skeletal fixators can be preferred for this purpose (12, 18, 23, 30, 31). A bone plate is a suitable fixation method but sometimes can not be affordable for owners or not to be applicable for some complicated intra-articular fractures. Fixation of these fractures with transcondylar screws and cross-pins is less traumatic, less costly, and strong enough to stabilize bone fragments; however, pin loosening and migration, skin laceration, and soft tissue and bone infection can sometimes happen as technical complications. Therefore, in this study, we preferred to connect cross-pins with an acrylic ESF to prevent any possible pin migration and subsequent complications. Besides, with this method, we aimed that animals could have an opportunity for early use of their extremity.

An additional immobilization with a splinted bandage can be preferred to support the fracture and its fixation postoperatively. Although a splint may help fracture union, it will prevent joint function, which is especially crucial for functional joint recovery in intra-articular fractures. It causes a significant decrease in the joint range of motion, loss of function, and permanent lameness (14, 36). To prevent these possible complications, cross-pins were included in the acrylic ESF; the stabilization was strong enough and encouraged our cases to use their extremities in the early recovery period. An additional immobilization method will not be necessary with this technique.

Fractures involving the articular surface should be reduced anatomically and fixed securely to protect the ideal reduction. Otherwise, deformity, joint stiffness, posttraumatic arthritis, and permanent pain will lead to constant lameness. Providing normal axial alignment and initializing early joint motion will allow excellent functional recovery. An immediate joint motion is also necessary to prevent joint stiffness and ensure articular healing and functional recovery (25, 28, 35, 36). In this study, cases #4, 7, 9, and 12 had intra-articular fractures. With this technique, an ideal anatomical reduction could be achieved in these cases with intra-articular fractures. Additionally, these patients used their limbs functionally, and we observed that the first time to use the limb was on the same day of the surgery in two cases (cases #4 and 9) and the day after surgery in cases #7 and 12. Early functional recovery was also recorded in the remaining cases. Deformity, joint stiffness, posttraumatic arthritis, pain, and lameness were not observed in any of these cases. Additionally, in the remaining cases without articular surface involvement, the day of first use of the extremity ranged from 0-3 days.

Several external fixator systems have been available for human and animal orthopedic fracture repair management for a long time, and the advantages and disadvantages of ESF are well known (9, 14, 16, 29, 33). ESF can be preferred for reconstructing intra/extra-articular epiphyseal/metaphyseal fractures, with or without additional techniques. For intra-articular fractures, the joint movements can also be temporarily blocked by the transarticular ESF. In this way, an orthopedic surgeon achieves intra-articular fracture stabilization until adequate bone healing but temporarily prevents joint functions. Following the removal of transarticular ESF, an inevitable decrease in joint range of motion and a weight-bearing lameness will resolve within weeks with additional physical therapy, and the joint will become functional (13, 16, 19, 20, 22). Possible complications of the tie-cross acrylic ESF are the same as the complications described for other forms of ESF. Disadvantages and advantages are the same; additionally, unlike transarticular ESF, tie-cross ESF allows joint functions. In our study, we observed that the tie-cross acrylic ESF encouraged animals to use their joints in the early healing period. Therefore no additional physical therapy was required after ESF removal. Besides, the joint range of motion in the affected extremity was within normal limits.

In the Tie-in ESF technique, an IM pin is included in the ESF system. This way, diaphysis or metaphyseal fractures (comminuted or not) can be successfully managed (1, 11, 14). In this study, cross pins were included in the ESF system, and it was called Tie-cross ESF. The advantages and disadvantages are the same for all ESF systems. However, an orthopedic surgeon will have extra benefits from tie-cross ESF, especially comminuted fractures close to the joint. Like other ESFs, tie-cross ESF will also provide bone integrity until fracture healing and allow patients to use their extremities. Furthermore, this technique will prevent cross-pin loosening and migration complications. Additionally, in case of comminution of epiphyseal or metaphyseal bone, the acrylic frame will also provide rigid external stability and will hold the fractured bone in reduction.

The mechanical properties of an acrylic external fixator vary according to its configuration; however, it is strong enough to bear weight even in large animals. Although its advantages and disadvantages are almost the

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same, unlike metallic fixators, it is quite inexpensive, easy to obtain, and easy to connect pins in different directions (5, 11, 34, 37). The acrylic bar is also used for the tie-in configuration (11, 27). Besides the high probability of pin migration in the cross-pin application, the successful results obtained in the tie-in technique and the use of acrylic as an external fixator for many years also encouraged us to develop this new technique. Additionally, we didn't encounter any acrylic frame failure in our cases in different weight scales. During the procedure, it was fairly easy to connect pins with the tube.

In conclusion, there are many techniques to manage epiphyseal and metaphyseal fractures with or without comminution, or articular involvement. This technique distinguishably differs from other ESFs because in this technique, cross-pins were included in an acrylic ESF frame (unilateral uniplanar, a Type 1a external fixator). Therefore, calling this new ESF configuration the "tie-cross ESF" was necessary. This technique can also be achieved with metallic ESF frames and connectors. However, an acrylic ESF will be cheaper and more flexible to include all pins. Additionally, tie-cross ESF will provide an early gain of joint functions, which is crucial for articular bone healing. Therefore, additional physical therapy will not be necessary after ESF removal.

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## Conflict of Interest

We declare that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

## Author Contributions

MAÇ came up with the idea of this technique and study design. GY and MAÇ carried out the surgical technique. GY and MAÇ performed the measurements and clinical assessments. GY and MAÇ evaluated and interpreted data and radiographs. GY and MAÇ contributed to writing and editing the original draft of the manuscript. GY and MAÇ reviewed and approved the final manuscript.

## Ethical Statement

This study was approved by the Near East University animal care ethics committee (No: 27.11.2020/121).

## Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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# Investigation of serum anti-Müllerian hormone levels at follicular phase and interestrus period in queens

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## ABSTRACT

The objectives of this study were: *i)* to report overall mean AMH serum concentrations in queens, ii) to determine relationship between serum AMH concentration with estradiol level, *iii*) to investigate the relationship between serum AMH with follicular phase and interestrus phase of the estrous cycle, *iv*) to discuss the usability of AMH as a biomarker to diagnosis of ovarian remnant syndrome in breeding season in queens. In the study, 64 healthy queens referred to clinics for routine ovariohysterectomy were used. After anamnesis, vaginal cytology, macroscopic examination of the uterus and ovaries and estradiol measurement, queens were divided into two groups: follicular phase (n: 45) and interestrus (n: 19). Estradiol and AMH levels in serum samples were determined by ELISA. In the evaluation of serum AMH concentrations, a statistical difference was determined between the follicular phase and interestrus ($P<0.05$). A negative correlation was found between age and estradiol in both follicular phase ($r = -0.171$) and interestrus ($r = -0.385$) groups. A positive correlation was found between age and AMH in the interestrus ($P<0.01$, $r = 0.696$). Serum AMH levels in the interestrus were found to be significantly higher than follicular phase ($P<0.05$). It was thought that this increase in serum AMH levels during the interestrus period, which is the stage where oocytes were selected for ovulation, may be an indicator of the role of AMH in oocyte selection in queens, as in many other mammalian species.

## Introduction

Clasically, the queen is described as a seasonally polyestrous, induced ovulator (11). However, some authors have reported cases of spontaneous ovulation in groups of queens housed together and in wild felids, some species may show alternatively induced or spontaneous ovulations (2, 7). Various studies are documented greatest frequency of cycling activity in queens in the Northern Hemisphere in January and February, with gradual frequency decline until September and October (3, 11).

Even in the absence of mating and ovulation, queens show successive proestrus, estrus, and interestrus under the influence of daylight. Proestrus and estrus, form the estrogen-dominated follicular phase of the estrous cycle (3, 7, 16, 25). Estrogen levels in cats are found to be above 20 pg/mL during the follicular phase (proestrus and estrus), and below 20 pg/mL during anoestrus and interestrus (5, 25). In particular, an estradiol level above 20 pg/mL is diagnostic for ovarian follicle activity (5, 22). The effect of estradiol on vaginal epithelium of the queen is to cause an increased number of epithelial cell layers and to cause vaginal cornification, resulting in change in the morphologic apperance of exfoliated epithelial cells (11).

Anti-Müllerian hormone (AMH) belongs to the Transforming Growth Factor-ß (TGF-ß) family and produces in females only in ovaries by the granulosa cells of growing follicles, it is known as a marker of ovarian follicular reserve and ovarian aging, tumor marker or tumor inhibitör (4, 6). The active form of AMH is made up of two identical monomer subunits connected by disulphide links and is synthesized as a prohormone. For

the molecule to function, both locations are critical (14). Measurement of AMH levels plays an important role for the diagnosis the presence or absence of the ovaries in bitch and queen (18, 19, 20) and also for diagnosis the ovarian pathologies (9) and ovarian remnant syndrome (1, 19, 20, 26). Although some studies have been carried to the determination of serum AMH levels in queens in recent years (1, 18, 20, 24) more studies are needed to understand if AMH is also a biochemical marker of follicular development in queens. It is also important to assessif AMH is a reliable diagnostic biomarker in ovarian pathologies or ORS cases, to avoid unnecessary exploratory laparotomy.

The aims of this study are: *i)* to report overall mean AMH serum concentrations in queens, ii) to determine relationship between serum AMH and estradiol levels, *iii*) to investigate the relationship between serum AMH with follicular phase and interestrus phase of the estrous cycle, *iv*) to discuss the usability of AMH as a biomarker to diagnosis of ovarian remnant syndrome in breeding season in queens.

## Materials and Methods

***Animals and Sample Collections:*** The queens were clinically healthy and were referred for routine ovariohysterectomy (OHE) by their owners with the request of spaying for population control at the Kırıkkale University Faculty of Veterinary Medicine Clinics. In mating season, queens who showed typical oestrus signs at least once according to the anamnesis before the surgery were included in the study. None of the queens had symptoms of systemic or gynecological disorders according to clinical and ultrasonographic examinations. Furthermore, all of the queens were unpaired, unmated, unstimulated and individually housed. Age, body weight, previous diseases and last estrus dates of queens were recorded as anamnesis information.

Ovariohysterectomy was performed on the animals under general anesthesia from the lateral side as stated previously (8); blood and vaginal smear samples were taken before the surgery. Vaginal smears were obtained from the anterior vaginal wall with a sterile cotton swab, stained with Diff-Quick (Bes-Quick) staining method and evaluated under a light microscope (Olympos CHKZ-F-GS). All epithelial cells were examined and classified as described previously (23). Blood samples were taken from *v. saphena medialis magna* into tubes without anticoagulant with 21 G needle and were centrifuged at 4000 rpm for 10 minutes (ElektromagCentrifuge M4808P). The serum samples stored in eppendorf tubes at -20°C until analysis.

The study was approved by the animal ethics committee, University of Kırıkkale, Türkiye (Approval Number: 2019/29).

***Macroscopic Examination of the Uterus and Ovaries:*** Uterus and ovaries obtained from all animals were examined macroscopically after surgery. The ovaries were examinated for the presence of fresh ovulation scars and were dissected longitudinally to investigate the presence of the corpora lutea. After macroscopic examination, the queen who had pathological condition, early pregnancy signs and corpus luteum or fresh ovulation scar in their ovaries were excluded. Finally, 64 queens aged between 8 months and 5 years were used in this study.

***Hormonal Analysis:*** AMH and estradiol levels in serum samples were determined by ELISA method using DRG Instruments Elisa Mat 2000 device. Anti-Müllerian Hormone level was determined using human specific ELISA kit (Beckman Coulter®, AMH Gen II, USA) and estradiol level was determined using ELISA kit (DRG Estradiol EIA-2693). We used a second-generation human-based AMH kit produced by Beckman Coulter Immunotech, that works based on a two-site immunoassay that targets the epitopes by utilizing two different monoclonal antibodies selectively in the mature area (14). We previously used the same kit for assessing AMH levels in queens (18). Aside from our research team, two independent researchers (1, 11) also chose to determine the AMH status of queens using human-based AMH kit. These studies revealed that both the sensitivity and specificity of the assay are higher than 90%. The linearity of the test was assessed by spiking two different samples, with the high-concentration AMH standard, followed by serial dilution for validation purposes.

***Grouping:*** Based on the anamnesis, vaginal cytology results, macroscopic examination of the ovaries and uterus expelled after surgery, and estradiol results, the operated queens (n:64) were divided into 2 groups as follicular phase and interestrus. Queens in estrus according to the anamnesis and vaginal cytology, with follicular development in their ovaries and with estradiol results above 20 pg/mL were included in “Follicular Phase Group” (n:45). Beside, queens those whose estrus ended at least 1 week ago according to the anamnesis, without corpus luteum in their ovaries, and also with estradiol results below 20 pg/mL were included in “Interestrus Group” (n:19).

***Statistical Analysis:*** All obtained variables were analyzed with Shapiro Wilk for normality and Levene test for homogenity of variance, before starting the significance tests. Statistical determination of the difference in parameters between the groups was evaluated with Student's T Test, and the correlation measurements of the parameters within the group were analyzed with the Pearson correlation test. The minimum margin of error was determined as 5% in all statistical analyses. Data was analyzed using the GLM for Repeated Measures procedure of SPSS 14.01 (SPSS Inc., Chicago, IL, USA).

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**Table 1.** Mean age, body weight, AMH and estradiol results of the queens (mean ± standard error).

| | n | Age (month) | Body weight (kg) | AMH (ng/mL) | Estradiol (pg/mL) |
|---|---|---|---|---|---|
| **Follicular Phase** | 45 | 21.31±2.01 | 3.15±0.12 | 5.92±0.57$^{a}$ | 115.05±36.78$^{c}$ |
| **Interestrus** | 19 | 18±2.44 | 3.05±0.10 | 9.44±2.01$^{b}$ | 16.58±1.10$^{d}$ |

$^{a,b}$ Different letters in the same column are statistically significant (P<0.05).
$^{c,d}$ Different letters in the same column are statistically significant (P<0.01).

## Results

Average age, weight, AMH and estradiol results of the follicular phase and interestrus groups were given in Table 1. Overall mean AMH levels of all queens was founded 7.28±0.74 ng/mL. When the age and weight of the queens were compared, no difference were determined between the groups (P>0.05). In the evaluation of serum AMH concentrations, a statistical difference was determined between the follicular phase (5.92±0.57 ng/mL) and interestrus groups (9.44±2.01 pg/mL) (P<0.05). Although it was not statistically significant (P>0.05), there was a negative correlation between age and estradiol in both follicular phase (r = -0.171) and interestrus (r = -0.385) groups. A positive correlation was determined between age and AMH in the interestrus group (P<0.01, r = 0.696).

## Discussion and Conclusion

Adult queens are seasonal polyestric animals, and their sexual activity begins in December, naturally continues until September or October (23). However, artificial light also affects ovarian activity in domestic cats. In queens exposed to artificial light for at least 10 hours, the sexual cycle does not end in September and can continue throughout the year (10). Estrous behavior in queens is associated with estrogen synthesis released from the follicles. Follicles that are smaller than 1 mm at the start of proestrus reach 1.5 mm in diameter at the beginning of estrus. Follicles with a diameter of 2-3 mm develop in the ovaries during estrus, and the plasma estradiol level, which is below 12-15 pg/mL during anoestrus and interestrus, rises above 20 pg/mL when follicular activity starts (22, 23). In this study, the mean estradiol value of the follicular phase group was 115.05±36.78 pg/mL, and the interestrus group was 16.58±1.10 pg/mL. In cow, during follicular waves, the development of a dominant follicle was paralleled by an increase in circulating estradiol level, as expected, confirming that estradiol is an endocrine marker of terminal follicular development (16, 21). Similar with cows, we suggest that estradiol levels could be a potential endocrine marker to determine the terminal follicular development in queens.

In this study, serum AMH levels were higher in interestrous (9.44 ng/mL) than follicular phase (5.92 ng/mL). In bitches, changes in AMH concentration throughout the oestrous cycle have been identified with many studies (17, 28) but in queens AMH studies have focused more on detecting the presence of ovaries and changes in AMH concentration over the entire oestrous cycle have not yet been clearly determined. In a study on serum AMH concentration and ovarian follicle population in queens (15), similarly with cows (21), anestrous bitches (13) and womens (4,6), serum AMH levels in queens were founded highly correlated with small antral follicle count and also higher in anestrus than follicular phase. In females, AMH has an inhibitory effect on follicular development. It plays a role in selection at the beginning of the recruitment period and reducing the sensitivity of antral follicles to FSH (Follicle Stimulating Hormone). Thus, by preventing excessive follicular recruitment and follicular development, it has a role in determining the physiological limits in follicle development. In the absence of AMH, the recruitment rate may increase, leading to rapid depletion of the follicular pool (27). Similarly to the study by Lapuente et al. (15), low AMH level in follicular phase obtained in this study could be due to the termination of the follicles development. Considering this new study, it was thought that the statistical difference between follicular phase and interestrus in this study may be due to developing follicles and especially the selection of dominant follicles in queens. At this moment, it is very difficult to explain and in order to better understand this, more detailed studies are needed on this hypothesis.

There were some AMH variations have been found, although cyclical changes may be different in different species. The FSH stimulus result in an increase in serum estradiol but did not affect serum AMH concentrations in queens (1). In our study, we found a negative correlation between AMH and estradiol concentrations. Thus, increasing in follicular growth towards the end of interestrus in queens and the high AMH concentration in this period suggested that oocyte selection was completed with the transition to oestrus. Furthermore, since exact physiological follicular size limits could not be determined, oocytes was thought to be selected before estradiol exceeded the 20 pg/mL limits. Further studies are

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required to demonstrate the cyclical changes in estradiol and AMH concentrations, serial samples from the same individuals are necessary.

Higher AMH levels were found in intact cats (7.28 ng/mL) than intact bitches (0.32 ng/mL) in our previous study (19). Although AMH is inversely correlated with age in prepubertal queens; along with folliculogenesis, AMH increases with age in sexually active queens in breeding season. Granulosa cells in primary, secondary and early antral follicles are producing AMH, but the main source of circulating AMH is early antral follicles (11). In the study conducted by Korkmaz et al. (13), they reported that with increasing age in bitches, the number of primordial and primary follicles, and especially the granulosa cell numbers in the secondary and preantral follicles are decreased in parallel with AMH hormone concentration.

In studies conducted in queens to date, the average of circulating AMH level has been detected in the range of 1.3 to 19 ng/mL (1, 11, 15, 18). In this study, similar to others, overal mean AMH was 7.28±0.74 ng/mL of all queens. However mean AMH levels in queens with follicular phase was lower than this level (5.92±0.57 ng/mL). In cases of feline (11) and canine (11, 19, 26) ovarian remnant syndrome, AMH concentrations were intermediate to those of spayed and intact queens and bitches. But, queens are reffered to veterinary clinics with the suspicion of ovarian remnant syndrome, mostly because they show signs of estrus and according to this study and Lapuente et al. (15), AMH levels were lower in follicular phase than interestrus or anestrus. Therefore, determining the reference limits of phsyolocigal AMH levels in queens, especially in follicular phase or others, is much more valuable for definitive diagnosis in feline ORS cases with unknown gyneacologic history.

In conclusion, in queens, changes of serum AMH and estradiol concentrations between follicular phase and interestrous period shows that AMH can play a role in determining the physiological limits in follicle development and also selection of dominant follicles as in other mammalian species. AMH gives a critical information regarding the presence of functional ovaries during the mating season or inactive period. Finally, it is extremly important to develop practical, economic, easy and also standart measurement methods developed for use by veterinary clinicians. Further studies are needed to confirm to role of AMH on reproductive physiology in queens.

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## Author Contributions

IPY and GS conceived and planned the experiments. IPY, GS, IMP and TBE carried out the experiments. IPY, GS, IMP and MP planned and carried out the simulations. IPY, GS and TBE contributed to sample preparation. IPY, IMP and MP contributed to the interpretation of the results. IPY, IMP and MP took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

## Ethical Statement

The study was approved by the Animal Ethics Committee, University of Kırıkkale, Türkiye (Approval Number: 2019/29).

## Conflict of Interest

The authors declare no conflict of interest.

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# A comparison of anesthesia induction by two different administration routes and doses of ketamine and medetomidine in red-eared sliders (*Trachemys scripta elegans*)

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## Abstract

This study aimed to assess the efficiency of ketamine and medetomidine by two different doses and routes on anesthesia depth and cardiac stability in red-eared sliders. Each turtle was anesthetized two times, with seven days wash-out period. Induction of anesthesia consisted of a bolus combination of ketamine (10 mg/kg) and medetomidine (0.2 mg/kg) administered in the left brachial biceps in the intramuscular protocol, or a bolus combination of ketamine (20 mg/kg) and medetomidine (0.2 mg/kg) administered in subcarapacial sinus after clear blood presence confirmation in the intravenous protocol. Vital signs, reaction on the skin palpation, manual mouth opening for endotracheal intubation, palpebral and cloacal reflex, and the withdrawal reflex of the front and hind limbs were measured and recorded every 5 minutes for 60 minutes after anesthesia injection. Atipamezole (1 mg/kg) was administered in the right brachial biceps one hour after ketamine and medetomidine administration. Needle insertion and possible painful reactions to drug administration were also evaluated and recorded. Obtained data were analyzed for normality and paired t-tests, Wilcoxon, or McNamar tests were performed where appropriate. The values of $P \leq 0.05$ were considered significant. A significantly less pronounced decrease in heart rate was observed with intravenous anesthesia protocol. Both protocols recorded complete anesthesia recovery 60 minutes after intramuscular atipamezole administration. A ketamine-medetomidine dose combination administered intravenously provides a more stable and consistent anesthetic plane in red-eared sliders than ketamine-medetomidine administered intramuscularly.

## Introduction

The growing popularity of turtles as pets has led to increased demand for veterinary care for these animals. Sedation and/or anesthesia are often required for clinical examination, diagnostic procedures, and surgery. The evaluation of anesthetic protocols in turtles is scarce and anesthetic regimens are primarily based on those commonly used in mammals. General anesthesia in chelonians can be challenging due to their unique anatomical and physiological characteristics. Most previous studies have focused on anesthesia-related mortality in chelonians, whereas only a few have investigated the physiological effects of anesthetics (6). Therefore, ketamine has been used at various doses, combinations, and routes of administration (3, 5, 17). However, the results of the studies are variable.

Ketamine (5-10 mg/kg) in combination with medetomidine (0.1-0.2 mg/kg) administered intramuscularly or intravenously can lead to superficial anesthesia (26). Greer et al. (11) demonstrated that intramuscular administration of a lower dose of ketamine (5 mg/kg) in combination with medetomidine (0.1 mg/kg) induces anesthesia deep enough for endotracheal intubation of all turtles. Moreover, the same study results demonstrated that the higher dose (10 mg/kg ketamine and 0.2 mg/kg of medetomidine) could produce sufficient anesthesia even

for surgical incisions and suturing of the skin. It was suggested previously that intravenous ketamine at a dose of 5-10 mg/kg, either alone or in combination with other anesthetics, can provide endotracheal intubation of turtles (21, 27). However, this method of administration of ketamine has not been thoroughly evaluated in chelonians (25). Dennis and Heard (6) reported that the intravenous combination of ketamine (5 mg/kg) and medetomidine (0.1 mg/kg) could provide short-term surgical immobilization in gopher tortoises (*Gopherus polyphemus*). Information on the cardiopulmonary effects of these drugs in reptiles is limited (6). Due to the aforementioned limited data, propofol has been favorable in reptile anesthesia because of the fast induction and fast recovery (7) but in some countries, propofol is almost unreachable. Dosenovic et al. (7) concluded that ketamine and medetomidine combination is more suitable than propofol in red-eared sliders' anesthesia regarding the degree of oxidative stress.

This study aimed to assess the efficiency of ketamine and medetomidine by two different doses and routes of their administration (intravenously and intramuscularly) on anesthesia depth and cardiac stability in red-eared sliders.

## Materials and Methods

The study was conducted at the Veterinary Faculty – University of Sarajevo, following approval by the Institutional Ethics Committee under approval number 01-02-153-2/21.

***Animals:*** Twenty clinically healthy red-eared sliders (*Trachemys scripta elegans*), with equally distributed sex, were included in the study. Observation of all animals started 10 days before the anesthesia protocol and finished 10 days after the last protocol testing. They were kept indoors in a turtle terrarium with a water temperature of 25.5 °C. The daytime air temperature was between 26.0 and 28.0 °C with a basking spot heating up to 35.0 °C over the land area, and a UVB light source was provided for 12 hours each day. Dark photoperiod with lower temperatures (18.0 – 21.0 °C) was allowed. Red-eared sliders were fed with commercial food (Tetra®, Germany) and water was regularly cleaned using terrarium filters (Aqua-Tech 30-60 Aquarium Power Filter, USA).

***Pilot study:*** Given the lack of existing literature on the intravenous ketamine and medetomidine combinations in red-eared sliders, it was deemed necessary to conduct a pilot study. A pilot study was conducted to assess the safety of the intravenous protocol and provide estimates for sample size calculation. Three healthy red-eared sliders underwent two different anesthesia protocols (intramuscular and intravenous protocol), with 7 days wash-out period in between. The intravenous protocol (IVP) was based on intravenous bolus administration of ketamine hydrochloride (*Ketaminol10*, MSD, Netherland) (10 mg/kg) in combination with medetomidine hydrochloride (*Sedastart*, Dechra, UK) (0.2 mg/kg), at doses recommended for intramuscular application (11). The effects of administered drugs were evaluated over 30 minutes. If adequate induction of anesthesia was not achieved, the lower ketamine doses were added incrementally in the subcarapacial sinus until the surgical plane of anesthesia was obtained as described by Bennett (3). The ketamine dose of 20 mg/kg and medetomidine dose of 0.2 mg/kg provided stable and effective induction of anesthesia, allowing endotracheal intubation of all animals, and it was defined as the final protocol dose. To determine the minimum required sample size for the final study, at which the significant difference between the anesthesia protocols' impact on vital stability would be found if such difference did exist, we performed an apriori power analysis using G power software (8). The desired significance level was set at 0.05, the desired effect size was set at medium, and the desired power level was set at 0.08.

***Study design and procedures:*** Based on the physical examination and complete blood cell count results, 20 clinically healthy animals were selected for the study. They underwent two anesthesia protocols in a randomized crossover design, with 7 days wash-out period. Anesthesia procedures were performed in a surgical room with a controlled air temperature of 25.0 °C. In intramuscular protocol (IMP), a combination of ketamine (10 mg/kg) and medetomidine (0.2 mg/kg) was administered in the left brachial biceps following aseptic skin preparation. After the muscle relaxation, all animals were placed on the electrical heat pad to maintain body temperature. Three ECG leads were placed on the carapace at the front left, front right, and left hind quarter level. The body temperature probe was placed into the cloaca after lubrication. The $SpO_2$ sensor was placed on the foot and a side-stream capnography line was attached to the intravenous cannula and placed intranasally. A room air thermometer probe was placed between the patient's plastron and a heating pad. Blood pressure measurement was not possible. In intravenous protocol, a combination of ketamine (20 mg/kg) and medetomidine (0.2 mg/kg) was administered in the subcarapacial sinus. A newly prepared drug combination was repeated during the blood confirmation procedure in case of lymph contamination. A side-stream capnography line was reattached to the endotracheal tube after intubation. The rest of the procedure was identical to the intramuscular protocol. Adequate muscle relaxation and absence of limb retraction

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were defined as time to induction after anesthesia injection.

Heart rate, body temperature, and thoracic impedance pneumography were measured and recorded every 5 minutes over the anesthesia monitor (Mindray iMEC8Vet, China) for 60 minutes following the administration of the anesthesia. Like in mammals, decreasing the heart rate by 30% of the basal values was considered as bradycardia (14). A masticatory muscle relaxation, palpebral and cloacal reflex, skin touch reaction, and withdrawal reflex using mosquito forceps on the front and hind limb were also evaluated and recorded every 5 minutes by an investigator blinded to the treatment. Relaxation and sensitivity were scored as described by Alves-Júnior et al. (1). Basal heart rate for each animal was obtained by an allometric scaling system (bpm=33.4 x $(BWkg)^{-0.25}$) before induction (34). Relaxation of the masticatory muscles was an indication for endotracheal intubation which was performed using intravenous cannulas (Mediplus Limited, India), and the presence or the absence of the gag reflex during the procedure was recorded. Endotracheal intubation was attempted each time the masticatory muscles were relaxed.

An hour after anesthesia injection, atipamezole hydrochloride (*Sedastop*, Dechra, UK), a dose 5 times greater than medetomidine (26), was administered in the right brachial biceps and the presence of painful reaction on the needle insertion and drug application was observed. During the recovery phase, monitoring the physiological parameters was continued until the patient's voluntary locomotion was observed.

***Statistical analysis:*** Experimental data were analyzed using PAST statistical analysis software (12). Recorded data were averaged across 13 measurements for each of the 20 turtles. Averaged data were tested for normality using the Shapiro-Wilk test. All data except the basal heart rate were found to be normally distributed. Box-plot inspection and IQR method were used to inspect for the presence of any outliers. Two outliers were identified in the IMP and IVP heart rate measurements, respectively, and thus excluded from the data analysis. Paired t-tests were conducted to assess differences in means of basal heart rate and heart rates obtained during monitoring in both groups. A mean percentage of heart rate reduction difference between intramuscular and intravenous anesthesia was also conducted. In light of the above, the t-test was chosen on the grounds of its sophistication in discerning between which conditions precisely the difference exists and how substantial is it.

Given that basal heart rate deviated from a normal distribution, to ensure results were not confounded by violation of parametric assumptions of the t-test, a non-parametric Wilcoxon test was also performed. In addition, log transformation was applied and transformed data were analyzed. There was no difference in the significance of results obtained using a non-parametric test, nor in comparison with those obtained on the transformed data. Therefore we will report the results obtained with a paired t-test, as the appropriate analysis method was determined. The mean percentage of heart rate reduction in intramuscular and intravenous anesthesia was also assessed. The difference in breathing frequency was tested using the McNamara test. Values of $P \leq 0.05$ were considered significant.

## Results

Twenty clinically healthy red-eared sliders (*Trachemys scripta elegans*) had a mean age of 5 (SD=4) years and a mean body weight of 0.43 (SD=0.29) kg. All animals' complete blood cell count was within referent values given by Heatley and Russell (15). The mean time of muscle relaxation in IMP was 9 (SD=2.6) minutes and the mean time of possible manipulation with the animal was 11 (SD=2.9) minutes. In IVP, the mean time of muscle relaxation and animal manipulation was 12 (SD=3.9) minutes.

A significant difference was observed between basal heart rate (M=46.4, SD=3.0) and heart rate during IVP (M=34.7, SD=1.2), (t (19)=4.302, P=0.0004). Similarly, a significant difference was observed between basal heart rate (M=46.4, SD=3.0) and heart rate during IMP (M=30.6, SD=1.0), (t (19)=4.775, P=0.0001). A significant difference was also observed in a direct comparison of the heart rate values recorded during IMP and IVP (t (19)=-3.155, P=0.005). In contrast, an IVP pulse is significantly higher (M=34.7, SD=1.2) than in IMP (M=30.6, SD=1.0) (Figure 1).

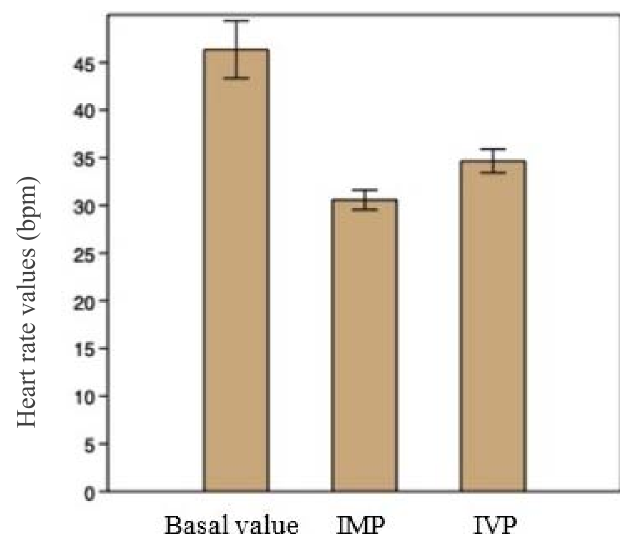


**Figure 1.** Difference between heart rate basal values and heart rate values in IMP and IVP groups.

A significant difference was observed in predetermined bradycardia values (decrease of the heart rate for 30% of basal values) between IMP and IVP (t (19)=-3.227, P=0.004). An average decrease of the heart rate for 31% (M=31.2; SD=3.8) of basal values was recorded during IMP, while during IVP was for 23% (M=23.4; SD=3.3) of preanesthetic values (Figure 2). In IMP, 95% of animals showed a decrease of the heart rate below 30% with a mean time of 29 (SD=19.8) minutes and only 55% of animals in IVP showed a heart rate decreasing below 30% with a mean time of 29 (SD=21.6) minutes.

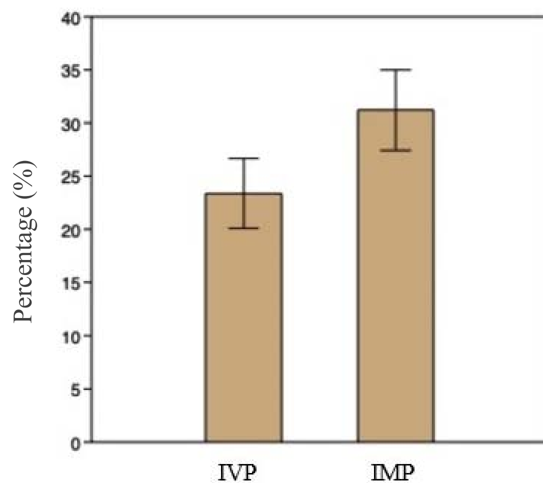


**Figure 2.** Heart rate reduction in IVP and IMP group during anesthesia expressed as a percentage.

No significant difference (P=0.451) was observed in respiratory rate between groups and arrhythmic breathing pattern was present in all animals. Pulse oximetry did not provide any saturation reading but indirect heart rate measurement was correlated with ECG values. An $EtCO_2$ by side-stream capnography line could not be obtained intranasally nor after endotracheal intubation, even after a probe by manual ventilation. Body temperature in all animals was successfully maintained between 24.0 and 26.0 °C.

In IMP, one animal achieved a surgical plane of anesthesia and endotracheal intubation was possible after 46 minutes. All animals in IVP reached the surgical plane of anesthesia and endotracheal intubation with a mean time of 47 (SD=15) minutes.

Reaction on the skin touch and withdrawal reflex was observed in all animals but the degree of response was pronounced in IMP. Spontaneous locomotion was not observed in IVP protocol during the anesthesia while it was recorded in 15% of animals in IMP, and it was recorded 30 (SD=2) and 55 (SD=3) minutes after ketamine and medetomidine administration.

Reaction on the needle insertion for atipamezole administration was increased in IMP. The mean time for spontaneous head movement was 6 (SD=2.9) minutes in both groups after atipamezole administration. The mean time for voluntary locomotion was 8 (SD=4.4) minutes in IMP and 9 (SD=4.2) minutes in IVP. Vomiting was observed only in the same turtle for both treatments 125 minutes after atipamezole administration in IMP and 30 minutes in the IVP protocol. An hour after atipamezole administration, all animals completely regained dorsal reflex which was a criterion for sufficient recovery to be returned to their enclosure. The difference in the recovery phase was not noticed between genders.

## Discussion and Conclusion

Ketamine hydrochloride is a common anesthetic drug used in reptiles with a wide safety margin (22). It can be administered in various routes and its anesthetic doses, as a sole agent, usually cause prolonged recovery time in reptiles (17, 22, 35, 42). Medetomidine has a high affinity toward α2 adrenergic receptors with a sedative and analgesic effect. It is commonly used for anesthesia induction in combination with anesthetic agents (22, 29). In chelonians, medetomidine alone usually causes severe cardiopulmonary depression with heart rate and respiratory rate reduction and hypotension (36). According to some studies, these drugs in combination can provide effective intramuscular anesthesia in red-eared sliders (11, 38). Bouts and Gasthuys (4), suggest that medetomidine (0.1-0.3 mg/kg) in combination with ketamine (10 mg/kg) administered intramuscularly in most reptiles may induce general anesthesia. Contrary to the decreasing effect of the sympathetic tone by α2-adrenoreceptor activation with medetomidine, dissociative anesthetics increase sympathetic activity resulting in cardiac output, blood pressure, and heart rate increases (39, 43). Ketamine shouldn't significantly affect ventilatory drive due to hypercapnia in mammals (16, 37). Furthermore, Dennis and Heard (6) suggest that ketamine in combination with medetomidine cause hypoxemia that is not severe enough to trigger a respiratory response in chelonians.

Propofol is the anesthetic of choice in reptile anesthesia, but a recent study suggests that propofol has a more significant oxidative stress effect on red-eared sliders than the ketamine-medetomidine combination (7). In their combination, a counterbalance is enabled (40). The quality of anesthesia induction by intravenous administration of ketamine has not been tested in chelonians (25). Dennis and Heard (6) investigated the hemodynamic stability of the gopher tortoises after intravenous bolus administration of ketamine and medetomidine. In the same study, the quality of anesthesia was not evaluated. In our research, intramuscular administration provided satisfactory muscle relaxation and enabled endotracheal intubation only in 5% of animals. Furthermore, 15% of animals showed

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spontaneous locomotion during anesthesia monitoring in the same protocol, and all had significant heart rate reduction. On the contrary, intravenous administration in all animals allowed endotracheal intubation, and heart rate reduction was much closer to basal values. These differences among protocols could be due to increased sympathetic activity caused by the higher dose and administration route of ketamine in the intravenous protocol. The same animal was used in both protocols, so individual differences were prevented. Furthermore, the carry-over effect was prevented considering the pharmacokinetic properties of these drugs (30, 31).

It is essential to understand that aquatic chelonians can develop anaerobic breathing by tolerating an anoxic environment very well (9, 18). Considering all chelonians' physiological characteristics, compensatory mechanisms, and monitoring parameters (9, 13, 19, 20, 22, 23, 41), none of our animals showed compromise. Furthermore, the inability to detail respiratory evaluation during the investigation was not concerning for patient safety. Besides, in reptiles pulse oximetry, arterial blood gas analysis, and capnography are not validated yet (24). Heart rate and ECG readings were considered essential parameters in our study to assess induced anesthesia quality. In mammals, a heart rate decrease of 20-30% below the low normal is regarded as bradycardia which requires treatment (14). To the best of the authors' knowledge, a decrease in heart rate considered bradycardia in reptiles is not defined. Our study suggests that induction of anesthesia results in a significant drop in heart rate, while heart rate frequency was less affected by intravenous protocol. This signifies that the average degree of reduction in heart rate was significantly lower in IVP, so much anesthesia can be deemed safer. The ECG interpretation is critical during anesthesia as it can be helpful for the assessment of anesthesia depth (2). This is especially useful in the absence of other methods such as Doppler or esophageal stethoscopes. An ECG interpretation is similar to a mammalian, but diagnostic reference parameters data is limited in reptiles (24). It showed valuable methods in our study, especially when other monitoring methods during anesthesia have failed. However, ECG as the only method for anesthesia monitoring in reptiles is not recommended (24).

All animals in our study showed a reaction in different degrees of withdrawal reflex testing induced reaction of a certain degree in all animals regardless of anesthesia protocol and more pronounced on the hind limbs. A similar observation was reported by Greer et al. (11). More pronounced withdrawal reflex of hind limbs could be explained by the constatation that the anesthesia effect in reptiles goes from the cranial to the caudal direction (3, 10). Touch reaction and withdrawal reflex were notably different between the two anesthetic protocols. All animals showed a reaction but it was significantly intense in IMP evaluating the time and intensity of limb retraction. This observation correlates with detected differences in anesthesia depth induced between the two protocols. Furthermore, the palpebral reflex was absent in IVP, and the cloacal reflex was present in both protocols. We used reflexes evaluation in correlation with obtained heart rate values to decide does the patient requires urgent treatment. None of the patients required treatment and all animals recovered well. The presence of painful reaction on the needle insertion and atipamezole application was increased in IMP compared to IVP also indicating that intramuscular protocol causes superficial anesthesia.

Intravenous anesthesia agent application is preferable because of predictability and animal recovery. Unfortunately, intravenous standard methods can cause technical difficulties (33). Using subcarapacial sinus is an effortless technique, but some studies warn of the possibility of accidental intrathecal drug application. In that case, permanent paralysis or even death is expected mainly if high volumes or irritant drugs are used (28, 32). In our study, none of the red-eared sliders showed neurological issues 10 days after treatment.

In conclusion, the intravenously administered bolus mixture of ketamine and medetomidine in investigated doses provided superior induction of the general anesthesia and stability of the red-eared sliders compared to previously described and recommended doses of the same mixture administered intramuscularly. Therefore, intravenous administration of this combination should be the preferred route for inducing surgical plane anesthesia in this animal species.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

## Author Contributions

LI made a substantial contribution to the conception and design of the study and took the lead in writing the manuscript. Also, carried out anesthesia procedures and recordings of vital signs. MA recorded animals' reactions and reflex evaluation; contributed to drafting the article

and critical revision of the manuscript. All authors read and approved the final manuscript.

## Ethical Statement

The study was conducted at the Veterinary Faculty – University of Sarajevo, following approval by the Institutional Ethics Committee under approval number 01-02-153-2/21, dated: 12.02.2021.

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Case Report

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# Anencephaly, bifid tongue, and cleft palate in a Pomeranian dog: GFAP and NeuN immunoreactivities

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## Abstract

Anencephaly is a congenital disease manifesting with the absence of the brain due to the failure of the cranial part of the neural tube to close during the embryonic stage. The disease may be accompanied by other anomalies and usually results in premature death. A stillborn puppy of a 2-year old female Pomeranian dog is examined in this case. The lack of brain tissue and accompanying abnormal skull formation was noted macroscopically. The eyes were protruding out of their normal position (protrusion), and a bifid tongue together with a secondary cleft palate was present. On serial sections stained with Haematoxylin-Eosin, only the medulla spinalis among the central nervous system structures could be inspected microscopically. Immunohistochemistry staining revealed GFAP immunoreactivity in the astrocytic glial cells. NeuN immunoreactivity was detected in the neurons in the medulla spinalis and spinal ganglions. Incomplete retinal layers were observed on the eye sections stained with Haematoxylin-Eosin and NeuN. The case was concluded to be coherent with skull and nervous system congenital malformations rarely observed in dogs. To the best of our knowledge, this represents the first description of a dog with anencephaly, bifid tongue and cleft palate.

Anencephaly is a type of neural tube defect (NTD) and affects the formation of brain and skull bones (8). Brain development occurs during embryonic development in animals (16). The leading cause of anencephaly is the failure of the neural tube to close properly during pregnancy (17). The neural tube originates from the neural plaque, which in the embryonic stage is fused with the neuroepithelium along the midline (16). A defect in the neural plaque may prevent the neural tube from closing completely, and the remaining gap in the neural tube can be observed as cranial, spinal, or both (8, 16). Defect in the cranial part of the neural tube leads to anencephaly (3, 8), and infectious agents, chemical substances, toxic plants and drugs, radiation, viruses, or nutritional disorders are the most important causes (16, 22). It is common in humans but rare in animals, and its etiology in dogs is not precisely known (8, 12, 16).

Bifid tongue and cleft palate are congenital defects rarely observed in humans and animals (9, 11, 13, 25). The bifid tongue is also defined as cleft tongue, accessory tongue, or double tongue (7, 9) and is reported to result from a fusion deficit in the embryonic stage. Persistent buccopharyngeal membrane, amniotic constriction bands in the region of the branchial arches, environmental factors, and large doses of vitamin A are described as some of the fusion deficit etiologies (18). The defect is believed to be caused by the failure of mesodermal migration into the midline structures of the mandibular portion of the first

branchial arch (18). Bifid tongue cases reported in mule foals, donkey foals, dogs, and a calf were often observed to have developed in conjunction with a cleft palate and a cleft mandible (5, 10, 19, 24, 25).

In this case, a stillborn puppy born to a 2-year-old female Pomeranian dog after her first labor is diagnosed with anencephaly, accompanied by a bifid tongue and a cleft palate, after macroscopic and microscopic examinations. The aim of this study is to show the presence of neurons and astrocytes by showing their immunoreactivity in the central nervous system.

The stillborn puppy with an anomaly born to a 2-year-old Pomeranian mother was brought to the Pathology Department of the Veterinary Faculty in Bursa Uludağ University for necropsy and histopathologic examination. Information obtained via anamnesis revealed that after 63 days of pregnancy, the mother gave birth to three puppies, of which two were normal, and one was stillborn. Incomplete skull formation of the stillborn puppy and a membrane cover over its skull was observed at necropsy (Figure 1). No cerebral hemispheres were found in the cavum cranii when this membrane was removed. Protrusion of the eyes out of their normal position (exophthalmos) was noted (Figure 2). It was observed in the examination of the mouth cavity that the tongue was split from the midline with an approximately 1.5 cm long cleft (bifid tongue), that there was also a 3 cm long cleft on the palate (palatoschisis) (Figure 3), and that the mandible protruded beyond the maxilla (brachygnathia superior). No pathological findings in the internal organs were found in the examination of the abdominal and thoracic cavities.

Specimens taken for microscopic examination were fixed in 10% neutral buffered formalin, subjected to routine procedures, and embedded into paraffin. 4-micron-thick sections were stained with routine H&E. Sections deparaffinized for immunohistochemical analysis were passed through the alcohol series. After washing in the buffers, it was incubated for 5 minutes in 3% $H_2O_2$ to suppress endogenous peroxidase activity after recovery of antigenicity for 30 minutes at pH 6.0 citrate buffer at 98°C. It was kept in Bloking Serum to prevent non-specific binding. As the primary antibody; were incubated with mouse anti-NeuN (1/500 dilution; Anti-NeuN Antibody, Clone A60, Sigma-Aldrich) and mouse anti-GFAP (1/200 dilution; Anti-GFAP Antibody, CloneG-A-S, Sigma-Aldrich) antibody for overnight at +4°C. The next day, incubation was performed with biotin-conjugated goat anti-mouse secondary antibody at a dilution of 1/300 for 2 hours at room temperature (Thermo Fisher Scientific). It was incubated with ABC enzyme for 1 hour. Sections were washed in buffers and then nuclear or cytoplasmic labeling was visualized with DAB. Negative control staining was performed for each staining. Microscopic examinations were made with Olympus BX50 microscope.

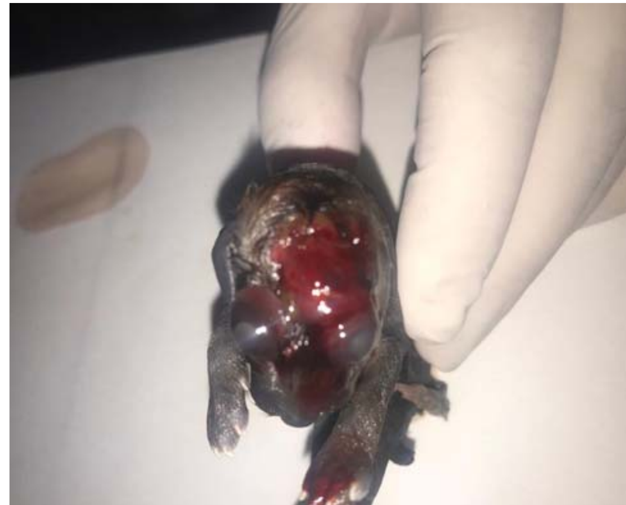

**Figure 1.** Presence of a membrane in place of the skull bones.

**Figure 2.** Protrusion (in the eyes) and anencephaly.

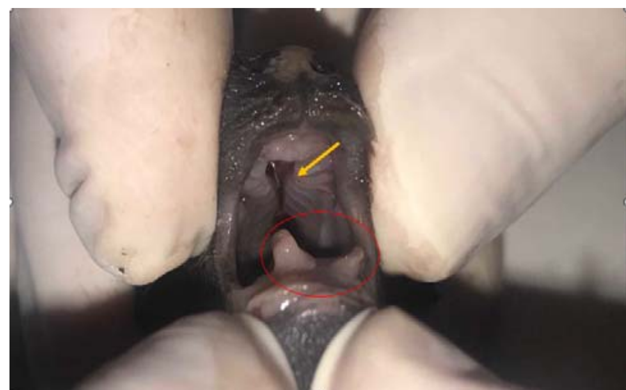

**Figure 3.** Cleft palate (palatoschisis) (yellow arrow) and cleft tongue (bifid tongue) (red circle).

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The medulla spinalis, ganglia, meninges from the nervous system, and the bony vertebrae were distinguished on the H&E-stained sections (Figure 4). No structures belonging to the brain or cerebellum tissues were found. Immunohistochemistry staining for GFAP was positive in the astrocytes of the grey matter around the canalis centralis and the glia limitans under the meninges (Figure 5-6). Neurons in the medulla spinalis and spinal ganglions were positive for NeuN (Figure 7-8). Retinal layers were not completely formed on the eye sections stained with H&E and NeuN (Figure 9-10).

In this case, the absence of the brain and cerebellum tissues, the skull is covered with a membrane, and the presence of only the medulla spinalis, both macroscopically and microscopically, are coherent with anencephaly in humans. Protrusion of the eyes, which develop from the forebrain during the embryonic stage (2), and the deficits in the retinal layers observed on histologic sections are consistent with anencephalic findings in humans and animals.

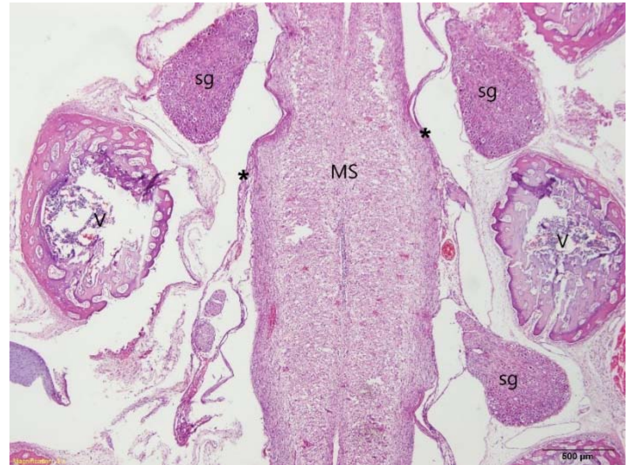

**Figure 4.** Medulla spinalis (MS), bony structures (V), meninges (*), and ganglia (sg) H&E. 4x objective.

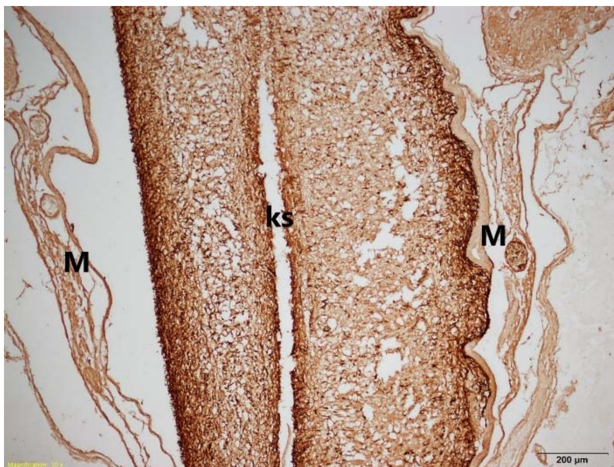

**Figure 5.** Dense GFAP positivity around the canalis centralis (ks) and in the glial limitans under the meninges (M) of the medulla spinalis. 10x objective.

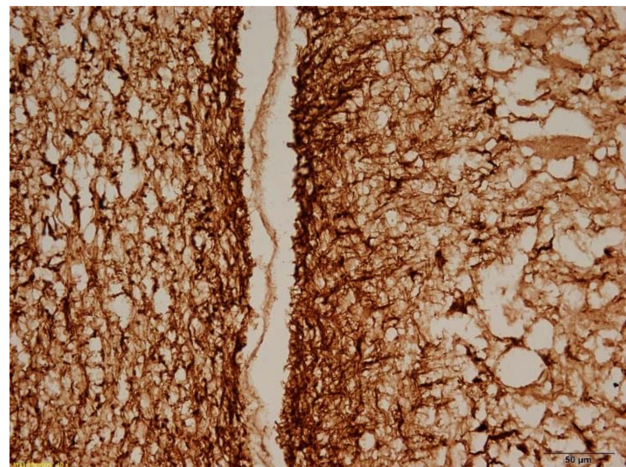

**Figure 6.** Dense GFAP immunoreactivity in the astrocytes around the canalis centralis. 40x objective.

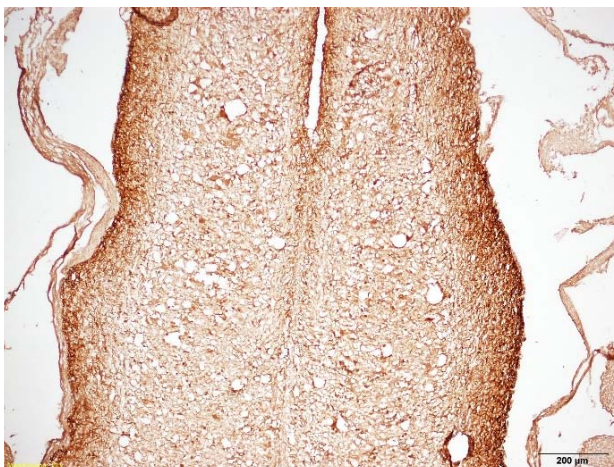

**Figure 7.** NeuN positivity in the neurons of the medulla spinalis. 10x objective.

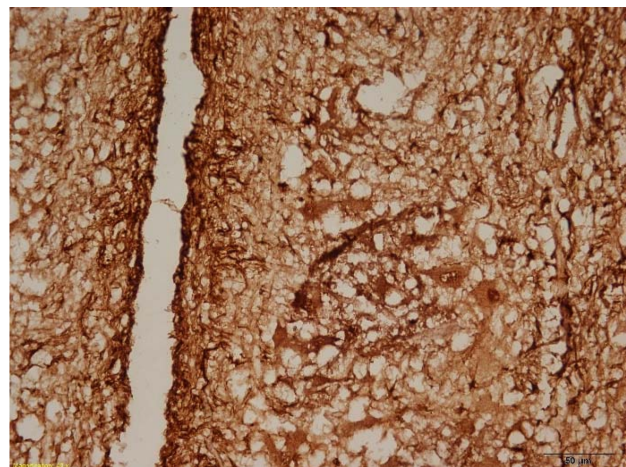

**Figure 8.** NeuN immunoreactivity in the neurons of the medulla spinalis. 40x objective.

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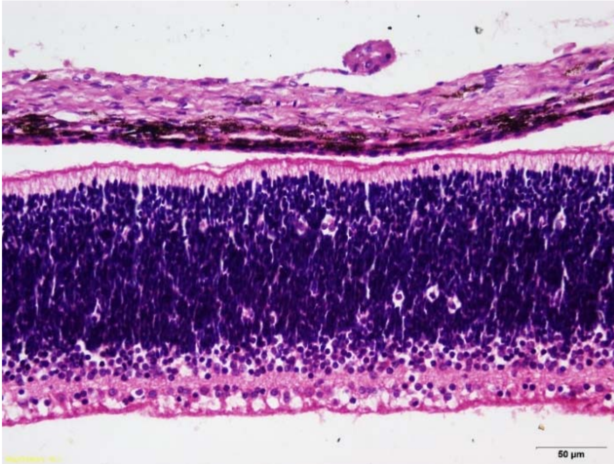

**Figure 9.** Incomplete retinal layers of the eye, H&E. 10x objective.

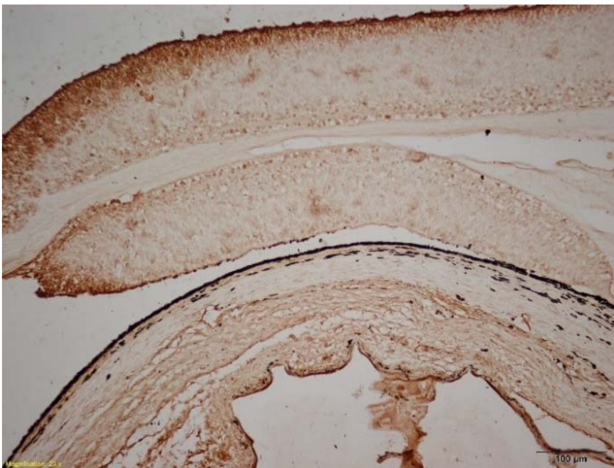

**Figure 10.** NeuN immunoreactivity in retinal layers of the eye. 20x objective.

In gross and microscopical examination, no cerebrum and cerebellum tissue were found. Immunohistochemical analyzes showed GFAP positive glial fibrils. This positive staining is as expected; it is intense in the glial limitans around the spinal canal and under the meninges, and it is lighter in gray matter. The immunohistochemically reactivity of glial cells which were positive for glial fibrillary acidic protein matches the expression profile of mature astrocytes of adult animals. Based on the presence of NeuN positive neurons and the positions of these neurons, this tissue was thought to be medulla spinalis tissue belonging to the central nervous system. In addition, there are spinal ganglia around the spinal canal and between the vertebral bones, where the presence of NeuN positive neurons is observed. The main purpose of these stainings is to show that the tissue in the area below the neck-cranial border is a tissue belonging to the central nervous system. The area of interest was stained with hematoxylin and eosin, but staining with the neuron marker NeuN and the astrocyte marker GFAP was necessary to clearly show the tissue of the central nervous system. We showed that in central nervous system malformations such as anencephaly, the histology of the spinal cord is normal and cells that can be stained with neuron and astrocyte specific antibodies are present in this tissue.

The development of the neural tube is a multi-step process controlled by various genes and environmental factors. NTDs are a group of complex and heterogenic central nervous system anomalies, including anencephaly, spina bifida, and encephalocele (17) and may occur under the influence of nutritional and environmental factors (15). Folic acid intake has an important role in preventing the development of NTD in humans and until the third month of pregnancy is reported to reduce NTDs significantly, and widespread folic acid consumption reduced the frequency of these cases by 25% to around 1/1500 (6, 14, 15, 20). While folic acid consumption is known to prevent NTDs in humans, only one study shows that folic acid intake reduces NTDs in dogs (4). Zinc deficiency is also reported as a possible cause of anencephaly (22). Safra et al. (21) discovered an NTD-related gene in dogs for the first time and reported that NTDs might be associated with a genetic disorder.

Toxic plants, viral infections, teratogenic agents, and genetic factors are thought to be the causes of malformation in animals (10). Some anticonvulsant drugs like valproic acid consumed in the first trimester have been reported to cause NTDs (15). While anencephaly is observed sporadically in cattle, it is seldom observed in other species (8). NTDs are rare in dogs (23).

The bifid tongue is a bifurcation formed in the tongue and is generally uncommon in animals. Millard et al. (13) reported that there had been only 46 cases of bifid tongue in the last 150 years. Rifai et al. (19) reported that while cleft lower lips were observed in mules, only one mule had a bifid tongue. Bifid tongue and mandibular cleft are usually observed together in animals. Bifid tongue composes nutritional problems for the animals born with it and requires surgery for reconstruction (10). In our case, accompanying anencephaly is also noted in addition to the bifid tongue and cleft palate (palatoschisis). The literature review reveals no previous cases of this combination of malformations in dogs, so it is concluded that this case is the first.

In our case, it is known that the parents did not have any previous health issues, and they both were fully vaccinated. Folic acid and zinc analyze could not performed in our case unfortunately. The birth is the first birth of the mother. The detected anencephaly, bifid tongue, and cleft palate formation are thought to result from an environmental insult the mother was exposed to.

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In conclusion, studies about fetal malformations and their etiologies in dogs are not sufficient. More advanced future studies are required.

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## Conflict of Interest

The authors declared that there is no conflict of interest.

## Author Contributions

OY, SE, SEY, ERY and DB conceived and planned the experiments. OY, SEY, SE, MOO planned and stained histochemically and immunohistochemically staining. OY, SEY, DB, SE and ZAK contributed to sample preparation. OY, SEY, ERY, AS and MOO contributed to the interpretation of the results. OY, ERY and SEY took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

## Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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# Mycobacterial infection in a Nile crocodile (*Crocodylus niloticus*) from Türkiye

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ABSTRACT

Mycobacterial infection in Nile crocodile tissues sent from a private zoo was characterized pathomorphologically and immunohistochemically in this case. Macroscopically, multifocal, greyish-white areas ranging in size from 1 mm to 5 mm were seen in the lung, liver, and spleen. Histologically, a large number of well-demarcated necrotic areas were seen. These areas included nuclei debris locally. Inflammatory cells along with a couple of multinucleated giant cells surrounded the necrotic cores. Numerous acid-fast bacilli were detected by Ziehl-Neelsen staining method. Immunolabelling for both *Mycobacterium bovis* and anti-BCG antibodies was positive in each tissue.

Mycobacterial infection is an important disease caused by *Mycobacterium* spp. in a wide range of species (5). There are more than 140 species of non-tuberculous mycobacteria in humans and animals (8). *Mycobacterium* spp. are the most common aetiological agent for necrotising granulomatous inflammation (11). It has been reported that mycobacterial infections are commonly seen in reptiles; however, this infection is extremely rare in crocodiles (5). Heterophilic, histiocytic and chronic granulomas associated with *Mycobacterium* were described in reptiles such as snake, chelonian and lizard (13). The most isolated mycobacterial pathogens in crocodilians are summarised in Table 1. Mycobacterial infections in crocodiles have been reported in The United Kingdom (4), South Africa (6), Australia (1, 2), Netherlands (7, 16), South Korea (10) and Czech Republic (12). In crocodiles, the source of this infection is not known clearly. Presumably, its origin was fish for *M. fortuitium* and pork for *M. avium* (6). There is no known successful treatment of this disease (5). Although Ziehl-Neelsen staining is usually sufficient for the diagnosis of *Mycobacterium* spp., many studies show that additional tests such as Polymerase chain reaction (PCR) method are also needed (13, 14). It was realised that the immunohistochemistry (IHC) technique was not performed in any of the past studies concluded in crocodiles for *mycobacterium* diagnosis. The purpose of the case was to evaluate mycobacterial infection in a Nile crocodile with histopathological and immunohistochemical findings.

A private zoo provided lung, liver, and spleen samples in 10% neutral buffered formalin solution for pathological analysis. The samples were taken from a four-year-old female Nile crocodile (*Crocodylus niloticus*) with no prior clinical signs. After routine tissue processing, 5 µm sections were stained with standard Haematoxylin & Eosin (H&E) and Ziehl-Neelsen (ZN)

method for detection of acid-fast bacteria (9). The accurate diagnosis of mycobacterial infection was confirmed by Avidin-Biotin Complex Peroxidase (ABC-P; UltraVision Quanto Detection System HRP Polymer, Thermo Scientific, Catalog#TL-125-QHL) method. After deparaffinization and rehydration, the sections were incubated with 0.1% trypsin for 10 minutes at 37 °C. Endogenous peroxidase was blocked using 3% Hydrogen peroxide-methanol for 20 minutes at room temperature. Protein blocking solution was applied to sections for 10 minutes at 37 °C. Sections were then incubated with both *Mycobacterium bovis* (dilution ratio: 1:500, Dako) and anti-BCG (dilution ratio: 1:1000, Dako) antibodies in humidity chamber for 1 hour at 37 °C (15). Subsequently, sections were incubated with biotinylated antibody and streptavidin-peroxidase for 15 minutes at 37 °C. Sections were covered using 3-Amino-9-EthylCarbazole (AEC) chromogen for 7 minutes. Mayer's Haematoxylin was used as the counterstain for 3 minutes. For negative control slides, the primary antibody was substituted with both Phosphate-Buffered Saline (PBS) and mouse anti-rabbit IgG (dilution ratio: 1:100, Santa Cruz Biotechnology). Except for protein blocking, sections were washed with PBS between each step.

Gross examination revealed multifocal, greyish-white foci ranging from 1mm to 5 mm in diameter on the cut sections of all tissue samples (Figure 1). In H&E stained, multiple well-demarcated necrotic areas consisting of nuclei debris and peripherally infiltrated by a few amount of lymphocytes and macrophages were noticed in the lung, liver and spleen (Figure 2). Around necrotic areas, multinucleated giant cells were observed. Additionally, abundant intralesional acid-fast bacilli were demonstrated with Ziehl-Neelsen staining (Figure 3). Positive staining for *M. bovis* in immunohistochemistry is mostly found around necrotic areas and in macrophages. (Figure 4). Besides, positive immunoreaction for anti-BCG was also observed.

Typical granuloma structures have a central core of cell debris surrounded by multinucleated giant cells, macrophages and lymphocytes, which are limited to a fibrous connective tissue. They are defined in mycobacterial infections in crocodiles as in humans and other domestic animals; on the other hand, necrosis and low cellularity were striking histologic features in the case. Similar to other authors' findings, dystrophic calcification was also not noticed in this case (1, 2, 7, 10).

**Table 1** Mycobacterial pathogens in crocodilians.

| Species | Affected tissues | Inflammation type | Methods | Agent isolated | References |
|---|---|---|---|---|---|
| *Caiman sclerops* | Lung, spleen, liver, kidney, pancreas, testis, epiglottis | Granulomatous | Bacterial culture | *M. marinum* | (4) |
| *Caiman sclerops* | Kidney | Granulomatous | - | - | (16) |
| *Crocodylus johnstoni* | Lung*, liver, spleen*, kidney | Granulomatous | Gram, PAS, ZN and PCR* | *M. ulcerans* | (1) |
| *Crocodylus porosus* and *Crocodylus johnstoni* | Skin (snout, conjunctiva, jaws, neck, thigh) | Granulomatous | ZN | *Mycobacterium* spp. | (2) |
| *Crocodylus niloticus* | Liver, several organs<br>Generalize<br>Lungs<br>Skin | Granulomatous<br>Granulomatous<br>Granulomatous<br>Ulceration | Bacterial culture | *M. avium cplx*<br>*M. terrae*<br>*Atypical M.*<br>*M. triviale* | (6) |
| Caiman | Fat | Granulomatous | Bacterial culture | *M. fortuitum* | (6) |
| *Crocodylus johnstoni* | Lung | Granulomatous | Fite's method, ZN and Nested PCR | *M. szulgai* | (10) |
| *Caiman crocodilus fuscus* | Liver, lung, spleen | Granulomatous | ZN and PRA | *M. szulgai*<br>*M. chelonae* | (12) |
| *Caiman latirostris* | Intestinal wall, liver, spleen | Granulomatous | ZN and PCR | *M. intracellulare* | (7) |
| Crocodile | Lung, heart | No obvious lesions | PCR | *M. szulgai* | (3) |

PAS, periodic acid-schiff; PCR, Polymerase Chain Reaction; PRA, PCR restriction analysis; ZN, Ziehl-Neelsen.

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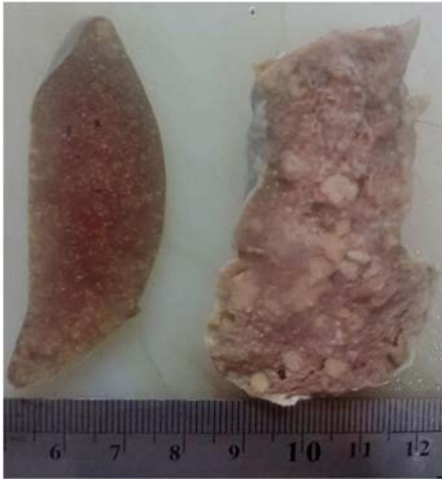


**Figure 1.** Multifocal, greyish-white areas in liver (left) and lung (right).

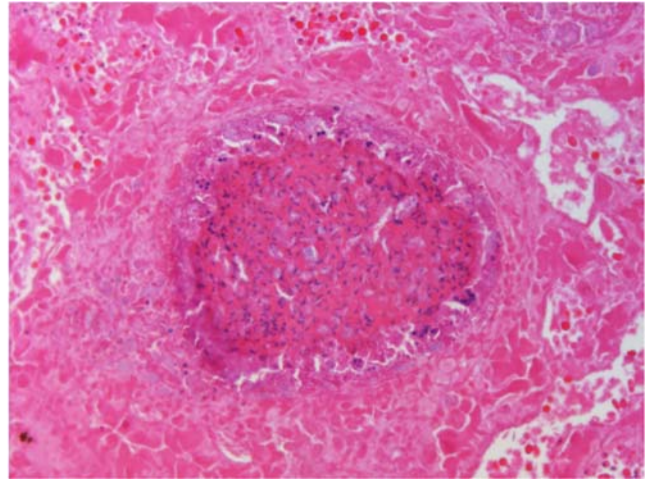

**Figure 2.** Well-demarcated necrotic area included nuclei debris and surrounded by lymphocytes, macrophages and multinucleated giant cell. Lung. H&E. X200.

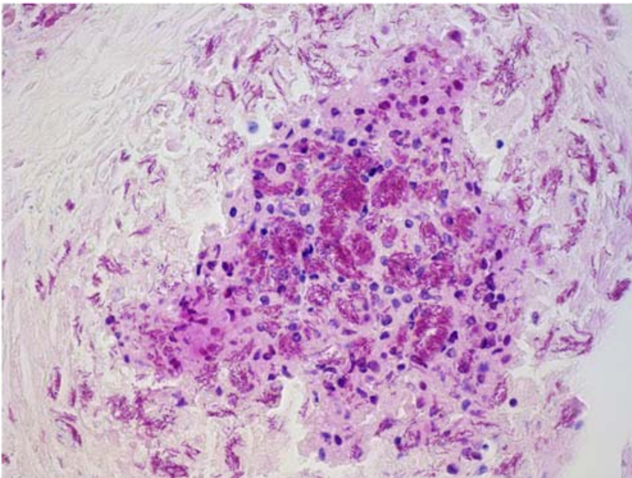

**Figure 3.** Numerous intralesional acid-fast bacilli. Liver. ZN. X400.

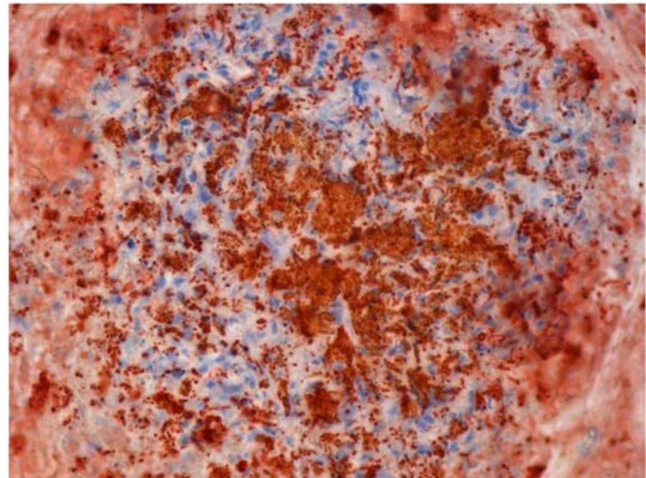

**Figure 4.** Prominent positive reaction for *M. bovis* both around the necrotic area and in macrophages. Lung. IHC. X400.

Generally, acid-fast bacteria have been found in the necrotic area and within the multinucleated giant cells (1, 2, 7, 10, 12). Roh et al. (2010) identified the microorganisms by Fite's method instead of Ziehl-Neelsen staining. However, in this study the bacteria were identified in the necrotic areas and in macrophages with Ziehl-Neelsen staining. In addition, other acid-fast bacteria, other than *Mycobacterium spp*., such as *Nocardia spp*., must always be remembered in Ziehl-Neelsen staining diagnosis. Reptiles including crocodiles are not vulnerable to *M. tuberculosis* and *M. bovis* infections due to their low body temperature (6). In this case we demonstrated the immunopositivity for *M. bovis*. Traditional bacterial culture and molecular techniques have been the gold standard for identifying the infectious agent. In the study, the tissues were sent within the formalin and we performed the immunohistochemistry method for demonstration of the causative agent. Because formalin-fixed tissues lack sensitivity and specificity, particularly for PCR, the study was based on immunohistochemistry to detect mycobacterial infection in crocodile, which resulted in the first diagnosis of mycobacterial infection in crocodile using the immunohistochemistry technique. Hereby, we also reported the first case of mycobacterial infection in a Nile crocodile in Türkiye.

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## Ethical Statement

No ethical approval was required in this case report.

## Conflict of Interest

The authors declared that there is no conflict of interest.

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