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- این مجله به استناد نامه شماره ۳/۲۹۱۰/۵۴۵ مورخ ۸۴/۵/۸ کمیسیون بررسی نشریات وزارت علوم، تحقیقات و فناوری دارای **درجه علمی ـ پژوهشی** می‌باشد.
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The effect of oral administration of encapsulated *Lactiplantibacillus plantarum* on the efficacy and immunogenicity of *Aeromonas hydrophila* vaccine in common carp

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Abstract

In the present study the effect of oral administration of probiotics *Lactiplantibacillus plantarum* in free form and microencapsulated with alginate/chitosan on immunogenicity and efficacy of Aeromonas *hydrophila* vaccine was evaluated in common carp. Three hundred and sixty common carps (48±5.1 gBW) were randomly divided into four equal groups in triplicates. Group 1 was vaccinated against *A. hydrophila*. Group 2 received the same vaccination and was also administered a diet supplemented with *Lactobacillus plantarum*. Group 3 was vaccinated and fed with encapsulated *L. plantarum*. Group 4, serving as the control, was fed with a basic diet without any supplementation. Biometrical measurement, blood and intestinal samples were taken on day zero, 30 and 60 of the experiment. Growth performance indices (Feed conversion ratio, specific growth rate, Protein efficacy ratio and food efficacy ratio) as well as immunological parameters (Antibody titer, serum lysozyme, complement and bactericidal activity, NBT reduction, globulin level and myeloperoxidase activity) were measured and compared among the groups. Meanwhile hematological parameters (Red Blood Cells, White Blood Cells, Hemoglobin and Hematocrit), intestinal enzyme activity (lipase, proteas, amylase and ALP). Antioxidant status (MDA level, SOD, GSH and catalase activity) and some serum biochemical indices (glucose, urea, Ca, Tg, ALP, CPK and Bilirubin) were measured and compared among the groups. On day 60 of the experiment the remained fish in each group were challenged with virulent *A. hydrophila* and cumulative mortality was recorded for 14 days. Results showed that the highest growth indices and intestinal enzyme activity were recorded in group 3 which were fed with encapsulated *L. plantarum*. Most of the immune indices evaluated in the study showed a significant increase in treatments 3 and 2 compared to the control group. The blood parameters and serum biochemical indices did not show significant differences among the groups. The mortality rate after the challenge was significantly lower in treatments 2 and 3 (30%) compared to the control group (60%). Overall, it can be concluded that not only the administration of *L. plantarum* play a role in improving the efficacy and immunogenicity of the injectable *A. hydrophila* vaccine in common carp, but also microencapsulation of this probiotic with alginate/chitosan enhances its effect on the vaccine's efficacy and immunogenicity. Therefore, the use of this microencapsulation method is recommended to improve the efficacy of the probiotic and the vaccine.

Key words: *Aeromonas hydrophila* vaccine, *Lactiplantibacillus plantarum*, Microencapsulation, Common carp, Immunogenicity

Introduction

Common carp (*Cyprinus carpio*) is one of the most widely cultivated fish species globally, with a significant role in aquaculture. Annually, global production of

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common carp exceeds 5 million tons, making it a cornerstone of freshwater fish farming, especially in Asia. The species is favored for its adaptability to various environments, fast growth rate, resistance to diseases, and its remarkability to many populations (FAO, 2023). This species is considered to be the most important species in Iraq aquaculture which dominates culture in fresh and brackish water with over 85% of the Iraqi aquaculture production (Esmaeili, 2021).

Cyprinid fish, particularly *Cyprinus carpio*, are highly susceptible to *A. hydrophila*, which causes fatal diseases like Aeromonas Hemorrhagic Septicemia. This severe infectious disease affects a wide range of aquatic animals, leading to extensive internal and external bleeding and resulting in a high mortality rate among susceptible species (Chen et al, 2020). *A. hydrophila* is one of the primary causes of the annual mortality of common carp in Iraq (Hossain and Heo, 2021).

One method of combating Aeromonas infections in aquaculture is through antibiotic therapy. However, the use of antibiotics in this setting comes with several disadvantages. A significant concern is the development of antibiotic-resistant bacteria, which can pose serious risks to both aquatic life and human health. Furthermore, the accumulation of antibiotics in the environment can disrupt ecosystems, harming beneficial microorganisms and potentially leading to bioaccumulation in fish that may be consumed by humans. Overreliance on antibiotics also tends to obscure underlying issues such as poor water quality or inadequate husbandry practices, which need to be addressed to ensure the sustainability of aquaculture. The high cost of antibiotics and the need for repeated treatments are also disadvantages of antibiotic therapy in aquatic animals (Gilani et al, 2024).

Vaccination is often considered the best and most logical method for preventing and controlling *Aeromonas* infections in fish (Schulz et al, 2020). Vaccination plays a significant role in reducing the incidence and severity of infections caused by this pathogenic bacterium; it boosts the immune response of fish, making them more resilient to *A. hydrophila* (Farias et al, 2020). With the implementation of proper vaccination protocols, the aquaculture industry can mitigate the economic losses associated with these infections, leading to sustainable fish production and improved food security (Nayak et al, 2022).

Extensive research has been conducted to prevent *A. hydrophila* infections in various aquatic organisms, including common carp by development of an extremely effective *A. hydrophila* vaccine (Abdul et al, 2022; Nayak, 2020). However, even after vaccination, fish can still exhibit symptoms of the disease when exposed to high levels of stress.

One of the problems with vaccines produced against Aeromonas infections is their relatively low efficacy and immunogenicity, which is largely related to the antigenic structure of these bacteria and the rapid changes in their surface antigens (Zhang et al, 2023). Therefore, the use of immune stimulants, such as probiotics, after fish vaccination against Aeromonas is strongly recommended to enhance immune responses (Wang et al, 2020).

Probiotics not only influence fish growth performance and health status by establishing and restoring balance in the gut bacterial flora, but they also enhance vaccine efficacy and immunogenicity by improving fish immune responses and health status (Hosainifar et al, 2020). Numerous studies have been conducted on the effect of oral probiotic administration on fish vaccine efficacy and immunogenicity. Guimarães et al, (2022) reported an improvement in the efficacy of the streptococcosis vaccine tilapia following Lactobacillus species administration.

Lactoplantibacillus plantarum is one of the most important probiotic bacteria, with its probiotic properties in fish well established. The effects of this bacterium have

been demonstrated in various fish species, including common carp, tilapia, and trout (Radkhah et al, 2024; Alishahi et al, 2022; Mohammadian et al, 2022). One challenge with administering oral probiotics in fish is the loss and degradation of these bacteria in the gastrointestinal environment, which diminishes their probiotic potency. To enhance the effects of this bacterium, it is crucial to protect the bacteria from gastrointestinal conditions. Various microencapsulation methods have been developed for this purpose. The use of biodegradable polymers, such as chitosan and alginate, for microencapsulating probiotics and shielding them from gastrointestinal conditions has recently gained increased attention from researchers (Ahmadmoradi et al, 2024; Hosseini et al, 2022). In addition to protecting probiotic bacteria in adverse gastrointestinal conditions, alginate and chitosan also act as immunostimulants, boosting the fish's immune response and overall health.

In the present study, based on our previous experience and research, alginate and chitosan were selected for the microencapsulation of the *Lactobacillus plantarum* using the emulsification method. Subsequently, the effect of the microencapsulated probiotic on the efficacy and immunogenicity of the *Aeromonas hydrophila* vaccine in common carp was evaluated.

Materials and methods

Bacterial strains

L. plantarum was selected from 30 lactic acid bacteria isolated from intestinal flora of wild and reared healthy cyprinid fish of cyprinid farms of Ahvaz, Iran, based on their in-vitro probiotic characteristics. The selected isolates were primarily identified microbiologically according to morphology of colonies, Gram staining, biochemical tests, and finally molecular identification via 16S rRNA gene sequencing (Mohammadian et al, 2016; Mohammadian et al, 2022).

Probiotic preparation

Lyophilized *L. plantarum* was inoculated in 10 ml Man Rogosa Sharpe (MRS) broth medium incubated at 37 $^{\circ}C$ for 48 hours using anaerobic jar. Following incubation of plates, the bacteria were harvested by centrifugation (10 min in 3000 g), and cells were washed tree times with PBS (pH = 7.2). The probiotic concentration was adjusted to 3×10^{8} CFU g food $^{-1}$ through OD absorption in 620 A by spectrophotometer

Microencapsulation of L. plantarum

Microencapsulation of *L. plantarum* with chitosan/alginate (MLCA) was done according to the emulsification method (Jiang et al, 2013; Hosseini et al, 2022). Briefly, the mixture of *L.plantarum* (10^{8} CFU g^{-1}), sodium alginate, and 15% (v/v) glycerol was dropped into 0.1 M $CaCl_2$ by passing through a cannula-like syringe in the presence of nitrogen gas pressure. The sodium alginate final concentration was 2% (w/v). Formed microcapsules were incubated for 30 min and then washed with 0.85% saline to remove unreacted $CaCl_2$. The chitosan (MW 10,000) solution 0.8% (w/v) was used to coat microcapsules for 30 min followed by two times washing. The microcapsules coated with chitosan-alginate were further coated with 0.1% (w/v) sodium alginate for 10 min followed by washing. Then microcapsules were stored at 4 $^{\circ}C$ until used. The control microcapsules without bacteria were also prepared by the same procedure.

Diet preparation

In this study, the experimental diet was prepared according to the method. The experimental diets were prepared based on Van Doan et al, (2016) as follows: Diet 1 basal diet without supplementation (for group1 and group 4), Diet 2 incorporated with 10^{8} CFU/g *L. Plantarum* (group 2), and Diet 3 incorporated with 10^{8} CFU/g encapsulated *L. bulgaricus* (group 3). To prepare feed containing probiotics at a concentration of 10^{8} CFU/g, first, the concentration

of the bacteria in initial stock was determined. The appropriate amount of bacterial stock for one kilogram of feed was mixed with 50 milliliters of PBS and sprayed uniformly over the feed. Then, liquid gelatin at 5 grams per liter and (55°C) was sprayed over the feed to protect the probiotic bacteria from dispersing in water. For the control group, all steps were repeated without the probiotic bacteria. To maintain high levels of probiotics, fresh batches of the diets were prepared every two weeks (Hosseini et al., 2022).

Vaccination against *A. hydrophila*

The high-virulence *A. hydrophila* of this experiment was selected from 12 pathogenic *A. hydrophila* isolated from diseased common carp referred to Fish Health Laboratory of Veterinary Faculty of Shahid Chamran University of Ahvaz, Iran. The severity of these isolates was evaluated based on in-vitro and in-vivo virulence assays and the highest virulent isolate was selected as a vaccine seed. Selected *A. hydrophilic* was identified using 16S RNA PCR- methods and confirmed through nucleotide sequencing.

Formalin killed *A. hydrophila* was prepared according to Abdy et al., (2017) briefly, *A. hydrophila* was first cultured in TSB medium and incubated for 36 hours at 30˚C. The bacteria were adjusted to 10^{10} cfu ml^{-1}, inactivated with 0.5% formalin for one hour. Formalin Killed Cells (FKC was washed twice (6000 g; for 30 min) with phosphate-buffered saline (PBS); then, it was cultivated in TSA plates and incubated at 30°C for 24h to guarantee outright bacterial inactivation. The immunization was conducted by injection in the peritoneal area 100 microliters per fish of *A. hydrophila* bacterin with a concentration of 10^{10} bacteria per milliliter, in the first day of experiment following the booster in day 21th of experiment.

Fish and experimental design

Three hundred and sixty healthy common carp (*cyprinus carpio*) fingerlings (48±5.1 g, Mean± SD) that had no previous history of parasitic infections and no signs of disease (gross and microscopic examination of gills, skin) were obtained from Azadegan cyprinid farm, Ahvaz, Iran. The fish were transferred to the laboratory of the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran acclimatized to laboratory conditions for 2 weeks in 500-L plastic quarantine tanks at 27±2 °C and fed with the control diet. The fish was randomly divided into 4 groups of 90 equal pieces, each group consisting of 3 replicates (30 fish each) stored for 60 days. The fish were divided into four equal groups with three replicates each (each replicate consisting of 30 fish). The first group was vaccinated with the injectable *A. hydrophila* vaccine. The second group was vaccinated and also fed with probiotics at a concentration of 10^8 CFU/g in the feed. The third group was vaccinated with the *A. hydrophila* and fed with microencapsulated probiotics. The fourth group served as the control group and was fed with a basal diet.

In each tank, approximately 25% of the water was exchanged daily, and 100% of the water was exchanged once a week, The fish were fed ad libitum twice daily, at 7:00 a.m. and 6:00 p.m., with the diets being hand-fed. Basic physicochemical parameters of the water were measured weekly to ensure optimal conditions. The O_2 concentration was maintained at no less than 5 mg $^{L-1}$ and pH ranged from 7.5 to 8.2 throughout the study period.

For the whole 60-day raising period, the feeding rate was ad libitum at 3% biomass and the uneaten feed was then siphoned away and dried separately in order to calculate the feed conversion ratio (FCR).

Sample collection

Sampling was performed on days zero, 30 and 60 of experiment and a total of 9 fish (3 fish from each replicate) were randomly

collected from each group for hemato-immunological and biochemical assays. After anesthetizing the fish with 2-phenoxiethanol (400 ppm), blood was collected from the caudal vein using a 1 mL syringe. Then, the blood samples were transferred to 1.5 ml microtubes with or without anticoagulant for hematological and immunological parameters respectively. Serum samples were collected by centrifuging at 3000 rpm for 10 minutes and stored at -20 ° C until used.

Growth performance

In the sampling points on days 0, 30 and 60, fish in each replica were weighed. The survival rate and growth performance of fish were calculated using the following equations:

Weight gain (WG) = final weight (g) - initial weight (g)

Specific growth rate (SGR %) = 100 - (ln final weight - ln initial weight)/Duration of experiment

Feed conversion ratio (FCR) = feed given (dried weight)/ weight gain (wet weight)

Survival rate (%) = (final fish number/ initial fish number) *100و

Food Efficacy Rate (FER) = 100 (final weight – Initial weight)/feed consumed,

Daily weight gain (DWG) = (final weight/initial weight)/time,

Protein efficiency ratio (PER) = Weight gain (g)/protein intake

Digestive enzyme activity

To analyze the digestive enzymes, the selected fish for sampling were euthanized after blood collection, and then intestine samples were taken following dissection. The intestine was dissected out using sterile technique at low temperature (on the ice-pack) and homogenized. Extracts utilized for enzyme assay were obtained after homogenization of intestine in cold 50 mM Tris–HCl buffer, pH 8.0 (1:9 v/w) followed by centrifugation (13.500 ×g; 30 min at 4 °C). The supernatant was then collected and kept at −80 °C in small portions for later determinations (Mohammadian et al., 2022). Bradford (1976) was used to assess the activity of total protein content in the gut using the diluted supernatant and bovine serum albumin as a reference. The α-amylase activity of the intestine was also measured using a soluble starch solution (Sigma– Aldrich) as the substrate, as described by Areekijseree et al. (2004). Amylase activity was measured in mol maltose generated per milligram of protein per minute. At room temperature, trypsin activity was determined using N-BenzoylL-arginine ethyl ester (BAEE) as a substrate in the presence of 0.1 mM HCl (Erlanger et al, 1961). The lipase activity was measured by quantifying the release of fatty acids from the enzymatic hydrolysis of triglycerides into glycerol in a stabilized olive oil emulsion (Fluka™) (Borlongan, 1990).

According to a modified approach (Otto et al., 1946), total ALP activity in homogenized tissue was measured at 410 nm and 37 °C using Pnitrophenyl phosphate as substrate and 2- amino-2-methyl-1-propanol buffer (0.84 mM, pH= 10.3). Casein (Sigma–Aldrich) was used as a substrate for measuring protease activity, and the result was subsequently reacted with Folin's reagent (Anson, 1938, with modifications). The absorbencies of each individual sample were determined using a spectrophotometer (UV-2802S; Unico, Shanghai, China), and the enzyme activities that were recorded as absorbance were modified and then reported as specific activity (U mg-1 protein min-1) (Erlanger et al, 1961).

Immunological parameters

Lysozyme activity assay

Serum lysozyme activity was determined turbidometrically according to the method described by Ellis. 1990. One hundred and thirty five µl of the *Micrococcus lysodeikticus* at a concentration of 0.2 mg ml^{-1} (w/v) in 0.02-M sodium phosphate buffer (SPB), pH 5.8 (Sigma- Aldrich) were mixed with 15 µl of each sample. Reduction of absorbance of 0.001 min^{-1} of samples was defined as one unit of lysozyme activity.

Alternative complement pathway activity

Alternative Complement Activity Pathway (ACP) of serum samples was measured and calculated based on Yano (Boshra et al., 2006) method using rabbit red blood cells (RaRBC).

Briefly, Veronal buffer used for the serum samples was diluted (5 times); then, 1% Rabbit RBC was gently poured into each well. Following 24 h incubation at 4 °C, the samples were centrifuged (five minutes in 3500 g). Afterward, 150 µl of supernatants were transferred to the wells of the microplate; then, the OD of each well was measured at 540 nm by an ELISA reader (Accu Reader, Taiwan).

Respiratory burst activity

The respiratory burst activity of leukocytes was evaluated using Nitro Blue Tetrazolium (NBT) according to the method suggested by Alishahi et al (2019) with minor modifications. In brief, 100 µl of blood samples were mixed with 100 µl of NBT (0.2 % in distilled water). The plate was well shaken and incubated for 30 min at 25 ˚C. Afterward, 2000 µl dimethylformamide was gently added to 100 µl of the prepared mixture; then, the final substance was centrifuged (at 3000 rpm for 10 minutes). Finally, the optical density of the supernatant was measured by a spectrophotometer (Shimadzu, Japan) at 620 nm.

Serum Bactericidal activity

The serum bactericidal activity was measured according to Yin et al (Dezfuly et al. 2020), with some modifications. The bacteria culture *(A.hydrophilla*) was pelleted (3000 g, 10 min) and washed 3 times with sterile PBS. A volume of 25 µL bacterial suspension (adjusted to 4×10^9 cells/ml) was added to 25 µL serum of fish in sterile Eppendorf tubes. Then, the tube was incubated at room temperature for 1 h. After that, plating the mixtures on TSA containing 1.5% NaCl was used to determine colony forming units (CFU)/ml.

Anti *A. hydrophila* antibody titer

A. hydrophila antibody levels in plasma were measured by ELISA with some modifications (Skov *et al*., 2018). Concisely, Microplate (Nunc, Denmark) was coated with 50 µL well ^{1} of formalin-killed and sonically disrupted *A.hydrophila* (100 µg/mL) antigen at a 1:15 dilution in bicarbonate coating buffer (pH=9.6) for 18 h at 4°C. After washing the plate; Common carp plasma samples (100 µL) were then, added at a 1:20 and 1:1 dilution respectively in PBS+0.05% Tween-20 (PBS-T) containing 0.1% skim milk. After 90 min incubation at 25°C, 100 µL of mouse anti common carp monoclonal immunoglobulin at a 1:4000 dilution in PBS-T containing 0.1% skim milk was added to all wells and then shaken for 60 min. After washing, 50 µL of goat anti-mouse IgG HRP conjugate (Sigma-Aldrich) at a 1:2500 dilution in PBS-T containing 0.1% skim milk was added and incubated for 60 min. Plates were washed as above and 50 µL TMB (3,3', 5,5; -tetramethylbenzidine - H2O2) chromogenic solution was added to each well for 10 min at 25°C. The reaction was stopped with 50 µL 2 N H_2SO_4. Lastly, serum and mucus antibody levels were read spectrophotometrically at 450 nm by an ELISA reader (Accu Reader, Taiwan).

Hematology and biochemical indices

RBC and WBC counts were determined using an improved Neubauer hemocytometer. Hemoglobin (Hb) concentration (g dl^{-1}) was estimated by cyano methemoglobin method using Drabkin's reagent. Hematocrit (Hct) was determined using microhematocrit capillaries filled with blood and centrifuged at 10000×g for 5 min and expressed as percentage of total blood volume (Thrall, 2004).

Serum biochemical indices, including levels of urea, calcium, glucose, triglycerides, alkaline phosphatase, creatine phosphokinase, and bilirubin, were measured using an autoanalyzer and commercial laboratory kits.

Antioxidant Status

Liver samples were obtained from each fish after euthanasia. After blood sampling and dissection of the fish, liver samples were collected, weighed, and then homogenized in ratio of 1–9 (w/v) of cold potassium phosphate buffer (0.1 M, pH =7.4, 4°C) at 10,000x g for 60 s. The homogenate was centrifuged (9,000x g, 30 min, 4°C); the supernatant was removed and aliquoted, then kept at −80°C. Catalase (CAT) (E.C. 1.11.1.6), superoxide dismutase (SOD) (E.C. 1.15.1.1) (McCord and Fridovich, 1969), and GSH level were determined according to the standard methods.

Intestinal bacterial flora

Samples of intestine were analyzed to quantify the total and Lactobacilli counts. Nine samples from each group were taken in each sampling point after blood sample collection and dissection. One gram of the samples was then homogenized by 9 ml of sterilized phosphate buffered saline (PBS, 0.1 M, pH=7.0) and stirred into 1 min in the stomacher (Heidolph instruments, Germany). Serial dilutions of each were then prepared under the sterile condition and spread on MRS and TSA plates. Following the 48 h incubation at 30 ℃, the number of colony on each plate was counted and reported as colony-forming units (CFU) per gram of sample.

Determination of LD 50

Before performing the challenge, the following steps were taken to calculate the median rate of lethality of the *A. hydrophila* for common carp (Alishahi et al., 2024). Briefly *A. hydrophila* was cultured in TSB culture medium for 48 hours at 37˚ C. The bacteria were adjusted to 10^8 cfu ml^{-1} after centrifugation (4000 rpm, 10 min). The 10-fold serial dilutions (10^5 to 10^8 CFU ml^{-1}) of the *A. hydrophila* were prepared in PBS and 0.1 ml of each concentration of *A. hydrophila* was injected intraperitoneally to 10 fish (each replicate) in a separate aquarium. The dead fish were netted and recorded daily for 10 days. The rate of mortality was analyzed and the LD50 was determined by Probit software using version 22 of SPSS. The LD50 of *A. hydrophila* in common carp calculated as 1.2×10^6. At the end of the study, the fish were challenged with this concentration of bacteria (Aramon et al, 2024).

Bacterial challenge

The remained fish in each group (at least 30 fish,10 fish from each replicate) were injected with live *A. hydrophila* via intraperitoneal route at day 60 of experiment. Firstly, the sedation of fish was done by 2-phenoxyethanol (300 mg l^{-1}) and 100 microliter of 1.2×10^6 cfu ml^{-1} of *A. hydrophila* (LD_{50} concentration) was injected intraperitoneally. After injection of the bacteria the fish were put to 100 L aquaria. The dead fish of each treatment were netted and checked two times daily for 10 days and the number of dead fish was recorded. The cumulative mortality rate (CMR) was calculated after mortality recording for10 days (Alishahi et al., 2018 and 2024). For confirmation of the cause of death re-isolation of *A. hydrophila* was done from the kidney and liver of the dead fish.

Statistical analysis

Before statistical analysis of data, their normality was determined using the Kolmogorov-Smirnov test. One-way ANOVA with Multiple Comparisons Test was used to compare the different groups, followed by Tukey's test ($P<0.05$) and then, the quantitative data were presented as mean ± standard deviation. All statistical analyses were performed using SPSS software (Version 24).

Results

Growth indices

The results of the growth indices comparison between the experimental goups are presented in Table 1. As shown in the table, nearly all growth indices including feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER), daily weight gain (DWG), and food efficiency ratio (FER) improved significantly in the

probiotic-fed treatments compared to the control and the vaccinated group (without probiotics) at both sampling times (P<0.05). The group vaccinated with *A. hydrophila* and fed with *Lactobacillus plantarum* microencapsulated with alginate and chitosan demonstrated the highest growth indices at both sampling stages compared to the other treatments (P<0.05). Survival rates were 100% across all the experimental treatments, with no mortality observed during the study.

Table 1: Growth performance indices of the experimental groups at days 30 and 60 of experiment

	Groups	SGR	FCR	PER	DWG	FER
Day 30	A	0.56±0.03 ^{ab}	2.08±0.13 ^{b}	1.51±0.10 ^{b}	0.34±0.04 ^{b}	48.24±3.16 ^{b}
	B	0.65±0.15 ^{a}	1.86±0.44 ^{ab}	1.49±0.31 ^{b}	0.40±0.08 ^{b}	47.66±9.96 ^{b}
	C	0.74±0.09 ^{a}	1.45±0.19 ^{a}	2.13±0.37 ^{a}	0.50±0.08 ^{a}	78.01±7.88 ^{a}
	D	0.47±0.14^{b}	2.23±0.52 ^{b}	1.45±0.30 ^{b}	0.31±0.07 ^{b}	46.37±9.73 ^{b}
Day 60	A	1.09±0.19^{ab}	2.31±0.07	1.35±0.14	0.91±0.23 ^{b}	39.96±4.6 ^{b}
	B	1.16±0.03^{ab}	2.06±0.15 ^{ab}	1.22±0.07 ^{b}	0.95±0.02	39.17±2.32 ^{b}
	C	1.24±0.05^{a}	1.87±0.45 ^{a}	1.31±0.19 ^{a}	1.02±0.21 ^{a}	43.42±2.22 ^{a}
	D	0.98±0.02^{b}	2.24±0.19 ^{b}	1.26±0.09 ^{b}	0.96±0.09 ^{b}	36.91±4.77 ^{b}

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

Digestive enzyme activity

The results of the comparison of digestive enzyme activities in the experimental groups at three sampling points are presented in Table 2. As shown in table 2, ALP and amylase showed a significant increase in both probiotic-treated groups (with and without microencapsulation) on days zero, 30 and 60 of the study, compared to the control treatment (P<0.05). However, the activities of protease, lipase, and trypsin were significantly increased only in the vaccinated treatment fed with microencapsulated probiotics compared to the control group in days 30 and 60 of experiment (P<0.05).

Table 2: The activity of intestinal digestive enzymes of the experimental groups at days zero, 30 and 60 of experiment

	Groups	ALP	Amylase	protease	Lipase	Tripsine
Day 0	A	14.54±3.07 ^{a}	47.48±11.30 ^{a}	0.12±0.02 ^{a}	7.59±2.84^{a}	65.41±18.5^{a}
	B	13.04±2.04 ^{a}	42.29±24.65 ^{a}	0.14±0.02 ^{a}	8.22±2.31^{a}	47.43±0.19^{a}
	C	13.79±2.55 ^{a}	44.88±17.97 ^{a}	0.13±0.02 ^{a}	7.91±2.58^{a}	56.42±19.7^{a}
	D	13.41±2.29 ^{a}	43.59±21.31 ^{a}	0.13±0.01^{a}	8.06±2.45 ^{a}	51.92±17.4^{a}
Day 30	A	12.20±4.18 ^{b}	43.70±21.9 ^{b}	0.12±0.03 ^{b}	8.11±1.93 ^{b}	45.87±16.3 ^{b}
	B	17.67±3.45 ^{a}	43.89±18.67 ^{b}	0.11±0.02 ^{b}	7.99±2.87 ^{b}	53.73±18.9 ^{b}
	C	16.71±4.04 ^{a}	64.83±20.33 ^{a}	0.16±0.01^{a}	11.8±3.65 ^{a}	92.49±26.8 ^{a}
	D	12.54±3.07 ^{b}	47.48±11.5 ^{b}	0.12±0.02 ^{b}	7.59±2.84 ^{b}	65.41±27.3 ^{b}
Day 60	A	14.41±4.28 ^{b}	47.62±11.23 ^{b}	0.13±0.02 ^{b}	8.13±2.26 ^{a}	52.55±19.2 ^{b}
	B	15.50±8.99 ^{a}	73.29±11.33 ^{a}	0.16±0.03 ^{ab}	9.79±1.83 ^{a}	63.50±12.6^{ab}
	C	17.79±3.74 ^{a}	80.55±24.49 ^{a}	0.17±0.03 ^{a}	9.54±2.74 ^{a}	87.81±30.5 ^{a}
	D	13.04±2.04 ^{b}	42.29±24.65 ^{b}	0.14±0.02 ^{b}	8.22±2.31 ^{a}	47.43±15.2^{b}

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

Immunological parameters

The data related to the serum immune response are summarized in Table 3 and figures 1 to 3. The comparison of immune indices between the experimental treatments at the three sampling stages showed that most immune indices, including anti-*A. hydrophila* antibody titer, lysozyme activity, NBT reduction, protein and globulin levels, and antitrypsin and myeloperoxidase activities, exhibited significant increases ($P<0.05$) in the probiotic-treated groups, particularly those treated with microencapsulated probiotics, compared to the control group at days 30 and 60 of experiment. However, some immunological indices, such as alternative complement activity, albumin levels, and serum bactericidal activity, did not show significant differences between the experimental groups ($P>0.05$).

Table 3: The immunological indices of the experimental groups at days zero, 30 and 60 of experiment

	Groups	complement	Bactericidal	Protein	Albumin	Globulin	Antitrypsin	mylopor
Day 0	A	5.77±0.46 ^{a}	0.27±0.09 ^{a}	3.81±0.27 ^{a}	0.96±0.11 ^{a}	2.85±0.32 ^{a}	0.91±0.17 ^{a}	0.31±0.09 ^{a}
	B	5.97±0.50 ^{a}	0.25±0.11 ^{a}	3.87±0.20 ^{a}	0.95±0.09 ^{a}	2.92±0.18 ^{a}	0.84±0.28 ^{a}	0.37±0.14 ^{a}
	C	5.87±0.48 ^{a}	0.25±0.12 ^{a}	3.84±0.13 ^{a}	0.96±0.10 ^{a}	2.88±0.25 ^{a}	0.88±0.23 ^{a}	0.34±0.11 ^{a}
	D	5.92±0.49 ^{a}	0.21±0.08 ^{a}	3.86±0.16 ^{a}	0.95±0.10 ^{a}	2.90±0.21 ^{a}	0.86±0.26 ^{a}	0.35±0.12 ^{a}
Day 30	A	6.27±0.46 ^{a}	0.24±0.09 ^{a}	4.01±0.47 ^{ab}	0.93±0.05 ^{a}	3.07±0.46 ^{ab}	0.78±0.26 ^{b}	0.40±0.12 ^{b}
	B	6.53±0.64 ^{a}	0.31±0.06 ^{a}	4.27±0.76 ^{a}	0.95±0.11 ^{a}	3.33±0.77 ^{a}	1.20±0.17 ^{ab}	0.49±0.11 ^{a}
	C	6.23±0.67 ^{a}	0.29±0.09 ^{a}	4.38±0.43 ^{a}	0.92±0.13 ^{a}	3.46±0.25 ^{a}	1.56±0.23 ^{a}	0.43±0.11 ^{ab}
	D	5.97±0.4 ^{a}	0.28±0.11 ^{a}	3.87±0.27 ^{b}	0.95±0.14 ^{a}	2.92±0.67 ^{b}	0.84±0.23 ^{b}	0.37±0.13 ^{b}
Day 60	A	6.00±0.87 ^{a}	0.26±0.13 ^{a}	4.01±0.44 ^{a}	0.94±0.12 ^{a}	3.06±0.44 ^{ab}	0.89±0.26 ^{b}	0.32±0.12 ^{a}
	B	5.67±1.05 ^{a}	0.21±0.14 ^{a}	4.14±0.32 ^{a}	0.93±0.11 ^{a}	3.22±0.28 ^{a}	1.08±0.34 ^{ab}	0.33±0.11 ^{a}
	C	6.20±0.54 ^{a}	0.24±0.09 ^{a}	4.15±0.65 ^{a}	0.93±0.08 ^{a}	3.22±0.83^{a}	1.31±0.22 ^{a}	0.33±0.10 ^{a}
	D	5.77±0.50 ^{a}	0.29±0.12 ^{a}	3.81±0.13 ^{a}	0.96±0.09 ^{a}	2.85±0.18 ^{b}	0.91±0.28	0.31±0.11 ^{a}

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences ($P<0.05$) within each sampling time).

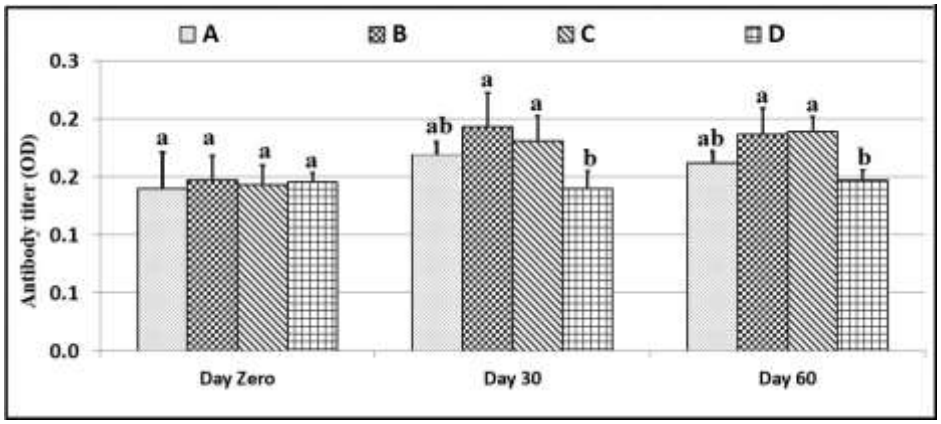

Figure 1: Anti *A.hydrophila* antibody titer of the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation bar indicate significant differences (P<0.05) within each sampling time).

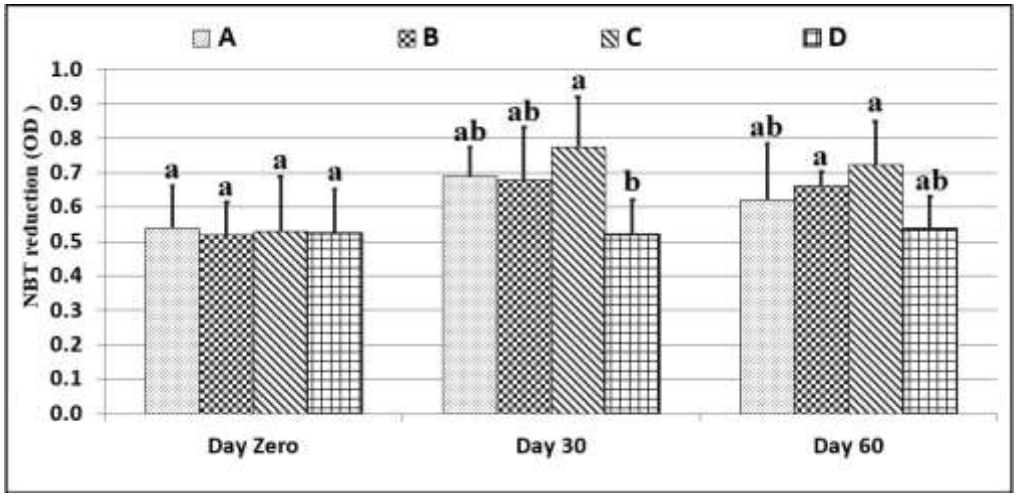

Figure 2: Nitro Blue Tetrazolium (NBT) reduction of the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation bar indicate significant differences (P<0.05) within each sampling time).

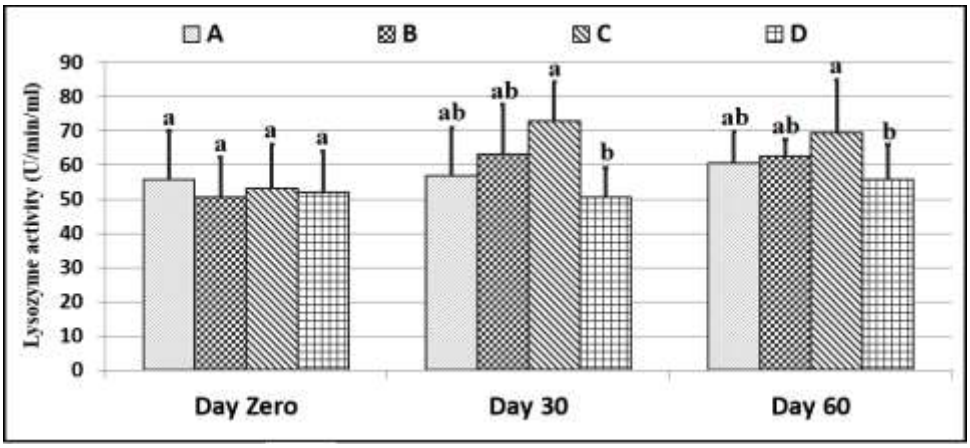

Figure 3: Lysozyme activity of the experimental groups at days zero, 30 and 60 of experiment. A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (different lowercase letters on the standard deviation bar indicate significant differences (P<0.05) within each sampling time).

Hematological and biochemical parameters

The data related to the serum Hematological parameters are summarized in Table 4. A comparison of the hematological parameters among the experimental groups at different sampling stages showed that the red globular-related indices (including red RBC, hemoglobin, and hematocrit) were not affected by vaccination or probiotic administration (P>0.05). However, the white blood cell counts significantly increased in the probiotic-fed treatments, particularly in those fed with microencapsulated probiotics, on days 30 and 60 of the study compared to the control group (P<0.05).

Table 4: The hematological parameters of the experimental groups at days zero, 30 and 60 of study

	Groups	Hb	PCV	RBC	WBC
Day 0	A	9.14±2.1[a]	35.20±3.03[a]	1.34±0.16[a]	21.2±6.2[a]
	B	8.74±1.91[a]	35.61±6.47[a]	1.42±0.16[a]	18.35±5.23[a]
	C	8.9±4.87[a]	34.46±6.43[a]	1.39±0.16[a]	20.26±7.2[a]
	D	8.86±1.34[a]	34.00±5.49[a]	1.39±0.17[a]	18.5±5.48[a]
Day 30	A	9.01±1.95[a]	35.40±4.51[a]	1.33±0.17[a]	20.5±4.86[b]
	B	8.65±2.71[a]	37.83±5.7[a]	1.35±0.21[a]	27.64±4.34[a]
	C	9.19±2.73[a]	37.20±3.84[a]	1.38±0.31[a]	31.76±4.24[a]
	D	9.14±1.94[a]	35.20±5.13[a]	1.34±0.19[a]	20.5±4.92[b]
Day 60	A	8.7±3.12[a]	35.60±4.86[a]	1.31±0.18[a]	22.1±4.81[b]
	B	8.71±2.94[a]	34.75±5.2[a]	1.39±0.21[a]	25±4.86[a]
	C	8.72±2.34[a]	33.00±4.65[a]	1.34±0.17[a]	28±4.34[a]
	D	8.94±2.67[a]	33.60±6.34[a]	1.41±0.19[a]	18.5±5.24[b]

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

The results of the comparison of serum biochemical indices between the experimental groups at different sampling points are presented in Table 5. The serum biochemical indices examined, including urea, calcium, glucose, triglycerides, ALP, Total and direct Bilirubin, and Creatine Phosphokinase were not affected by probiotic (with or without microencapsulation) administration (P>0.05).

Table 5: The serum biochemical parameters of the experimental groups at days zero, 30 and 60 of study

		UREA	CA	GLU	TG	ALP	CPK	OT-B	PT-B
Day 0	A	10.97±2.52[a]	8.97±1.02[a]	157.3±40.77[a]	270±70.5[a]	336±75.8[a]	81.83±25.7[a]	269.6±44.81[a]	1.97±0.18[a]
	B	13.33±2.34[a]	8.53±1.02[a]	146.6±40.77[a]	213.6±72.21[a]	331.3±75.8[a]	82.33±25.9[a]	228.3±41.8[a]	1.85±0.17[a]
	C	12.15±2.05[a]	8.75±0.29[a]	152±35.3[a]	241.8±11.1[a]	333.6±29.72[a]	82.08±7.65[a]	249±46.61[a]	1.91±0.32[a]
	D	12.74±2.28[a]	8.64±0.66[a]	149.3±38.06[a]	227.7±40.82[a]	332.5±18.45[a]	82.21±16.71[a]	238±46.16[a]	1.88±0.24[a]
Day 30	A	11.83±2.17[a]	8.90±0.47[a]	144.6±36.70[a]	221.6±56.81[a]	314.3±52.76[a]	91.00±12.18[a]	266±45.71[a]	1.91±0.27[a]
	B	12.00±0.76[a]	9.10±0.20[a]	120.3±40.02[a]	240±25.98[a]	371±41.24[a]	83.67±19.31[a]	226.6±33.41[a]	2.1± 0.17[a]
	C	12.67±1.73[a]	9.07±0.10[a]	122.3±17.47[a]	257.6±21.73[a]	342.6±25.72[a]	83.67±9.87[a]	212.6±49.66[a]	1.9±0.35[a]
	D	10.97±1.53[a]	8.97±0.5[a]	157.3±26.27[a]	270.5±26.7[a]	336±29.72[a]	81.83±17.6[a]	269.6±46.9[a]	1.9±0.30[a]
Day 60	A	12.33±2.05[a]	9.07±0.29[a]	139±35.35[a]	234.4±51.7[a]	359±42.98[a]	91.50±7.65[a]	222.3±41.04[a]	1.94±0.32[a]
	B	12.67±2.31[a]	8.47±0.55[a]	124±20.66[a]	250.3±79.32[a]	332.3±45.3[a]	92.33±35.8[a]	237.3±38.8[a]	1.9±0.36[a]
	C	13.33±2.08[a]	8.97±0.93[a]	129±33.78[a]	220.6±11.14[a]	378.6±84.24[a]	88.33±18.01[a]	255.6±41.5[a]	1.7±0.39[a]
	D	13.33±0.58[a]	8.53±0.40[a]	146.6±22.61[a]	213.6±93.26[a]	331.3±30.37[a]	82.33±22.59[a]	228.3±56.2[a]	1.8±0.33[a]

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

Antioxidant status

The antioxidant status in the liver of the fish from the experimental treatments is presented in Table 6. The level of MDA (malondialdehyde) in the probiotic treated groups, especially in the microencapsulated probiotic group, was significantly lower than in the control group (P<0.05). In contrast, the activities of superoxide dismutase

(SOD) and GSH (glutathione) in the probiotic treated groups showed a significant increase compared to the control group on days 30 and 60 of the study. The level of catalase enzyme did not show significant differences among the groups across the three sampling stages.

Table 6: The antioxidant status of the experimental groups at days zero, 30 and 60 of study

	Mean	MDA	SOD	GSH	catlase
Day 0	A	94.81±46.29 [a]	1.97±0.21 [a]	0.28±0.05 [a]	0.04±0.012 [a]
	B	105.48±20.92 [a]	2.58±0.45 [a]	0.29±0.04 [a]	0.03±0.011 [a]
	C	100.14±33.61 [a]	2.27±0.33 [a]	0.29±0.04 [a]	0.04±0.013 [a]
	D	102.81±27.26 [a]	2.42±0.39 [a]	0.29±0.04 [a]	0.03±0.012 [a]
Day 30	A	89.85±11.38 [b]	2.11±0.66 [b]	0.31±0.12 [a]	0.05±0.014 [a]
	B	79.21±15.97 [b]	3.63±0.54 [a]	0.37±0.13 [a]	0.05±0.011 [a]
	C	74.85±23.92 [b]	4.87±0.9 [a]	0.43±0.07 [a]	0.06±0.02 [a]
	D	106.81±46.29 [a]	1.97±0.21 [b]	0.28±0.05 [b]	0.04±0.012 [a]
Day 60	A	94.67±22.03 [ab]	2.73±0.60 [b]	0.31±0.04 [b]	0.03±0.014 [a]
	B	85.13±12.19 [b]	2.87±0.36 [ab]	0.38±0.08 [a]	0.04±0.013 [a]
	C	79.89±9.19 [b]	3.27±1.09 [a]	0.39±0.08 [a]	0.05±0.014 [a]
	D	117.48±20.92 [a]	2.58±0.45 [b]	0.29±0.04 [b]	0.03±0.011 [a]

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

Intestinal Bacterial flora

The total bacterial count and the count of lactic acid bacteria in the intestines of experimental groups were compared in sampling points (Table 7). The results indicated that, although the total bacterial count did not differ significantly (P>0.05) between the treatments, the number of lactic acid bacteria was significantly higher in the treatments fed with probiotic-containing diets compared to the other groups (P<0.05).

Table 7: The bacterial flora of intestineof the experimental groups at days zero, 30 and 60 of study

	Groups	Heterotroph bacteria	Lactic Acid bacteria
Day 0	A	165±26.06 [a]	122.33±33.15 [b]
	B	169±25.16 [a]	131.00±9.07 [b]
	C	167±25.61 [a]	126.67±21.11 [b]
	D	168±25.38 [a]	128.83±15.09 [b]
Day 30	A	171.3±55.9 [a]	141.00±43.71 [b]
	B	180.6±44.81 [a]	170.00±42.3 [ab]
	C	175.3±46.32 [a]	180.33±44.66 [a]
	D	169±26.06 [a]	131.00±33.1 [b]
Day 60	A	173.3±45.35 [a]	144.00±56.29 [ab]
	B	162.3±53.72 [a]	156.67±48.01 [a]
	C	171.3±30.44 [a]	156.67±31.56 [a]
	D	165±25.16 [a]	122.33±9.07 [b]

A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group. (Different lowercase letters on the standard deviation indicate significant differences (P<0.05) within each sampling time).

Challenge

The results of the challenge with *A. hydrophila* of treated fish showed that the highest mortality rate (60%) occurred in the control group, while the lowest mortality rate (20%) was observed in the both vaccinated groups fed with encapsulated probiotic-containing diets (Figure 4).

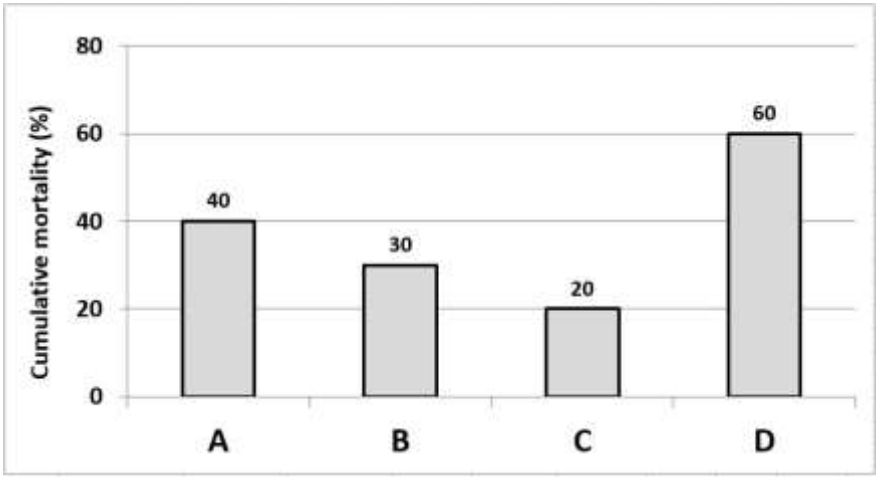

Figure 4: Mortality rate after challenge with *A.hydrophila* in the experimental groups at days60 of experiment. A: vaccinated group, B: vaccinated and *L.plantarum* treated group, C: vaccinated and encapsulated *L.platarum* treated group, D: control group.

Discussion

Improving growth indices is one of the primary goals in aquaculture. The results of the current study showed that the highest growth indices were observed in the fish vaccinated with *A. hydrophila* and fed with *L. plantarum* microencapsulated with alginate/chitosan. The microencapsulation of the probiotic likely enhanced the probiotic effects of probiotic, leading to improved growth in this treatment. The ability to improve growth performance of encapsulated probiotics has previously been demonstrated in other aquatic animals such as Oriental Bream Fry (*Abramis brama orientalis*) (Asadi et al, 2016), green terror (Neissi et al, 2013), sea bass (Ashouri et al., 2018), basa fish *(Pangasius bocourti*) (Van et al, 2014) and Nile tilapia (Van et al, 2017).

It is well established that the use of dietary probiotics can positively affect the growth of fish through stimulation of appetite, elevation of digestive enzymes activity, regultion of the population of the gut microflora, modification of the intestinal morphology, promotion of feed utilization and provision of micronutrients (Pinpimai et al, 2015; Mohammadian et al, 2022). In this study, protecting probiotics through microencapsulation techniques enhances their efficacy. Similarly, significant improvement of growth parameters has been reported in Nile tilapia (*Oreochromis niloticus*) and rainbow trout fed with diets containing free or encapsulated *Saccharomyces cerevisiae* and *L. rhamnosus*, respectively (Pinpimai et al, 2015; Hooshyar et al, 2020). The improvement of growth indices in probionts supplemented groups could likely be owing to the increase in digestive enzyme activities, induced by probiotics (Jang et al, 2019). The increase in digestive enzyme activities and therefore, improved feed utilization through the use of probiotics has also been reported in *O. mykiss* as the results of other bacterial strains, like *L. casei* and *L. plantarum* or even in other fish species, like *Sparus aurata*, fed with *Lactobacillus* sp. (Assan et al, 2022). The obtained results suggested that higher amylase, trypsin, ALP and lipase activities, in the *L. plantarum*-

treated fish, might be responsible for improved growth performance. The higher intestine ALP activity indicates the intensity of nutrient absorption in the enterocytes of fish, which it can be responsible for more carbohydrate and lipid uptake (Gawlicka et al, 2000). The previous studies explained how probiotics (especially *L. plantarum*) are able to stimulate this enzyme activity within the brush border of fish enterocyte (Mohammadian et al, 2017). The improved intestine protease activity was in line with the increased PER in our study. According to the results, fish vaccinated with *A. hydrophila* and fed with encapsulated probiotic had significantly higher activities of intestinal trypsin, α-amylase, lipase and ALP. Therefore, the enhancement of digestive enzymes activity was apparently one of the main reasons for growth-stimulatory effects of the probiotic used. The elevated activities of digestive enzymes have been reported to enhance the digestion of macromolecules and therefore facilitate the absorption of nutrients within the gut lumen (Assan et al, 2022).

In this study, the vaccinated fish demonstrated improved antioxidant defense after the administration of microencapsulated probiotics at both sampling stages. The higher activities of serum SOD, CAT and GSH and the lower levels of MDA were observed in the probiotic treated groups, in particular in the microencapsulated group. In agreement with our results, the protective effects of encapsulated or non-encapsulated Lactobacillus strains have been reported previously against oxidative stress caused by stressors in aquatic animal models (Giri et al, 2018).The previous studies have revealed that there are insufficient levels of endogenous antioxidants in cultured fish to cope with external stressors, and improvement of antioxidant defense capacities of fish is highly important (Ghanei-Motlagh et al, 2020). On the other hand, *Lactobacillus plantarum* possess direct scavenging activities against active oxidants by production of enzymes or metabolites with potent antioxidant abilities such as SOD, GSH, and butyrate (Wang et al, 2020). Likewise, Administration of Lactobacillus spp. can positively alter the antioxidant defense system of fish through regulation of antioxidant-dependent signaling pathways (Hoseinifar et al, 2020). SOD and CAT are involved in the disproportionation of superoxide anion radical, and the degradation of hydrogen peroxide, respectively (Yousefi et al, 2019; Ghanei-Motlaghet al, 2021). GSH is a tripeptide non-enzymatic antioxidant which plays an important role in the balance of intracellular redox reactions (Haddad and Harb, 2005). MDA is a secondary product of lipid peroxidation reflecting the cell membrane injury mediated by free radicals.

The results of the current study showed that common carp vaccinated with *A. hydrophila* and fed with probiotics (with or without microencapsulation) did not exhibit significant differences in blood indices and serum biochemical parameters with control group ($P>0.05$). Hemato-biochemical tests are important tools to assess the health status of fish (Fazio, 2019). Irianto and Austin reported no change in the number of RBC in *O. mykiss* fed with probiotics for 14 days. Similarly, The *Micrococcus luteus* administrated fish showed no increase in the number of hematological parameters in *O. niloticus* (El-Rhman et al, 2009). Contrary to our finding, Firouzbakhsh et al, (2011) reported the improved hematological indices in *Astronotus ocellatus* treated by probiotic mixed diet. They suggest that the increase in hematological parameters may be due to the higher growth rates, which lead to increased hematopoiesis and oxygen-carrying capacity. However, this conclusion is limited as changes in hematological indices due to nutrient manipulation often reflect ion regulatory or respiratory issues, indicating higher energy demands to maintain homeostasis rather than supporting growth.

Out results showed that vaccination against *A. hydrophila* and the administra-

tion of probiotics (with or without encapsulation) had no significant effect on serum biochemical indices. The lack of impact on blood and serum biochemical parameters suggests that these treatments do not adversely affect the health of the fish. However, it is possible that a longer administration period or a higher concentration of probiotics could improve blood and serum biochemical indices. Nonetheless, under the conditions used in the current study, vaccination and probiotic administration did not affect these parameters.

Groups 3 and 2, vaccinated and fed with *L. plantarum* with and without microcapsulation, exhibited a significant increase in WBC at days 30 and 60 of trial. In similar works, elevated WBC demonstrated in *O. mykiss* received dietary probiotics. (Mohammadian et al, 2017). The increase in WBC count in the probiotic fed fish seems to be the result of induced activities in the anterior part of the head kidney.

In this study, most immune indices, including anti-*A. hydrophila* antibody levels, serum lysozyme activity, NBT reduction, serum globulin and protein levels, anti-trypsin, and myeloperoxidase, showed a significant increase in group 3 (vaccinated fish with *A. hydrophila* and fed with diet containing *L. plantarum* microencapsulated with chitosan/alginate) compared to the control group ($P<0.05$). However, some indices, such as serum complement activity and serum antimicrobial power, did not show significant differences among the treatments ($P>0.05$). Elevated lysozyme activity in groups 2 and 3 suggests that these applied probiotics can likely provoke the immunity system of common cap. In agreement with our finding, higher level of serum lysozyme in *O. mykiss* fed with *L. casei, L. plantarum*, and *C. divergens* was reported previously (Mohammadian et al, 2019).

The alternative complement activity is accounted as another indicator of innate immune response in the case of infectious disease (Bavia et al, 2022). In the present study, NBT reduction was elevated in group 3. Consistent to our finding, Andani et al, (2012), and (Mohammadian et al, 2019) showed that administration of Lactobacillus bacteria increases the serum complement and NBT activity in *O. mykiss*. On the other hand, contradictory findings were also reported (Mozanzadeh et al, 2023), attributing the possible difference in experimental procedure and even bacterial strains. The obtained results showed that when fish recieved *L. plantarum*, the NBT reduction was higher than the control group. NBT reduction is an indicator for respiratory burst activity of immune-related cells in fish (Zhu & Su, 2022). The findings of respiratory burst activity following the probiotics treatment in fish are often contradictory, while some studies indicated that probiotics did not have any significant impact on this nonspecific defense mechanism of fish (Mozanzadeh et al, 2023). Several *in vitro* and *in vivo* studies showed a significant increase in respiratory burst activity by various probiotics in many aquatic animals including fish (Zhu and Su, 2022). This study further confirmed that the probiotics might be responsible for degrading free radicals production by host phagocytic cells.

Myeloperoxidase and anti-trypsin, as two immunological indices, showed a significant increase in groups 2 and 3 compared to the control group. The probiotic administration in fish has been shown to enhance the activity of anti-trypsin and myeloperoxidase (Sahu et al, 2013). These effects are attributed to the stimulation of the immune system, which improves the ability to respond to pathogens of the fish. Anti-trypsin activity is linked to the regulation of protease enzymes, while myeloperoxidase is involved in the production of reactive oxygen species during the immune response, both contributing to increased disease resistance in the fish (Hoseinifar et al, 2016).

The intestinal bacterial count and flora of fish in the different treatment groups were influenced by the treatments. Although the number of heterotrophic bacteria did not show significant differences among the

groups (P>0.05), the number of lactic acid bacteria significantly increased in group 3 (vaccinated fish fed with microencapsulated probiotics) and group 2 (vaccinated fish treated with probiotic) compared to the control group (P<0.05). In similar studies, the effect of probiotic administration on altering the intestinal bacterial flora and increasing lactic acid bacteria in the gut has been reported. Mohammadian et al, (2019) reported an increase in the proportion of lactic acid bacteria in the intestines of rainbow trout fed with a diet containing *Lactobacillus bulgaricus.* It is likely that the probiotic bacteria established in the gut, after proliferating, led to changes in the intestinal bacterial flora, replacing other bacteria, particularly Gram-negative bacteria, with beneficial lactic acid bacteria. A healthy gut microbiota can prevent colonization by pathogenic bacteria, reduce inflammation, and improve nutrient absorption, all of which contribute to a more effective immune response.

Overall, it can be concluded that the administering *L. plantarum* probiotic to the fish immunized with the *A. hydrophila* vaccine enhanced the efficacy of the vaccine and immunogenicity, as well as improved growth and health indices in common carp. Additionally, microencapsulation of this probiotic with alginate and chitosan microparticles significantly improved its positive effects on vaccine efficacy, growth indices, and fish health status. Therefore, this microencapsulation method is recommended for improving probiotic and vaccine efficacy and fish health. Further studies are suggested to refine the probiotic microencapsulation technique and its effect on the efficacy of highly demanded vaccines in aquaculture industry.

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Conflict of Interest

The authors declare no conflict of interest.

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The effect of quorum quenching probiotics on modulated digestive enzymes activity, growth performance, gut microfora and biochemical parameters in Common carp (*Cyprinus carpio*)

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Abstract

In this study, two main bacteria with probiotic ability (*Citrobacter freundii* and *Bacillus foraminis*) with autochthonous quorum quenching (QQ) were isolated from the intestine of *Cyprinus carpio* and their effects on growth performance, gut microbial flora, biochemical indices and digestive enzymes activities (i.e., α-amylase, lipase, trypsin, chymotrypsin, and alkaline phosphatase) of *C. carpio* were determined. Juveniles of *C. carpio* (n=450, weighing 50±10 g) were randomly divided into 6 equal groups (with 3 replications) and fed on diets containing 1×10^9 cfu g^{-1} of *C. freundii* (QQ1, G1), *B. foraminis* (QQ2, G2), *Lactiplantibacillus plantarum* (without characteristics QQ, WQQ, G3), QQ1 + QQ2 (G4), QQ1 + QQ2+WQQ (combine, G5), and a control diet (without probiotic) for 60 continuous days. Results showed that probiotic supplementations had generally significant effects on growth performance. The G5 and G3 had the best effect on specific growth rate (SGR) and feed utilization efficiency in *C. carpio* at days 30 and 60, respectively. The trypsin, protease, and chymotrypsin activities, on day 30 after feeding, significantly increased in G5 when compared with those in the control and the other groups. Significant changes in bacterial intestinal flora were observed in all probiotic groups compared with the control. These results highlighted the potential use of *Bacillus foraminis* (QQ2, G2) alone or in combination with other probiotics (G5) as additive in *C. carpio* diets but are not recommended in the long term. The results indicated that supplementation of isolated bacteria from the intestine of *C. carpio* (i.e., G3) can efficiently improve growth performance, intestinal microbiota and some digestive enzyme activities in juvenile *C. Carpio* in the long term culture. Therefore, it can be used as a growth enhancer like the commercial probiotics.

Key words: *C. carpio*, Intestine bacteria, Growth performance, Digestive enzyme activity, Microbial flora

Introduction

One of the most significant objectives of contemporary aquaculture is the substantial increase in production and the optimisation of profitability (Denev et al, 2009). Over the

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past few decades, the aquaculture industry has developed as a result of the use of a variety of fish species, the implementation of intensive culture systems, and the enhancement of the metabolic assimilation of dietary nutrients. The common carp is one of the most economically significant freshwater fish, typically reared in earthen ponds. The previous investigations have indicated that feed costs account for 84% of the total costs associated with the production of freshwater fish (Saravanan et al, 2021). Carp species can be produced even in lower quality waters, which is an especially valuable characteristic in the Asian and Middle East region. From the aspect of sustainability achieved in carp pond farms, it is essential to perform fish meal-independent and cereal-based fish meat production which provides increasing production of this fish species in the long run (Mohtashamipour et al, 2022). In this context, it has been reported that the highest cost paid for carp production in Iran is related to feed. Therefore, it is important to improve the feed effciency (FE) and decrease the total cost required for carp production per unit of surface (Mohammadian et al, 2024). The concept of functional feeds formulated by probiotics represents a novel approach within the aquaculture industry. The application of probiotics as an alternative to therapeutics in aquaculture is not a novel concept (Panigrahi et al, 2005). However, there has been a notable increase in interest in the safe and highly effective functions of such probiotics (Gatesoupe, 1999). A variety of microorganisms have been assessed as probiotics in aquatic animals (Mohammadian et al, 2024; Xia et al, 2019; Kuebutornye et al, 2019; Mirbakhsh et al, 2013; Faeed et al, 2016). However, quorum quenching bacteria (QQBs) represent the most prevalent probiotic used in aquaculture (Ghanei motlagh et al, 2021). It has been demonstrated that application of functional supplements such as probiotics can effciently improve growth performance, nutritional value and physiological responses of aquatic animals (Mohammadian et al, 2018). Incorporation of probiotics to diet can also improve the activity of digestive enzymes which in turn increase the absorption of nutrients from the gut and provide required energy for fish growth (Mohammadian et al, 2017). Likewise, use of probiotics is an eco-friendly and sustainable approach that reduces the use of harmful chemical compounds particularly antibiotics in aquaculture (Gatesoup,1999; Wang et al, 2008). The *Aeromonas hydrophila* is one of the most important gram-negative pathogenic bacteria in fish that causes hemorrhagic septicemia, ascites and mortality at different ages and in different species of fish (sha et al, 2005). There are several reports available regarding the beneficial influence of bacterial probiotics including live yeast, bifidobacters, *Lactobacillus*, feeding on growth performances and gut microbiota in some aquatic animals such as Shabot (*Tor grypus*) (Mohammadian et al, 2017), Asian Seabass (*Lates calcarifer*) (Mozanzadeh et al, 2023), European sea bass (*Dicentrarchus labrax*) (Tovar et al, 2002; Tovar-Ramırez et al, 2004), sea bream (*Sparus aurata*) (Suzer et al, 2008; Sáenz de Rodrigáñez et al, 2009), beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) (Askarian et al, 2008), Tilapia *(Oreochromis niloticus*) (Standen et al, 2013), Rainbow trout *(Oncorhynchus mykiss*) (Korkea-Aho et al, 2012; Ramos et al, 2013), *Salmo trutta caspius* (Mohammadian et al, 2022). Quorum sensing (QS) is a process by which bacteria monitor their population in a cell-density dependent manner through the synthesis, exchange, and the detection of small intracellular signals (autoinducers) (Xavier and Bassler, 2005). Among the various types of signals that are produced in bacteria (Autoinducer-1, Autoinducerprobably the best studied. Bacteria produces the QS molecules, and when the intensity of these signals reaches a threshold, they diffuse back into the bacterial cell and regulate the expression of QS-related genes such as those involved in biofilm formation and production of virulence determinants (Galloway et al, 2011). Moreover, the isolation of

autochthonous bacteria with QQ potential has frequently been reported from freshwater fish. On the other hand, despite the high mortalities caused by freshwater *Aeromonas*, QQ strategy has not been adopted against commonly occurring *Aeromonas* pathogens in fish, particularly, *C. carpio*, an adaptive freshwater fish with high economic importance that has gained much attention from researchers and farmers in the last decade. However, there is no comprehensive investigation of the real probiotic characterization of QQ bacteria prior to their administration to fish farm culture and their application on a commercial scale. There are little reports available regarding the beneficial influence of QQ bacteria including Bacillus, Citrobacter, Bifidobacter, feeding on growth performances and gut microbiota in some aquatic animals such as Rainbow trout *(Oncorhynchus mykiss*) (Torabi et al, 2019), Zebrafish *(Danio rerio*) (Chen et al, 2019), Asian Seabass (*Lates calcarifer*) (Ghanei-Motlagh et al, 2021). In the present study, QQ bacteria with a potential to degrade the dominant range of AHL molecules produced by several significant and prevalent pathogenic *Aeromonas* spp. in fish, were isolated from the intestine of common carp and characterized and their efficacy as autochthonous probiotics was tested for the first time. Due to the lack of information on QQ probiotic application in carp, the goal of this study was to evaluate the effects of two QQ, *Citrobacter freundii* and *Bacillus foraminis*, isolated from the intestine of *C. carpio* and a *Lactobacillus* without characteristics QQ *(Lactobacillus plantarum* WQQ) on growth indices, gut microbiota, and digestive enzymes (α-amylase, lipase, trypsin, chymotrypsin, and alkaline phosphatase) activities in juvenile Common carp (*C. carpio*).

Materials and Methods

Bacteria

Bacterial isolates were recovered using a previously described method (Irianto and Austin, 2002). Briefly, the entire digestive tracts of *C. Carpio* captured from natural water resources of Khuzestan province in Iran were removed and their contents were discarded. The quorum quenching potential of *C. freundii* QQ1 and *B. foraminis* QQ2 was confirmed in our previous study using the agar well diffusion and thin layer chromatography methods. In this study, their QQ activity was also tested against *Yersinia ruckerie* by the degradation assay on Luria-Bertani agar as suggested by Chu et al, (2011). The tested *Y. ruckerie* was able to induce *Chromobacterium violaceum* CV026. This biosensor respondes to exogenous AHLs with *N*-acyl side chains from C_4 to C_8 in length with production of purple pigment violacein. *Pseudomonas fluorescence* P3/pME6863 and *Pseudomonas fluorescence* P3/pME6000 were respectively used as positive and negative controls in AHL degradation assay. The strains CV026, P3/pME6863 and P3/pME6000 were kindly provided by Dr. Torabi Delshad. The *L. plantarum* strains used in this study as none QQ character were primarily identified based on colony and cell morphology, Gram staining, biochemical characteristics, and 16S rRNA gene sequencing (GenBank accession number EU520326 and EU520327) (Mohammadian et al, 2016). These strains were grown for 30 h at 37°C in MRS broth (BD Difco, Sparks, MD, USA).

Diet preparation

The control diet was formulated using the ingredients as subsequently described. The proximate analysis of the basal diet according to the AOAC method was: 37.1% for crude protein, 8.8% for crude lipid, 9.6% ash and 390 Kcal per 100 g for gross energy. Probiotic bacterial suspensions were prepared by centrifuging (15min., 4000 rpm) the 72h TSB cultured bacteria and resuspending them in PBS at the concentration of Macfarland grade 3 (1.2×10^9 cfu mL^{-1}). The probiotic-enriched diets were prepared by gently spraying of the prepared bacterial suspension on the control and mixing that part by part in a drum mixer to obtain a final probiotic concentration of 1×10^9 $cfug^{-1}$. They were packed in sterile propylene containers and stored at 4°C for viability studies for a week.

This dose was chosen based on a previously recommended dose (Takafoyan et al, 2024). Final concentrations of probiotic bacteria in the diet were confirmed by suspending one gram of food in sterile PBS and culturing the serial diluted food suspension in TSA media. Counted bacteria in the food were almost the same as added probiotic bacteria in all batches of probiotic-enriched diets.

Experimental design

Juveniles of *C. carpio* (50±10 g) (Mean±SD) were transferred from a private cyprinid farm in Khuzestan Province, Iran, to the Lab of Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz. The fish were acclimated for 2 weeks in indoor 300 L fiberglass tanks and were fed with a standard diet (37.1% crude protein, 8.8% crude lipid, 9.6% ash, and 390 Kcal $100g^{-1}$ gross energy). Then, after verifying the health status of the fish, they were distributed randomly into 12 tanks at an initial density of 25 fish per tank were then divided into 6 treatment groups, including control (n=25), QQ1 (G1, n=25), QQ2 (G2, n=25), *L. plantarum* (G3, n=25 as a whithout characteristics QQ), QQ1 + QQ2 (G4, n=25), QQ1 + QQ2+W QQ (G5, n=25). Final concentration of each probiotic was about 1×10^{9}cfu g^{-1} of the diet (Table 1) (Nikoskelainen *et al*., 2001). The aquaria were supplied with water from external Biofilteres (Athmann, China), at a temperature of 25.9±1.2˚C. The fish were fed with probiotic-contained diets for 60 days (twice a day). During the experimental period, the temperature ranged from 24.5 to 28.5°C, salinity was from 0.6±0.11 % and the dissolved oxygen was 5.9±1.3mgL^{-1}.

Table 1: The experimental design and treatment setting up, applied in this study.

Treatment	G1	G2	G3	G4	G5	Control
Probiotics category	QQ1	QQ2	*L. plantarum* (W QQ)	QQ1 + QQ2	QQ1 + QQ2 + W QQ	Normal saline
Additive quantity (g kg−1)	1×10^{9}	1×10^{9}	1×10^{9}	1×10^{9}	1×10^{9}	0.0

Sampling and analysis of biological parameters

In order to determine growth performance, the weight of all fish in each treatment was measured at the fiberglass tanks. The fish, then, were fed with a standard diet (37.1% crude protein, 8.8% crude lipid, 9.6% ash, and 390 Kcal $100g^{-1}$ gross energy). Growth performance at 30 and 60 days after the feeding was assessed in terms of Body Weight Growth (BWG), Specific Growth Rate (SGR), Food Efficiency Ratio (FER), Food Conversion Ratio (FCR) and Protein Efficiency Ratio (PER). The calculations were performed using the following formulae: BWG %= 100× (FBW–IBW)/ IBW, SGR%= 100× (lnŦFBW–lnIBW)/ days, FCR=feed consumed/ (FBW–IBW), FCE%=(FBW–IBW)/feed consumed×100, PER=IBW/protein intake. IBW is initial body weight, FBW is final body weight and days are days of feeding.

Digestive enzyme activity

To analyze the activity of digestive enzymes, on days 30, and 60 following probiotic feeding, the fish were starved for 24 h. Then, 3 fish were taken randomly. The intestine was dissected out under sterile conditions and at low temperature (around 4ºC, near icepack). After the samples were homogenized in a cold homogenizing buffer containing 50 mMTris–HCl, pH 8.0 (1:9v/w) followed by centrifugation (13.500 ×g; 30 min at 4°C). The supernatant was collected and kept at −80°C in small portions for later determinations (Rungruangsak-Torrissen et al, 2002; Rungruagsak-Torrissen and Fosseidengen, 2007). Total protein content of the supernatant was assayed according to a

Ŧ Natural logarithm having based 10

(Bradford, 1976) method using bovine serum albumin as standard. Trypsin activity was measured using N α -Benzoyl- L - arginine ethyl ester N α -Benzoyl- L - arginine ethyl ester (BAEE) as the substrate (Erlanger et al, 1961). Banzoyl-L-Tyrosine ethyl ester Ester (BTEE) was used as a substrate to determine enzyme activity of chymotrypsin (Hummel, 1959). ALP activity was kinetically measured using 4-nitrophenyl phosphate (PNPP) as substrate by a commercial kit (Pars Azmoon Co., Tehran, Iran). α-amylase activity was measured according to the modified Bernfeld method as described previously (Areekijseree et al, 2004) using starch solution as substrate. Amylase specific activity was expressed as μmol maltose produced h^{-1} mg $protein^{-1}$. Lipase activity was determined based on the measurement of fatty acids release due to enzymatic hydrolysis of triglycerides in stabilized emulsion of olive oil (Borlongan, 1990). Enzyme activities were measured as the change in absorbance using a spectrophotometer (UV-2802S; Unico, Shanghai, China) and expressed as specific activity (U mg^{1}protein) (Sun et al, 2012).

One of the objectives of this study was to select a suitable modified method for improving nutrient utilization. Feeding habit and metabolic flexibility of carbohydrate–protein utilizations to reach this objective was measured in probiotics fed on *C. carpio* according to Hofer and Schiemer (1981) and Thongprajukaew et al, (2011). To do this, α-mylase/Trypsin ratio was calculated for each treatment.

Intestinal bacterial community

The specimens of posterior intestine were aseptically removed and homogenized with sterilized PBS (1:10 w/v). The homogenates were serially diluted and 100 μL of tenfold diluted suspensions were spread on different agar media including tryptone soy agar (TSA), MRSA (de Man, Rogosa and Sharpe) and mannitol egg yolk polymyxin (MYP) in triplicates to determine the populations of total aerobic heterotrophic bacteria, LAB spp. and *Bacillus* spp. respectively. All plates were incubated for 48 h at 29 °C and the counted colonies were expressed as denary logarithm of colony forming units (CFU) per ml homogenized suspension (Ghanei-Motlagh et al, 2021b).

Statistical analysis

All statistical tests were performed using SPSS software (SPSS, Release 16.0, SPSS, Chicago, IL, USA). Two-way analysis of variance (ANOVA) and general linear model were used to evaluate the effect of time and treatments on each variable. One-way analysis of ANOVA was done to determine the differences between different variables. Differences were considered statistically significant when $P<0.05$ and the results are expressed as mean ± SD.

Result

Growth performance

The final weight, weight gain, daily weight gain, relative growth rate and specific growth rate of *C. carpio* significantly increased when they were fed with diets containing *B. foraminis* (Q2) *and* Combine group (Q1+Q2+WQ) at 30 days and *L. Planturum* (WQ) for 60 days (Table 2). The minimal FCR and higher protein efficiency ratio (PER) was observed in fish fed with dietary Combine group (Q1+Q2+WQ) and control group while the highest FCR was observed in the Q1 and Q1+Q2 fed groups at 30 days and the minimal FCR and higher PERwas observed in fish fed with dietary WQ group for 60 days (Table 2). The lowest protein efficiency ratio was found in the fish fed Q1+Q2 containing food at 30 days and Combine group (Q1+Q2+WQ) for 60 days.

Table 2: Growth performance of *C. carpio* fed either regular feed or feed supplemented with probiotics for 60 days

Parameters	Groups	Day 30	Day 60
IW	Q1	59.2 ± 1.4^{a}	70.1 ± 2.7^{b}
	Q2	65.96 ± 1.11^{a}	80.36 ± 3.52^{a}
	WQ	65.1 ± 2.52^{a}	78.3 ± 2.5^{ab}
	Q1+Q2	64.7 ± 2.2^{a}	75.3 ± 1.5^{ab}
	Q1+Q2+WQ	66.54 ± 1.32^{a}	81.73 ± 4.15^{a}
	Control	61.8 ± 2.22^{a}	69.36 ± 1.5^{b}
FW	Q1	70.1 ± 2.7^{b}	102.5 ± 8.7^{b}
	Q2	80.36 ± 3.52^{a}	104.07 ± 2.56^{b}
	WQ	78.3 ± 2.5^{ab}	119.43 ± 3.55^{a}
	Q1+Q2	75.3 ± 1.5^{ab}	105.5 ± 4.9^{b}
	Q1+Q2+WQ	81.73 ± 4.15^{a}	105.16 ± 5.55^{b}
	Control	69.36 ± 1.5^{b}	103.03 ± 3.9^{b}
FCR	Q1	$3.3\pm0.26^{b,A}$	$2.07\pm0.12^{a,B}$
	Q2	$2.9\pm0.11^{ab,B}$	$4.4\pm0.6^{b,A}$
	WQ	$2.7\pm0.32^{ab,A}$	$1.62\pm0.01^{a,B}$
	Q1+Q2	$3.6\pm0.2^{b,A}$	$2.21\pm0.25^{a,B}$
	Q1+Q2+WQ	$2.41\pm0.22^{a,B}$	$4.04\pm0.1^{b,A}$
	Control	$2.44\pm0.12^{a,A}$	$2.098\pm0.05^{a,A}$
SGR	Q1	$0.56\pm0.04^{ab,B}$	$1.26\pm0.1^{a,A}$
	Q2	$0.65\pm0.09^{a,A}$	$0.87\pm0.05^{b,A}$
	WQ	$0.61\pm0.02^{ab,B}$	$1.4\pm0.01^{a,A}$
	Q1+Q2	$0.51\pm0.06^{ab,B}$	$1.12\pm0.1^{a,A}$
	Q1+Q2+WQ	$0.68\pm0.02^{a,A}$	$0.85\pm0.01^{b,A}$
	Control	$0.43\pm0.06^{b,B}$	$1.01\pm0.01^{ab,A}$
PER	Q1	$0.93\pm0.16^{ab,B}$	$1.51\pm0.2^{a,A}$
	Q2	$1.07\pm0.11^{ab,A}$	$0.8\pm0.08^{b,A}$
	WQ	$1.14\pm0.22^{a,B}$	$1.93\pm0.12^{a,A}$
	Q1+Q2	$0.88\pm0.02^{b,B}$	$1.4\pm0.334^{a,A}$
	Q1+Q2+WQ	$1.33\pm0.15^{a,A}$	$0.97\pm0.02^{b,B}$
	Control	$1.28\pm0.12^{a,A}$	$1.13\pm0.134^{ab,A}$
DWG	Q1	$0.36\pm0.08^{b,B}$	$1.071\pm0.02^{ab,A}$
	Q2	$0.47\pm0.017^{ab,B}$	$0.87\pm0.01^{b,A}$
	WQ	$0.44\pm0.02^{ab,B}$	$1.37\pm0.02^{a,A}$
	Q1+Q2	$0.35\pm0.03^{b,B}$	$1.076\pm0.044^{ab,A}$
	Q1+Q2+WQ	$0.5\pm0.02^{a,A}$	$0.95\pm0.02^{b,A}$
	Control	$0.28\pm0.03^{b,A}$	$1.017\pm0.044^{ab,B}$
RGR	Q1	$18.6\pm0.25^{ab,B}$	$46.5\pm0.75^{a,A}$
	Q2	$21.15\pm0.61^{a,A}$	$29.16\pm0.26^{b,A}$
	WQ	$20.3\pm1.21^{a,B}$	$52.6\pm0.55^{a,A}$
	Q1+Q2	$16.1\pm0.37^{ab,B}$	$40.27\pm0.76^{ab,A}$
	Q1+Q2+WQ	$22.3\pm1.21^{a,A}$	$33.6\pm0.55^{b,A}$
	Control	$13.1\pm0.37^{b,B}$	$42.27\pm0.76^{ab,A}$
FER	Q1	$29.3\pm2.7^{b,B}$	$48.6\pm1.2^{ab,A}$
	Q2	$34.33\pm0.47^{b,A}$	$27.2\pm0.44^{b,A}$
	WQ	$36.7\pm0.8^{aa,B}$	$61.76\pm1.18^{a,A}$
	Q1+Q2	$28.12\pm0.09^{b,B}$	$47.08\pm3.02^{ab,A}$
	Q1+Q2+WQ	$42.7\pm0.8^{a,A}$	$31.76\pm1.18^{b,A}$
	Control	$41.12\pm0.09^{a,A}$	$49.08\pm3.02^{ab,A}$

* For each parameter, values (Mean ± SD) bearing different uppercase letters or different lowercase letters represent significant differences within each column or each row, respectively ($P<0.05$).

* Abbreviations: IW, Initial Weight; FW, Final Weight; %WG, FCR, Feed Conversion Ratio; SGR: Specific Growth Rate; DWG, Daily Weight Gain; PER, Protein Efficiency Ratio, RGR, Relative Growth Rate; FER, Feed Efficiency Ratio.

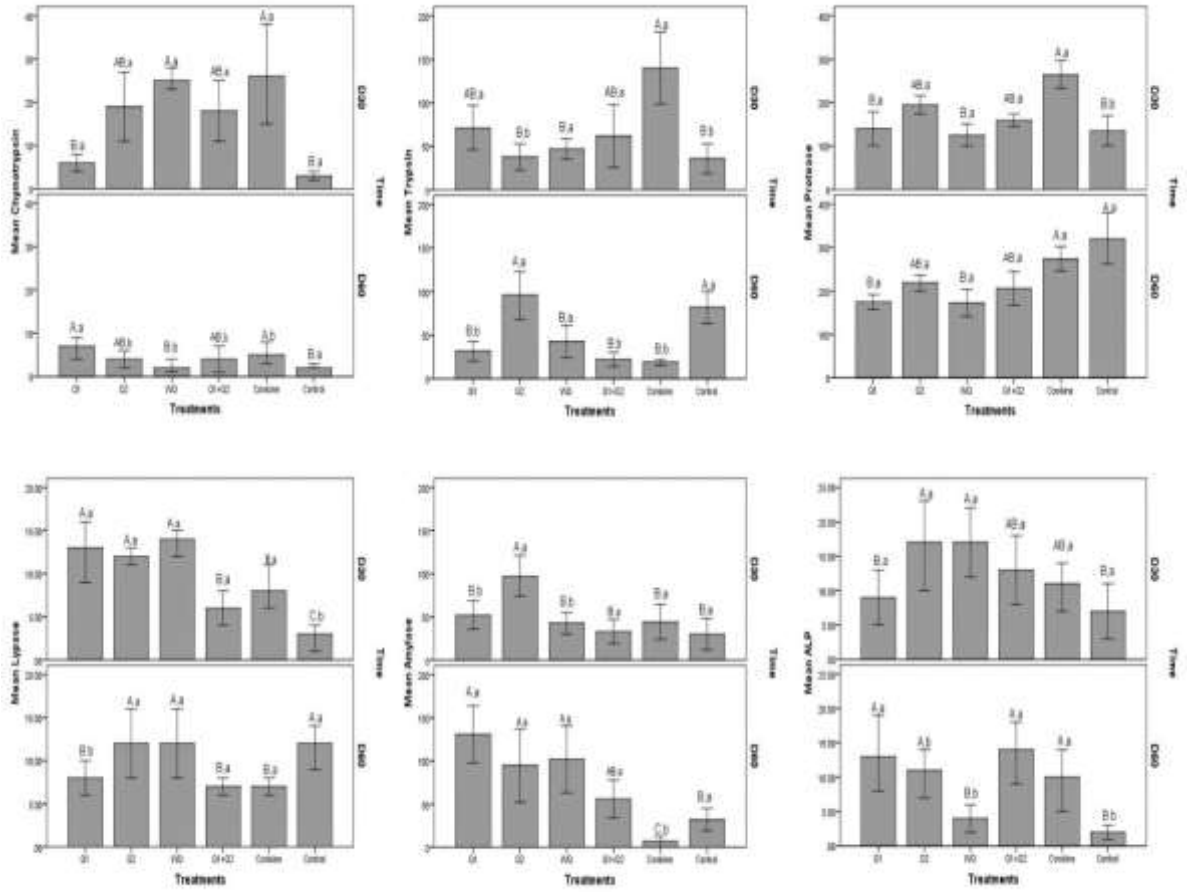

Figure 1: Digestive enzyme activity in *Cyprinus caroio* **treated with QQ1 (*C. freundii* ~1×10^9 cfu g^{-1}), QQ2 (*B. foraminis* ~1×10^9 cfu g^{-1}), WQQ (*Lactobacillus planturum* ~1×10^9 cfu g^{-1}), QQ1+QQ2 (*C. freundii* and *B. foraminis* ~1×10^9 cfu g^{-1}), QQ2 (*B. foraminis* ~1×10^9 cfu g^{-1}), QQ1+QQ2 +WQQ (*C. freundii* and *B. foraminis and Lactobacillus planturum* ~1×10^9 cfu g^{-1}) and without probiotic (control). Values is shown as means±standard error (n = 9). Legends as mentioned in Fig. 1.**
***For each parameter, values (Mean ± SD) bearing different lowercase letters or different uppercase letters represent significant differences within each column or each row, respectively (P<0.05).**

Digestive enzyme activity

The specific activity of trypsin was increased during the 30 days of treatment in Combine group (QQ1+QQ2+WQQ) group, while its activity declined at the end of the experiment. The highest specific activity of trypsin was determined in QQ2 group on the 60th day of the experiment (Figure 1). No significant differences were found among the other groups on days 30 or 60 of feeding (P>0.05).

The specific activity of α-amylase increased in all experimental groups during the 30 days of probiotic feeding and thereafter slightly increased (except combine group) until the end of the experiment. Its highest activity was found in QQ2 group on day 30 of the experiment (Figure 1). The highest was found in QQ1, QQ2 and WQQ groups on day 60 of the experiment (Figure 1).

The specific activity of ALP was significantly elevated on the 30th day of the feeding in G1 and G2 groups (P<0.05). The highest activity of ALP was observed on day 30 past the feeding in QQ2 and WQQ groups (Figure 1). We found changes in ALP activity of QQ1 group, 60 days after the probiotic feeding. The highest activity of ALP was observed on day 60 past the feeding in QQ1 and QQ1+QQ2 groups.

Chymotrypsin activity was influenced by probiotic administration, so that on the 30th day of the test, Combine group (Q1+Q2+WQ) and WQ groups had the highest chymotrypsin activity and a significant difference with the control group (P<0.05). The results on the 60th day of the feeding showed that all experimental groups had declined chymotrypsin activity.

Lipase activity had no significant (P>0.05) differences among the probiotic-fed groups during the feeding period (Figure 1). The highest activity of Lipase was observed on day 30 past the feeding in QQ1, QQ2 and WQQ groups (Figure 1). We found changes in Lipase activity of QQ2 and WQ group, 60 days after the probiotic feeding. Protease activity was influenced by probiotic administration, so that on the 30th day of the test, combine group had the highest Protease activity and a significant difference with the control group (P<0.05). The results on the 60th day of the feeding showed that all experimental groups had declined Protease activity except the control group.

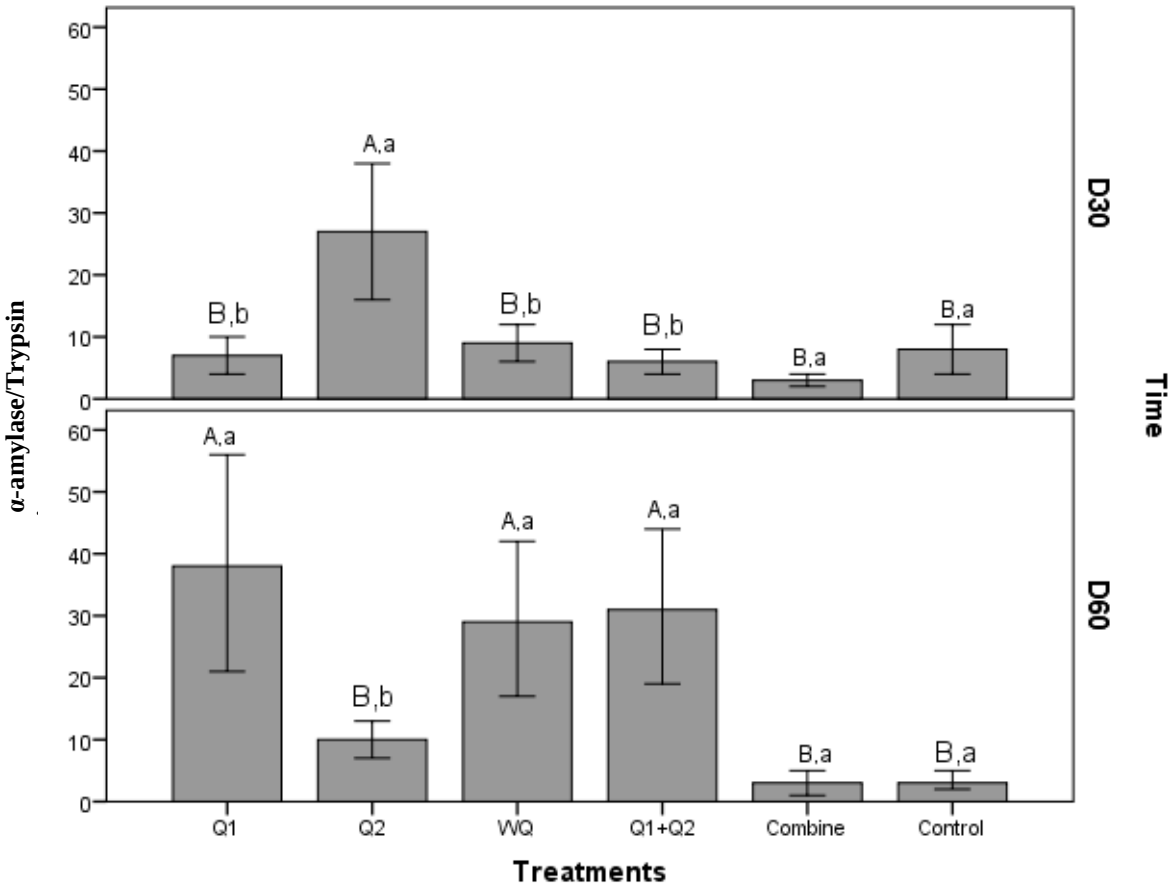

Figure 2: α-amylase/Trypsin ratio in *Cyprinus caroio* **treated with QQ1 (*C. freundii* ~1×10^9 cfu g^{-1}), QQ2 (*B. foraminis* ~1×10^9 cfu g^{-1}), WQQ (***Lactobacillus planturum*** ~1×10^9 cfu g^{-1}), QQ1+QQ2 (*C. freundii* and *B. foraminis* ~1×10^9 cfu g^{-1}), QQ2 (*B. foraminis* ~1×10^9 cfu g^{-1}), QQ1+QQ2 +WQQ (*C. freundii* and *B. foraminis* and *Lactobacillus planturum* ~1×10^9 cfu g^{-1}) and without probiotic (control). Values is shown as means±standard error (n = 9). Legends as mentioned in Fig. 1.**

***For each parameter, values (Mean ± SD) bearing different lowercase letters or different uppercase letters represent significant differences within each column or each row, respectively (P<0.05).**

The highest amylase to trypsin ratio (A/T ratio, P<0.05) was found in fish-fed QQ2 diet on the 30th day of the treatment. The highest activity of A/T ratio was observed

on day 60 past the feeding in QQ1, QQ1+QQ2 and WQQ groups (Figure 3) (Figure 2).

Microbiological assay

Before the probiotic feeding, the fish had low detectable lactobacilli level in the entire intestines. The viable count of LABs significantly increased in a time-dependent manner in the intestine of fish-fed WQQ and combine (QQ1+QQ2+WQQ) group probiotics-contained diets. Although fish-fed QQ2 supplemented diets increased viable counts 60 days after the probiotic feeding, the number of viable LABs in QQ1 was significantly reduced on the 60th day of the experiment (Table 3). On the 30th day of the experiment, the total count of bacteria was significantly increased in the control group compared to the other group ($P<0.05$). After 60 days of feeding with diets containing probiotic, no significant alteration in the total count of bacteria was observed between the groups ($P>0.05$). Moreover, *Bacillus* cultivable bacterial counts were found at significantly higher numbers in the groups fed with diets containing QQ2 and combine (Q1+Q2+WQ) group than in the control group at days 30 and 60 ($P<0.05$).

Table 3: Total viable counts (means ± SEM (n = 9)), total lactic acid bacteria (LAB) and *Bacillus* from the digestive tract of *C. carpio*.

Parameters	Groups	Day 30	Day 60
Total Count Bacteria ($\times10^5$ CFU/g)	Q1	176.6±53.5 ^{Aa}	156.3±47.5 ^{Aa}
	Q2	196.3±19.5 ^{Bb}	238.2±35.3 ^{Aa}
	WQ	169.0±27.16 ^{Aa}	206.0±66.1 ^{Aa}
	Q1+Q2	136.6±19.6 ^{Aa}	179.0±33.5 ^{Aa}
	Q1+Q2+WQ	180.0±22.64 ^{Aa}	194.0±25.5 ^{Aa}
	Control	246.0±19.6 ^{Aa}	253.0±33.5 ^{Aa}
Lactic Acid Bacteria ($\times10^2$ CFU/g)	Q1	54.0±7.63 ^{Ba}	46.0±33.5 ^{Ca}
	Q2	65.0±7.63 ^{Bb}	199.45±26.2 ^{Ba}
	WQ	233.0±1.15 ^{Aa}	268.66±3.2 ^{Aa}
	Q1+Q2	72.0±14.3 ^{Ba}	102.3±10.4 ^{BCa}
	Q1+Q2+WQ	237.0±10.3 ^{Aa}	276.33±10.4 ^{Aa}
	Control	18.0±10.3 ^{Ca}	14.33±10.4 ^{Ca}
Bacillus spp. ($\times10^4$ CFU/g)	Q1	ND	ND
	Q2	4.8 ± 0.11^{Ab}	13.31 ± 0.08^{Aa}
	WQ	ND	ND
	Q1+Q2	4.74 ± 0.1^{Aa}	3.77 ± 0.06^{Ba}
	Q1+Q2+WQ	4.8 ± 0.11^{,Ab}	8.81 ± 0.08^{Aa}
	Control	3.34 ± 0.1^{Aa}	5.87 ± 0.06^{Ba}

* ND: No detected. Different lowercase letters indicate statistically significant differences between each of the experimental groups at various sampling time points (row) ($P<0.05$). Different capital letters denote significant differences between the experimental groups at a specified time point (column) ($P<0.05$).

Discussion

Probiotics are microbes that have positive effects on fsh welfare when they are administered in adequate numbers in diet or water. Probiotics should be compatible with their target species to colonize the host's intestine and establish their positive effects (Mohammadian et al., 2019c). In this study, two autochthonous QQ bacteria (*C. ferundi* and *B. foraminis*) with probiotic action were isolated from the intestine of *C. carpio* and their effects on growth performance, gut microbial flora, biochemical indices. Digestive enzyme activity was determined during a 60-day feeding. We also compared their properties with a WQ LAB strain *(L. plantarum*). Our results showed that *B. foraminis* and Combine group improved the growth performance of *C. carpio* more effectively when compared with other tested group on

day 3o. The promotion of the growth rate, FER and PER in *B. foraminis* fed group occurred concomitantly with increasing protein turnover. *C. carpio* fed WQQ supplemented diets showed higher growth performance, SGR, RGR, PER, FCR and FER in relation to other experimental groups on day 60. This result was in agreement with those observed by Liu et al, (2013) who observed the highest growth performance in hybrid tilapia fed two different *Lactobacillus* strains and Reda et al. (2018) who observed the highest growth performance in African catfsh (*Clarias gariepinus*) fed with host-associated *B.cereus* 39HN compared to fish fed with a commercial probiotic (*B. amyloliquefaciens*). Different actions of probiotics on growth performance of treated fish, found in our experiment support the suggestion that each probiotic strain may interact with the host in a different way (Bomba et al, 2002; Sun et al, 2012; Mohammadian et al, 2017). It may also be explained by the greater adaptive capacity of *B. foraminis* in aquatic environments in comparison to *C. freundii* and *L. Plantarum* at short time culture (30 days). At this time of culture, it was also found that supplementation of food with *B. foraminis* could improve the feed utilization of *C. carpio* in higher rates than other probiotics. In the previous investigations, the positive effects of probiotics on growth performance have been attributed to a variety of factors., intestinal bacteria shared in the decomposition of nutrients, such as enzymes, minerals and vitamins, and thus, facilitate feed utilization, digestion and absorption. Growth indices such as PER, FCR and FER increased among *C. carpio* fed on a diet containing WQ (*L. Plantarum*) for 60 days. These findings are similar to that obtained by (Lin et al, 2017; Adorian et al, 2019; Mohammadian et al, 2022). It has been indicated that probiotics in feeds with a certain concentration display a growth promoting effect and can be beneficial for commercial fish production. In practical terms, this means that probiotic can decrease the amount of feed necessary for animal growth resulting in production cost reduction. Another probable reason of this difference can refer to autochthonous characteristic of *B. foraminis* which may provide higher digestible nutrients for the host and digests higher dietary protein/amino acids when compared to the allochthonous bacteria. Considering these findings, we concluded that different QQ bacteria especially *B. foraminis* isolated from intestine of *C. carpio* can improve the growth performance of cultured juvenile *C. carpio* when administrated as a food additive. Such probiotics are recommended to be used as a commercial growth promoter to facilitate extensive culture of *C. carpio* in future.

Probiotics by increasing digestive enzymes activity can enhance growth previously confirmed in different fish species, such as gilthead sea bream (*Sparus aurata*) (Suzer et al., 2008), silver pomfret (*Pampus argenteus*) (Gao et al, 2016), common carp (*C. carpio*) (Mohammadian et al, 2022), Rohu (*Labeo rohita*) (Saravanan et al, 2021) and as well as in olive flounder (*Paralichthys olivaceus*) (Ye et al., 2011). Administration of probiotics isolated from the gut of *C. carpio* had an effective action on the activities of different digestive enzymes. On day 30 of the probiotic feeding, the specific activities of lipase were higher in all probiotics-fed fishes in relation to the control group. In the current study, QQ2 and combine group had higher proteases and chymotrypsin and ALP activities than other groups, suggesting that using only bacillus with blend of all probiotic bacteria strains in the combine group had more stimulatory effect on digestive enzymes compare to those fed with QQ1. It seems that the presence of *B. foraminis* may be provided with wide range of stimulatory bioactive compounds or enzymes that enhanced enzyme activities in this group. In agreement with results from the previous studies (Francis et al, 2001;

Lara-Flores et al, 2010), we found higher ALP activity in *C. carpio* treated with QQ2 and WQQ containing diets for 30 days. The increase in the activity of AP reflects a possible development of brush border membranes of enterocytes that can be stimulated by the probiotics (Cuvier-Péres and Kestemont, 2001). Activities of this brush border enzyme have been reported to be indicators of the intensity of nutrient absorption in the enterocytes of fish (Gawlicka et al, 2000). High ALP activity also has been reported to be an indicator of carbohydrate and lipid absorption (Calhau et al, 2000; German et al, 2004; Lalles, 2019). Taken these findings, it has been concluded that higher growth performance rate in *C. carpio* fed probiotics may be due to the improvement of enterocytes function as well as better conversion and utilization of feed in brush border.

The trypsin, α-amylase, and chymotrypsin in the *C. carpio* digestive system may not only be secreted from ingested bacteria but may also be derived from indigenous origin, as in grouper (*Epinephelus coioides)* (Sun et al, 2012), barramundi (*Lates calcarifer*) (Siddik et al, 2022) and in beluga (*Huso huso*) (Askarian et al, 2008). Because gram-positive bacteria like members of the genus *Bacillus, Citribacter* and *Lactobacillus*, secrete a wide range of exoenzymes, the origin of enzymatic activities in fish-fed probiotics could not be distinguished (Moriarty, 1996; Moriarty, 1998; Suzer et al, 2008). It has been confirmed that relatively higher activities of digestive enzymes result in growth performance improvement. It is believed that probiotics influence digestive processes by enhancing the population of beneficial microorganisms, microbial enzyme activity, improving the intestinal microbial balance, consequently improving the digestibility and absorption of food and feed utilization (Mohapatra *et al*., 2012; Askarian *et al*., 2008). Our results were in agreement with the study in sea bass (*Dicentrarchus labrax*) larvae (Tovar-Ramírez *et al*., 2004), common carp (*Cyprinus carpio*) (Yanbo and Zirong, 2006), Indian white shrimp (*Fenneropenaeus indicus*) (Ziaei-Nejad et al, 2006), shrimp (*Litopenaeus vannamei*) (Wang, 2007), Skrodenyte-Arbaciauskiene (2007); gilthead sea bream (*Sparus aurata*, L.) (Suzer et al, 2008), grouper (*E. coioides*) (Sun et al, 2011), rainbow trout (*Oncorhynchus mykiss*) (Andani et al, 2012) Kuebutornye and Abarike (2019) demonstrating the improvement of survival rate, growth parameters, and digestive enzyme activities.

Regarding the above results, we encountered contradictory findings because α-amylase, lipase and ALP activities were generally lower in *C. carpio* in combine group than those in fish of QQ1, QQ2 and QQ1+QQ2 group at days 30, while combine group had the highest growth performance. On the other hand, Proteolytic activities (The trypsin, protease, and chymotrypsin), were consistent with the growth results. One possibility is that different probiotics in the diet may affect the gut microbiological and biochemical parameters independently (Balcázar and Rojas-Luna, 2007). According to these findings, it has been concluded that higher growth performance rate in *C. carpio* fed probiotics may be due to the improvement of protein turnover function as well as better conversion and utilization of feed in gut of omnivorous fish. Additional studies are required in order to clarify this hypothesis in detail. A/T ratio had no association with fish growth because it was at the highest level in QQ2 group, while the highest fish growth was observed in combine group at days 30 but in days 60, it was consistent with the growth results. The significantly higher growth performance and A/T ratio in WQQ groups respectively, may indicate higher energy requirement for protein utilization and growth in WQQ group than the other groups at long time culture (Thongprajukaew et al, 2011; Ghanei motlagh et al, 2022).

The gut microbiota can play an important role in the health and growth of the aquatic animals (Vine et al, 2004). Our results showed that feeding of *C. carpio* with diet containing *L. plantarum* could increase counts of viable LABs. These results were in agreement with the previous reports that probiotics have been used as growth promoters in Atlantic salmon and rainbow trout (Robertson et al, 2000), Tilapia (Ferguson et al, 2010), Rainbow trout (Merrifield et al, 2010) , Shrimp (Castex et al, 2008), zebrafish (Alavinejad et al, 2022; Zang et al, 2019) and Siberian sturgeon (Geraylou et al, 2013a; Geraylou et al, 2013b). Findings of this study clearly demonstrate that the probiotic-contained feed must be given to fish continuously to retain the probiotic-bacteria level in the gut. In our study when fish were fed on *L. plantarum* containing diets, changes were less apparent in the diversity of the microbiota in 60 days, compared to those in fish of QQ1 and QQ2 groups. In the present study, the highest numbers of LABs concomitant of the highest growth rate were found in the intestine of *C. carpio* fed *L. Plantarum* (WQQ). It suggests that the numbers of viable LABs are more efficient than enzyme activity in enhancing the growth parameters of *C. carpio*. To confirm this hypothesis we found that combine group could increase counts of the microbiota in the intestine compared to the control and other probiotic groups at days 30, but these changes were not translated into increased enzyme activity in the gut. Taking these findings into consideration, we concluded that feeding of *C. carpio* with combination of QQ1, QQ2 and *L. plantarum* may balance intestinal microbial flora resulting in improvement of food absorption and enhancement of growth performance at short time culture (Irianto and Austin, 2002).

In this study, isolation of *Bacillus* spp. from the intestine of common carp was possible after feeding with QQ2 probiotics for 4 weeks compared to the other groups in which no isolation occurred. Moreover, total cultivable bacterial counts were significantly lower in the probiotic groups than the control group ($P<0.05$). This could be associated with high survival of these strains within the gastrointestinal tract from which they isolated and their appropriate abilities to adhere on mucus as described in our previous study (Ghanei-Motlagh et al, 2020). After 60 days feeding with QQ2 probionts, significant alteration in populations of *Bucillus* spp. was observed between the treatments ($P>0.05$).

In conclusion, the ability of *B. foraminis* and some deal of *C. ferundi* isolated strains to augment growth performance and enzyme activity, colonize and modify the intestinal microbiota as a potential probiotic strains on day 30 were confirmed. But application of WQQ (*L. plantarum*) probiotics is recommended as supplementation for other cultural fish because the diet with these probiotic bacteria increases digestion, absorption of protein, and other nutrients in the gastrointestinal tract due to the increase of intestinal proteolytic enzyme activity. Finally we recommend a similar study be done on cultured shrimp species, because this industry needs to pay attention to health management.

The study suggests that incorporating QQ1, QQ2 and *Lactobacillus plantarum*, as dietary supplements in common carp feed can significantly enhance growth indicators and the food conversion ratio, making them viable candidates for use in aquaculture practices at short time culture.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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Effects of fish oil supplementation against formaldehyde-induced congenital skeletal anomalies in Wistar rats

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Abstract

It has been shown that formaldehyde (FA) as a common organic compound causes developmental anomalies and fetal defects. Fish oil is recommended for the normal growth and development of the fetus due to its fatty acid docosahexaenoic acid. The aim of this study was to investigate the potential of FO in protecting fetal growth and preventing skeletal anomalies caused by treatment of pregnant rats with FA. For this thirty pregnant Wistar rats were randomly categorized into five groups of control, sham (Normal saline; Orally and intraperitoneally), fish oil (0.5 mg/kg/bw; Orally), formaldehyde (10 mg/kg/bw; intraperitoneally), formaldehyde + fish oil. The treatment period was from day 0 to 20 of pregnancy. On the 20th day of gestation, the animals underwent anesthesia, and followed by a laparotomy to determine the weight and crown-rump length (CRL) of the fetuses. Skeletal stereomicroscopic assessments were conducted on the fetuses using the alizarin red / alcian blue staining method. Additionally, the expression of Runx2 and BMP4 was assessed through qPCR. The findings indicated that exposure to formaldehyde (FA) during prenatal development significantly reduced fetal weight and CRL, as well as the expression of Runx2 and BMP4 genes. Furthermore, FA heightened the occurrence of congenital skeletal abnormalities, including cleft palate, spina bifida, and non-ossification of fetal bones. Nevertheless, co-administration of fish oil (FO) with FA injection in pregnant rats improved fetal bone growth and mitigated skeletal anomalies. Fish oil demonstrated the potential to alleviate the teratogenic effects of exposure to formaldehyde by enhancing the expression of genes related to osteogenesis.

Key words: Congenital skeletal anomalies, Formaldehyde, Fish oil, Fetus, Rat

Introduction

Formaldehyde (FA) is a simple organic compound that belongs to the aldehyde family of compounds. It is the simplest member of this family, with the chemical formula CH_2O (El-Maghrabey et al, 2022). Low levels of formaldehyde can be detected in food, fruit and water. On the other hand, exogenous sources of formaldehyde include vehicle exhaust, power plants, fire and cigarette smoke, and photochemical

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oxidation of hydrocarbons (Zhang et al, 2021). Formaldehyde has a wide range of applications, including its use in the manufacture of building materials, such as particleboard, plywood, and fiberboard. It is also used in many household products, such as paints, adhesives, and cleaning agents (Gonzalez-Rivera et al, 2020). Furthermore, formaldehyde is used as a disinfectant and preservative in pathological laboratories, anatomy mortuaries, hospitals, and plastic industries (Soltanpour et al, 2022). Despite its many uses, exposure to formaldehyde at high levels can cause adverse health effects. The International Agency for Research on Cancer (IARC) and National Agencies have classified formaldehyde as carcinogenic to humans (group 1) (McGregor et al, 2006). Populations exposed to formaldehyde include not only adult workers who are occupationally at risk, but also the elderly, pregnant women, and young children (Lam et al, 2021). Recently, it has been found that the exposure of women to formaldehyde during pregnancy causes damage to the body of the pregnant woman and disrupts the development of the fetus (Zhang et al, 2021).

There is strong evidence that exposure to formaldehyde during pregnancy may be associated with an increased risk of birth defects in infants (Haffner et al, 2019). Studies have shown evidence of an association between formaldehyde exposure among pregnant women who worked in jobs such as embalmers, pathologists, or histology technicians, and an increased risk of miscarriage (Duong et al, 2011). Another study found that formaldehyde in pregnant women affects fetal development and causes congenital heart anomalies (Zhang et al, 2021). Exposure to formaldehyde during pregnancy can lead to oxidative stress in the fetus. Oxidative stress caused by formaldehyde can lead to adverse effects on fetal development, including impaired growth, developmental anomalies, and changes in gene expression (Pidoux et al, 2015). It has been suggested that exposure to formaldehyde during the first trimester of pregnancy can have the most adverse effects on fetal development and increase the possibility of congenital anomalies leading to spontaneous abortion (Thrasher and Kilburn, 2001). The results of studies have shown that the adverse effects of formaldehyde during pregnancy lead to a variety of congenital anomalies, including congenital heart disease, obstruction of the digestive tract, organ defects, and cleft palate, which ultimately lead to low birth weight and fetal death (Motoki et al, 2019). While these studies provide some evidence for a link between formaldehyde exposure and birth defects, they have not definitively established a causal relationship.

During pregnancy, the developing fetus needs significant amounts of omega-3 and other essential fatty acids to support the growth of rapidly dividing cells. These fatty acids are important for the development of the fetal brain, nervous system, and other organs and tissues (Nnamonu et al, 2020). Fish oil is recommended for the normal growth and development of the fetus due to its fatty acid docosahexaenoic acid (Massari et al, 2020). Docosahexaenoic acid is a family of long-chain polyunsaturated fatty acids (LC-PUFAs) or omega-3 fatty acids found in the phospholipid membrane of most cells. This fatty acid is one of the essential fatty acids that the body is not able to synthesize and must enter the body through diet (Roszkos et al, 2020). These fatty acids are transferred from the mother to the fetus through the placenta during pregnancy and are essential for fetal growth. Most reports indicate that a diet rich in these fatty acids during pregnancy prevents placental abruption, fetal growth retardation and preterm delivery (Basak et al, 2020). These fatty acids protect the tissue against free oxygen radicals by preventing lipid peroxidation. Fish oil with omega-3 fatty acids, such as eicosapentaenoic acid and docosahexaneoic acid, has antioxidant

properties and its consumption in rats reduces MDA activity (Gould et al, 2013).

PUFAs are important for cellular signaling, gene expression, and synthesis of second messengers that affect membrane function. They also act as ligands for transcription factors and other intracellular proteins, regulating gene expression, bone formation, and cellular product production (Basak et al, 2020). PUFAs are important for bone growth and development as they regulate inflammation in bone tissue, which is crucial for bone regeneration and repair. Additionally, PUFAs are known to enhance the absorption of calcium and other minerals essential for bone mineralization (Martyniak et al, 2021). They can also regulate osteoblast and osteoclast activity, affecting the equilibrium of bone formation and resorption. Adequate intake of long-chain PUFAs is therefore crucial for maintaining optimal bone health and preventing conditions such as osteoporosis (Bao et al, 2020).

Therefore, this study aimed to evaluate the effect of prenatal formaldehyde injection on fetal development and congenital skeletal anomalies and the protective role of fish oil against formaldehyde - induced developmental toxicity.

Materials and Methods

Study design

In this experimental study, 30 adults female Wistar rats and 10 adults male Wistar rats weighing 220± 20 g were used for the experiments. Animals were purchased from the authorized laboratory animal breeding center of the Faculty of Veterinary Medicine (Laboratory Animals House, Shahid Chamran University of Ahvaz, Ahvaz, Iran). The rats were kept in a controlled environment that was free of pathogens and maintained at a temperature of 22.00 ± 2.00 ˚C, with a relative humidity of 50.00 ± 10.00% and a light cycle of 12 h light/dark. Rats were fed with a standard pellet diet and had free access to water. All experimental assays were conducted in compliance with Guidelines for the Humane Care and Use of Laboratory Animals using protocols approved by the Shahid Chamran University of Ahvaz (EE/1401.2.24.204168/scu.ac.ir). Two weeks before the experiment, the rats were placed in the polypropylene rat cage to adapt to the experimental situation. Three female and one male adult rat were kept overnight in a cage for mating. The next morning when a vaginal plug was seen; so that day was considered as the zero day of pregnancy.

Animals grouping

A total number of 30 pregnant Wistar rats were randomly divided equally into five groups. Group 1 served as the control with no treatment. In group 2, sham group, normal saline was administered by gavage and IP. Group 3 was treated orally with fish oil at a dose of 0.5 mg/kg/bw via intragastric gavage (Albert et al., 2017). Group 4 received formaldehyde (Merck, Darmstadt, Germany) at a dose of 10 mg/kg/bw IP (Monfared, 2014). Group 5 received formaldehyde IP along with fish oil via intragastric gavage. The treatment period of the animals was from days 0 to 20 of gestation.

Fetus preparation and morphometric analysis

On the 20th day of gestation, the pregnant rats anesthetized through intraperitoneal injection of a ketamine-xylazine combination, followed by laparotomy. Subsequently, the uterus was dissected, and embryos were excised from the amniotic sac. The weight and Crown-rump length (CRL) as well as count of viable and absorbed fetuses were evaluated in both the right and left uterine horns. Thorough examination for visible congenital anomalies was performed.

Evaluation of the fetal skeletal anomalies

Typically, fetal skeleton staining involves using Alizarin Red S for bones and Alcian Blue for cartilage (Banaei et al., 2024). In the examination of congenital skeletal anomalies, the fetuses were skinned and soaked in acetone for one day, followed by 96% ethanol for seven to ten days. The specimens were then immersed in a solution containing 0.12% Alizarin Red S and 0.14% Alcian Blue in ethanol and glacial acetic acid. Subsequently, fetuses underwent maceration in 2.00% KOH, followed by clearing and hardening in a mixture of 1:1 glycerin and distilled water, and were finally stored in pure glycerin (Mahabady et al, 2016). The occurrence of congenital skeletal anomalies was meticulously assessed using a stereo-microscope. The evaluation of congenital skeletal anomalies, including cleft palate (CP), spina bifida (SB), fused ribs, non-ossification of the sternum (NO-St), delayed ossification of the forelimb (DO-FL), and delayed ossification of the hindlimb (DO-HL), was conducted using a stereomicroscope (Nikon, SMZ 800, Japan).

Expression of BMP4 and Runx2 genes

Total RNA isolation and cDNA synthesis

RNA extraction and cDNA synthesis procedures were conducted on fetuses located in the left uterine horn to evaluate the expression of BMP4 and Runx2 genes. For this study, total RNA was extracted (RNXTM) according to the manufacturer's protocol. The concentration of RNA was determined using a nanodrop device at 260 and 280 in 260/280 nm wavelength absorbance ratio. To assess RNA purity, the optical density ratio of 260/230 was photometrically checked, and samples with a ratio exceeding 1.8 were selected for cDNA synthesis. The synthesis of cDNA was carried out using 1 µg of total RNA following the instructions of the cDNA synthesis kit (Yekta Tajhiz, Iran). The GAPDH gene served as a calibrator for data analysis. The primers of BMP4, Runx2 and GAPDH genes were obtained from the NCBI database, and primer sets were designed using BatchPrimer3 software (BMC Bioinformatics, USA) and analyzed using nBLAST web browser (https://www.ncbi.nlm.nih.gov/geo/query/blast.html) to check the target template specificity (Table 1).

Table 1: List of primers employed for quantitative real-time RT-PCR in rat target genes.

Gene	Purpose	Forward primer (5′→3′)	Reverse primer (3′→5′)	Product (bp)	GenBank accession number
BMP-4	qPCR	GGAGTTTCCATCACGAA GAACATC	GAGATCACCTCATTCTCTG GGAT	124	NM_012827.2
Runx-2	qPCR	ACTCTGCCGAGCTACGA AAT	AAGTGAAACTCTTGCCTCG TC	105	XM_006244550.3
GAPDH	qPCR	AGTTCAACGGCACAGTC AAG	TACTCAGCACCAGCATCAC C	119	XM_017593963.1

Quantitative real-time RT-PCR

Real-time PCR reactions were carried out following the YTA SYBR Green qPCR Master Mix 2X kit protocol (Yekta Tazehiz, Iran). Each reaction, with a volume of 12.5 µl, comprised 6.25 µl of Syber Green qPCR Master Mix 2x, 0.25 µl of each primer at a final concentration of 200 nM, 3 µl of cDNA with a final concentration of 200 nM, and 2.75 µl of ddH2O. The temperature cycle involved an initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 15s. and annealing/extension at 60°C for 30 s. Result analysis was performed using LightCycler SW1.1 software, and calculations were based on the $2^{-\Delta\Delta Ct}$ formula.

Statistical analysis

Statistical analyses were conducted using the GraphPad Prism software (Graph Pad Software Inc. San Diego, California, USA). The findings regarding fetal lengths, weights, and the expression of BMP4 and Runx2 genes were presented as mean ± SD. A significance threshold of $P < 0.05$ was applied for statistical significance.

Results

Morphometry of fetal growth

The results of the cesarean section are depicted in Fig 1. The fetal weight and CRL in the FA group exhibited a significant decrease compared to the control and other groups ($p<0.05$). Co-administration of FO with FA significantly increased the weight and CRL of fetuses as compared to the FA group ($p<0.05$). The reduction in the weight and CRL of fetuses in the FA + FO group was significant compared to the control ($p<0.05$; Figure 1). No evident congenital anomalies were observed in the fetuses from the control, sham, FO, and FA + FO groups. In contrast, the FA group exhibited various apparent congenital anomalies, including subcutaneous hemorrhage at the neck and thorax, as well as the presence of undeveloped fetuses.

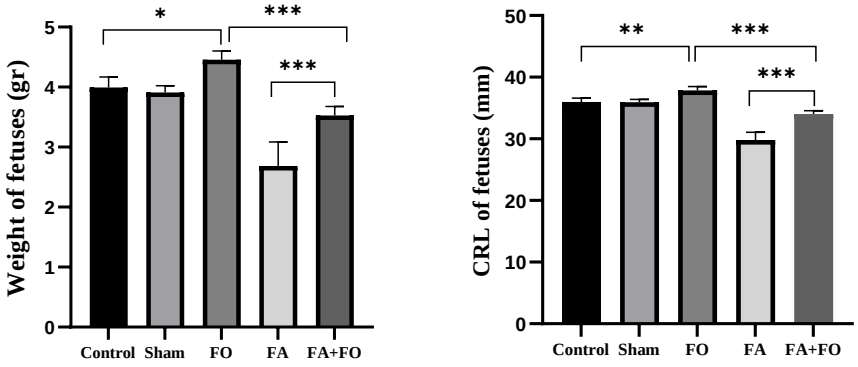

Figure 1: Mean ± standard deviation of fetal weight and crown-rump length (CRL) in different groups. FO: Fish Oil; FA: Formaldehyde. *p< 0.05, **p< 0.01, ***p< 0.001 indicates significant difference between groups.

Live and resorbed fetuses

The injection of formaldehyde into pregnant rats did not result in any mortality among the pregnant rats throughout the study duration. The total number of fetuses obtained from six mothers in the control, sham, FO, FA, and FA + FO groups was 75, 73, 78, 38, and 68, respectively. Examination of the percentage of these values revealed a decrease in the percentage of live fetuses in the FA group (65.78%) compared to the control group (93.33%). Additionally, the percentage of resorbed fetuses in the FA group (34.21%) increased in comparison to the control group (6.66%). While the percentage of these values in the FA + FO group was 88.23% and 11.76%, respectively, which indicated the positive effect of FO on improving the number of live and resorbed fetuses (Table 2).

Table 2: Total number (N) of live and resorbed fetuses, as well as the incidence (%) of fetal congenital anomalies in different groups

	Groups				
Variables	Control	Sham	FO	FA	FA + FO
NF	75	73	78	38	68
LF (%)	93.33	91.78	96.15	65.78 ***	88.23 *
RF (%)	6.66	8.21	3.84	34.21 ***	11.76 *
CP (%)	-	-	-	36.84 ***	10.29 **
SB (%)	-	-	-	21.05 ***	4.41 *
FR (%)	-	-	-	39.47 ***	7.35 **
NO-St (%)	5.33	5.47	2.56	44.73 ***	11.76 *
DO-FL (%)	-	-	-	31.57 ***	5.88 **
DO-HL (%)	-	-	-	34.21 ***	8.82 **

*p< 0.05, **p< 0.01, ***p< 0.001 indicates significant changes between groups. FO: Fish Oil; FA: Formaldehyde. NF: number of fetuses, LF: live fetuses, RF: resorbed fetuses, CP: cleft palate, SB: spina bifida, FR: fused ribs, NO-St: non-ossification of the sternum, DO-FL: delayed ossification of the forelimb, DO-HL: delayed ossification of the hindlimb.

Congenital skeletal anomalies

As presented in Table 2, congenital skeletal anomalies such as SB, CP, FR, DO-FL, and DO-HL were not detected in the control, sham, and FO groups. In contrast, the FA group exhibited a notable prevalence of congenital skeletal anomalies, including CP, SB, FR, NO-St, DO-FL, and DO-HL was observed. Administration of FO along with FA injection to pregnant rats reduced congenital skeletal anomalies compared to the FA group. However, the percentage of congenital skeletal anomalies of SB, CP, FR, NO-St, DO-FL, and DO-HL in the FA + FO group increased compared to the control group (Table 2, Figures 2 and 3).

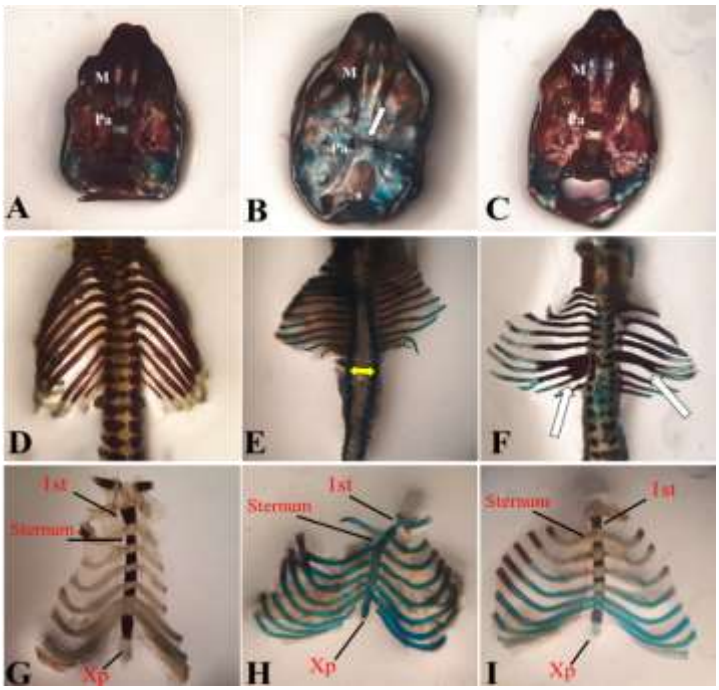

Figure 2: The ventral view of the fetal skull and the dorsal view of the vertebral column of Wistar rat fetus stained with Alizarin red S and Alcian blue. A) Normal palatine bone in the control group; B) Formaldehyde-induced cleft palate (white arrow); C) Normal palatine bone in formaldehyde + fish oil group. D) Normal vertebral column and ribs (control group); E) Spina bifida (yellow arrow) induced by formaldehyde. F) Fused ribs (white arrow) induced by formaldehyde. G) Normal sternum in the control group; H) Sternum with non-ossification of all sternebrae in the formaldehyde group; I) Normal sternum in the formaldehyde + fish oil group, (M: Maxilla; Pa: Palatine; Xp: Xiphoid process; 1st: First sternebra).

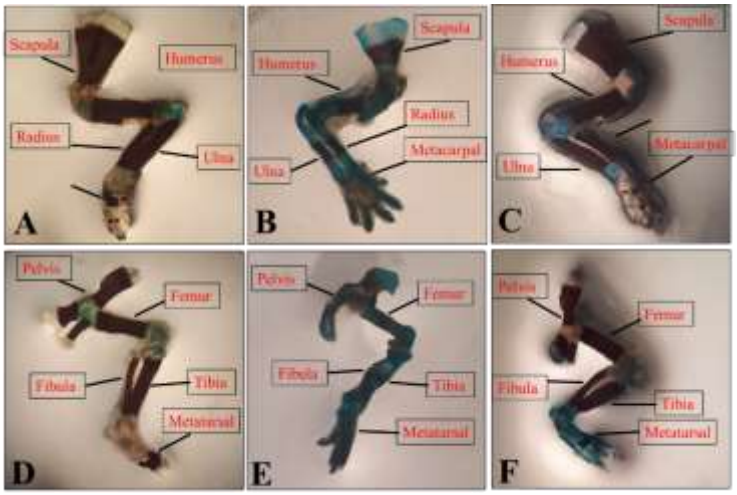

Figure 3: Lateral view of forelimbs and hindlimbs of Wistar rat fetus, stained with Alizarin red S and Alcian blue. A) Normal forelimb in the control group; B) Delay ossification of forelimb in the formaldehyde group; C) Normal forelimb in formaldehyde + fish oil group. D) Normal hindlimb in the control group; E) Delay ossification of hindlimb in the formaldehyde group; F) Normal hindlimb in formaldehyde + fish oil group.

Expression of osteogenesis-related genes

The outcomes of analyzing the expression of BMP-4 and Runx2 genes in various groups are depicted in Fig 4. The expression of BMP-4 and Runx2 genes in fetuses from the FA group exhibited a significant decrease compared to the control group ($P<0.05$). Co-administration of FO with FA significantly elevated the expression of BMP-4 and Runx2 genes in comparison to the FO group ($P<0.05$). No significant difference in these values was observed between the FA + FO and control groups (Figure 4; $P>0.05$).

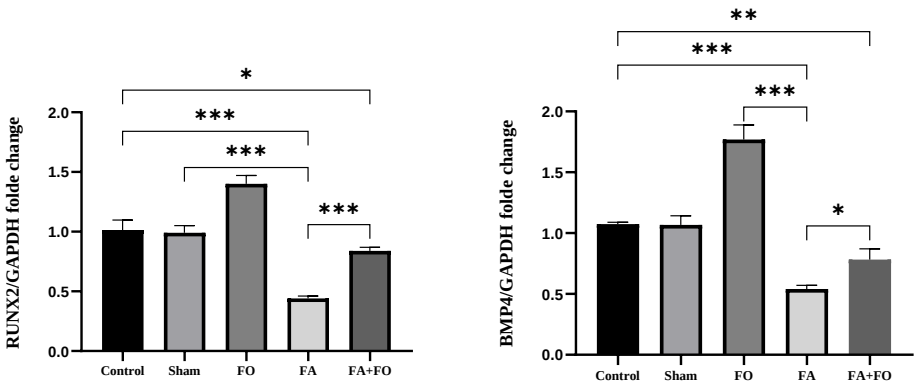

Figure 4: The expression of BMP4 and Runx2 genes in control and treatment groups. FO: Fish Oil; FA: Formaldehyde. *p< 0.05, **p< 0.01, ***p< 0.001 indicates significant difference between groups.

Discussion

Formaldehyde is a highly useful chemical compound that has a wide range of industrial and medical applications. However, exposure to formaldehyde at high levels is known to carry a series of health risks to individuals (Zhang et al, 2020). There have been limited studies on the effect of formaldehyde on the

morphometric and skeletal structure of the fetus in pregnant rats, but no study has been done on the effect of fish oil and its protective effects against the destructive properties of formaldehyde. Therefore, the present study determined the protective role of fish oil against developmental toxicity caused by treatment with formaldehyde on fetal morphometry and skeletal system. The findings of this study suggest that formaldehyde injecting into pregnant rats throughout days 0 to 20 of pregnancy disrupts the skeletal development of rat fetuses. This disruption is evident in the diminished expression of osteogenesis-related genes, ultimately leading to shortened body length, reduced skeletal area, and an increase in congenital skeletal anomalies. Furthermore, the oral administration of fish oil to pregnant rats treated with formaldehyde resulted in enhanced fetal growth and reduced congenital skeletal anomalies by upregulating the expression of osteogenesis-related genes in rat fetuses.

In prenatal developmental toxicity studies, the evaluation of the fetus is a critical aspect of the study. This evaluation includes the assessment of external, visceral, and skeletal endpoints. External endpoints include examination of the fetal body for the presence of any external apparent anomalies. This can include an examination of the fetal head, eyes, limbs, and other areas for any visible signs of defects (Hougaard et al, 2015). Skeletal endpoints involve the examination of the fetal bones and joints for any anomalies or malformations that may be present. It may include an evaluation of the skull, spine, limbs, and other areas for any structural or functional deficiencies (Rodríguez and Mandalunis, 2018). Prenatal developmental toxicity studies are typically conducted in laboratory animals such as rats to determine the harmful effects a chemical may have on fetal development. These studies help identify any morphological, physiological, and functional abnormality in the fetus, as well as anomalies in the external, visceral, skeletal, or reproductive systems. Chemical substances have the potential to cross the placenta and enter the developing fetus, which may interfere with the normal growth and development of the fetus (Scialli et al, 2018). It has recently been shown that maternal exposure to environmental chemical pollutants is the second leading cause of infant mortality in developing countries (Basal et al, 2020).

The developing fetus is highly vulnerable to the harmful effects of environmental toxins, and exposure to formaldehyde during pregnancy can have adverse effects on fetal growth and development (Webb et al, 2014). Formaldehyde can easily penetrate the placental barrier and cause fetal defects in humans and various animal species (Pidoux et al, 2015; Franklin et al, 2019). Studies have shown that formaldehyde exposure during pregnancy can lead to a range of developmental anomalies and fetal defects, including craniofacial defects, neural tube defects, and limb anomalies (Tung and Winn, 2011). Furthermore, individuals may vary in their susceptibility to the harmful effects of formaldehyde exposure, with some populations, such as pregnant women, children, and the elderly, being more vulnerable than others (Herrero et al, 2022). The findings of the current study showed that formaldehyde decreases fetal weight and CRL index in rat fetuses. These changes were probably associated with skeletal degeneration. A developmental toxicity study in FA-exposed rats showed that exposure to FA for 6 h/day from days 6 to 20 of pregnancy had reduced fetal body weight. However, they did not observe a significant change in the average implantation site, average embryo loss, average absorption sites, average live embryo and embryo sex ratio in FA-exposed rats (Nielsen et al, 2013). Another study was conducted on rats to determine the developmental toxicity of formaldehyde. The rats were exposed to 2,

5 or 10 ppm formaldehyde for six hours a day from the 6th to the 15th day of pregnancy. The results of the study showed that exposure to formaldehyde had no adverse effects on live fetuses, dead fetuses and resorptions, fetal weight, mortality before and after implantation. However, this study showed that the incidence of reduced ossification of the pubic and ischial bones was significantly higher in the 5 and 10 ppm treated groups compared to the control group (Martin, 1990). In addition, embryo culture studies have shown that formaldehyde can cause severe embryotoxicity, including death (Hansen et al, 2005). However, the absence of these effects in trials using doses up to 40 ppm suggests that FA does not enter the foetus through inhalation (Nielsen et al, 2013).

One of the mechanisms of the toxic effects of formaldehyde is the production of reactive oxygen species. It has been shown that formaldehyde can stimulate the production of ROS in cells, which can lead to oxidative stress and cellular damage (Zerin et al, 2015). Indeed, exposure to formaldehyde and the resulting oxidative stress are cytotoxic, which can lead to developmental toxicity in the fetus. Formaldehyde can also disrupt fetal DNA synthesis and repair, leading to genetic damage that can manifest as developmental anomalies or other health problems later in life (Zhang et al, 2021). In men, oxidative stress caused by environmental chemical agents may cause suppression of spermatogenesis, decreased testosterone levels, and apoptosis in the testis (Khazaeel et al, 2022). In females, congenital anomalies and reproductive effects can occur due to environmental factors and chemicals, depending on the dosage and timing during pregnancy. This is often attributed to the disruption of the equilibrium between the production of reactive oxygen species and the body's antioxidant defenses (Laforgia et al, 2018). The expression of Runx2 and BMP4 genes, which play important roles in the regulation of bone development and growth, can be affected by oxidative stress (Sadeghi et al, 2023; Rai et al, 2022) The synchronized functioning of BMP-4 and Runx2 is crucial for the process of ossification in the developing fetus. BMP-4, a protein with a pivotal role, stimulates the differentiation of cells into osteoblast cells, responsible for bone matrix production. Meanwhile, Runx2 regulates gene expression necessary for bone formation. A decline in Runx2 expression hinders cell differentiation into osteoblasts, thereby impairing bone formation (Rahman et al, 2015) The present study reveals that prenatal exposure to formaldehyde (FA) has the potential to diminish the expression of BMP-4 and Runx2 genes in the fetus, resulting in abnormal skeletal growth and skeletal malformations. Other studies have shown that oxidative stress can downregulate the expression of Runx2 and BMP4 genes, leading to decreased bone formation and growth (Lee et al, 2021). Oxidative stress inhibits Runx2 and BMP4 signaling pathways and DNA epigenetic changes lead to the inactivation of transcription factors that regulate the expression of Runx2 and BMP4 genes (Zhu et al, 2018).

Omega-3 fatty acids are a type of polyunsaturated fatty acid that cannot be synthesized by the body and must be obtained through the diet. Fish oil contains high levels of omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid, which are essential fatty acids required by the human body for normal growth and development.(Ghotbeddin et al, 2022). Studies have shown that omega-3 fatty acids, including EPA and DHA, increase the expression of genes that code for antioxidant enzymes such as glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) in fetal cells and tissues (Garrel et al, 2012; Shabani et al, 2019). These antioxidant genes encode enzymes and other proteins that reduce oxidative stress and protect cells from ROS-induced

damage. In addition to increasing the expression of antioxidant genes, it has been reported that omega-3 fatty acids enhance the osteogenic differentiation of mesenchymal stem cells (MSCs) through signaling activation (Levental et al, 2017). The underlying mechanisms involved in the osteogenic effects of omega-3 fatty acids are thought to involve several signaling pathways, including the Wnt/beta-catenin and PI3K/Akt pathways. These pathways are involved in regulating osteoblast differentiation, proliferation, and mineralization, and can be activated by omega-3 fatty acids to promote bone formation (Sadeghi et al, 2023; Sharma and Mandal, 2020). This study showed that fish oil has beneficial effects on the congenital skeletal anomalies caused by prenatal exposure to formaldehyde in rat fetuses. Following the administration of fish oil to formaldehyde-treated rats, the weight, CRL and number of viable fetuses were significantly increased compared to formaldehyde-treated rats. Moreover, the administration of fish oil to formaldehyde-exposed fetuses significantly reduced the number of congenital anomalies. In line with our findings, recently Shrestha et al showed that the administration of Omega-6 and Omega-3 fatty acids in rat fetuses improves the growth, development and the overall fetal health and prevent preterm labor in rats (Shrestha et al, 2020). The study by Zararsiz et al demonstrated that omega-3 fatty acids reduced formaldehyde-induced neurotoxic effects, maintained the levels of SOD, GSH-Px and MDA enzymes, and reduced formaldehyde-induced cell damage, indicating the potential of their neuroprotection against formaldehyde toxicity (Zararsiz et al, 2006). Research indicates that antioxidants like vitamin E can counteract formaldehyde's toxicity. Antioxidants scavenge reactive oxygen species generated by formaldehyde, mitigating oxidative stress, apoptosis and cellular damage. This suggests a potential protective role for antioxidants against formaldehyde-induced toxicity, expanding therapeutic avenues for mitigating environmental toxin exposure (Wu et al, 2017; Bakar et al, 2015). In addition, other studies have shown that in rat fetuses, the administration of fatty acids increases the weight, length and head circumference of the fetus, as well as the gestational age and fetal viability. Also, these studies have shown that Omega-3 fatty acids during pregnancy is associated with reducing the risk of fetal congenital anomalies (Akerele and Cheema, 2016). This shows that adequate consumption of fatty acids during pregnancy is important for the optimal growth and development of the fetus.

In conclusion, the findings of the current study demonstrated the effects of fish oil administration on the elimination of formaldehyde-induced teratogenicity for the first time. Prenatal exposure of rat fetuses to formaldehyde disrupts skeletal development and an increase in congenital anomalies including spina bifida, cleft palate, fused ribs, non-ossification of the sternum, delayed ossification of the forelimb and hindlimb in rats. These congenital skeletal anomalies were associated with a decrease in the expression of Runx2 and BMP4. Moreover, the study highlights that the antioxidant properties of fish oil alleviate the teratogenic effects of prenatal formaldehyde exposure by enhancing the expression of osteogenesis-related genes and preventing formaldehyde-induced oxidative stress.

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Conflict of interest

The authors declared no conflict of interest.

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Morphologic and morphometric study of the lumbosacral vertebrae in guinea pig (*Cavia porcellus*) based on CT scan images

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Abstract

Computed tomography (CT) is an accurate diagnostic imaging technique used to evaluate the vertebral column in exotic and small animals. The present study aimed to investigate the morphology and morphometric of the normal lumbosacral vertebrae in guinea pigs (Cavia porcellus) using CT scan images. This cross-sectional descriptive study utilized 10 healthy adult guinea pigs (*Cavia Porcellus*) (5 males and 5 females) with a mean age of 12 ± 1.20 months and an average weight of 1.04 ± 0.15 kg. Following anesthetization with a cocktail of xylazine (4 mg/kg) and ketamine (60 mg/kg), CT scans of the lumbosacral vertebrae were performed in the sagittal, transverse, and dorsal planes, from the cranial part of the first lumbar vertebra to the caudal extremity of the sacrum. Based on the results of this study, all parts of the lumbosacral vertebrae and intervertebral joints of guinea pig (*Cavia porcellus*) can be observed and evaluated in computed tomography images. The spinous process of the lumbar vertebrae in the sagittal plane and the cranial and caudal articular processes in the sagittal and transverse reconstruction planes were more identifiable. The mammillary processes and the cranial and caudal vertebral notches were better observed in the dorsal plan. Two lateral recesses were visible in the caudal vertebral foramina of L_6 at the junction of the pedicle and the vertebral body, a feature reported here for the first time. The interarcuate spaces of guinea pig lumbar vertebrae were very narrow, but this space was wide and large between the L_6 and S_1 vertebrae. For epidural anesthesia, surgeons can perform cerebrospinal fluid puncture and anesthetic drugs injection from this location. In this study, morphometric measurements of different parts of the lumbosacral vertebrae were subjected to statistical analysis. The results of this research can be employed in teaching computed tomographic anatomy of lumbosacral vertebrae, interpretation of CT scan images, as well as in clinical and treatment decisions of guinea pig (*Cavia porcellus*).

Key words: Computed tomography, Guinea pig (*Cavia porcellus*), Lumbosacral vertebrae, Morphology, Morphometric

Introduction

Guinea pigs are mammals from the order of rodents and the family of *Caviidae*. Currently, 13 species of these rodents have been identified. The most common breeds are the American, Abyssinian and Peruvian (Pignon and Mayer, 2020). All types of guinea pigs are social animals and tend to live in groups. Domestic species of guinea pigs (*Cavia porcellus*) are not found in nature (Zipser et al, 2014). This rodent is considered as a popular pet as well as a valuable laboratory animal. The guinea pig

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was the first animal used in medical research, and its name is synonymous with the experimental animal (Mähler et al, 2014).

The skeleton of the guinea pig is almost entirely bony, divided into axial and appendicular portions. The axial skeleton comprises the skull, vertebral column, ribs, and sternum, while the appendicular skeleton consists of the limbs and limb girdles. The vertebral column is composed of approximately thirty-seven vertebrae, which are separated from one another by intervertebral discs. The guinea pig (*Cavia porcellus*) has 7 cervical, 13 thoracic, 6 lumbar, 4 sacral and 5-7 caudal vertebrae (Barbera et al., 2019). A typical vertebra consists of a body, an arch and processes. The vertebral arch comprises of right and left pedicles and laminae which form the lateral walls and roof of the vertebral canal. Vertebrae are connected to each other through intervertebral joints which are of the diarthrodial type and do not contain joint fluid (Moarabi et al, 2024).

The lumbosacral vertebrae of guinea pigs (*Cavia porcellus*) are among the most complex vertebrae in the spine due to their multiple functions. Complications that may be observed include dysplasia, spondylosis, herniation and mineralization of the intervertebral disc, spinal canal stenosis, fracture, luxation, arthrosis, arthritis and neoplasia (Proks et al, 2021; Shomer et al, 2015). These vertebrae may have complications such as the hemivertebrae, wedge vertebrae, symmetrical hypoplasia, transitional and butterfly vertebrae, spina bifida, herniation and mineralization of the intervertebral disc, spinal canal stenosis, fracture, luxation, arthrosis, arthritis and neoplasias (Segal et al, 2018; Munif et al, 2023).

Various imaging techniques can be useful in diagnosing these injuries. DaCosta and Samii (2010) reported that CT can be a suitable method for evaluating spinal diseases in exotic and small animals. Additionally, CT is preferable to myelography because it is a non-invasive method. Furthermore, they have stated that although the diagnostic sensitivity of MRI is higher than CT, this method cannot be used in cases such as injuries caused by gunshots to the vertebral column. Therefore, in such cases, CT scan will be considered as a suitable selection method (Da Costa and Samii, 2010).

Witkowska et al, (2014), by examining the micro-computed tomography (micro CT) of guinea pig bones, have described some of its bone characteristics and species differences. Proks et al, (2021), by conducting a radioanatomical study on the vertebral column of 240 guinea pigs, stated that 12.5% of them had congenital anomalies related to the vertebral column, with the cervical and lumbosacral vertebrae having the least and the most complications, respectively. In a study, Soroori et al, (2022) reported that there was no significant difference in the height of the extremity plate of lumbar and coccyx vertebrae based on a morphometric evaluation of rabbit lumbosacral vertebrae using the CT method. However, other parameters such as height of the vertebral body and spinous process, length of transverse process and vertebral body were significantly different. Furthermore, they stated that the depth of the spinal canal was uniform from the first lumbar vertebra to the third sacral bone, decreased in the fourth sacral vertebra and remained constant until the second coccygeal vertebra.

McDougall et al, (2009) investigated the relationship between back pain and pathology of intervertebral discs by performing micro-CT of the vertebral column of young (2-5 months) and old (17-37 months) guinea pigs. According to the study, micro CT findings are consistent with histopathology results, and the clinical symptom of pain is a poor predictor for evaluating the degree of degeneration and damage of vertebrae and intervertebral discs. In this context, micro CT indications have greater diagnostic accuracy. Since the

guinea pig (*Cavia porcellus*) is used in a wide range of biomedical research, it is necessary to have accurate knowledge of its tissues. The computed tomography images of different tissues of guinea pig can provide valuable information to researchers (Carrera et al, 2022; Wills et al, 2021). Assessment of the tomographic features of the lumbosacral vertebrae of guinea pig (*Cavia porcellus*) can be beneficial in identifying anatomical characteristics and conducting pathological examinations. Therefore, it is necessary to accurately evaluate the normal anatomical, morphological and morphometric details of these vertebrae.

The accurate anatomical information is essential for safe intervention in the rodent lumbosacral region, and knowledge of the morphometry of this region will help reduce surgical risks and complications. Since the surgical techniques of the vertebral column in guinea pigs and/or rodents involve the utilization of bony anatomical landmarks, the morphometric data of the various parts of the vertebrae are essential (Pignon and Mayer, 2020). The precise anatomical dimensional knowledge is important to understand the etiopathogenesis of the complications and disorders of the lumbosacral region (Liau et al, 2017). The bony landmarks like the transverse and spinous processes, body length and height are important during the internal fixation of the lumbar spine (Sun et al, 2022). The anatomical studies help in understanding the detail complex morphometric of the vertebral column. There is an assumption that the morphometry of the vertebrae play a role in degenerative diseases of the vertebral column. The measurements are essential while choosing a suitable implant and this may avoid the intervertebral space exceeding and subsequent injury of the blood vessels. In addition, these studies provide necessary information to designers and manufacturers of spinal devices (Martz et al, 1997). Also, complications such as hemivertebrae, wedge-shaped vertebrae, symmetrical hypoplasia, butterfly vertebrae, transitional vertebrae, and hypoplasia/aplasia of the caudal articular process can cause mild to severe deformity of the lumbosacral vertebrae of the guinea pig. Sometimes, these mild deformities are difficult to detect in the lumbosacral vertebrae (Proks et al, 2015). In order to reach a definite diagnosis, it is necessary to have a normal range of the sizes of these vertebrae to compare suspicious cases with them.

Currently, radioanatomical studies of the lumbosacral vertebrae of guinea pigs (*Cavia porcellus*) are limited and there are no documented and detailed reports in this field. Therefore, this specific study was conducted on these vertebrae, investigating and determining their different parts and normal values.

Material and methods

Study design and animals

This cross-sectional descriptive study utilized 10 healthy adult guinea pigs (*Cavia Porcellus*) (5 males and 5 females). The mean weights of males and females were 1147±80 and 921±58 grams, with average ages of 12±1.01 and 12±0.94 months, respectively. Throughout the study, these animals received proper nutrition and were kept in an isolated room with a temperature of 25°C, relative humidity of 65%, and a 12-hour light/dark cycle (Pignon and Mayer, 2020).

Anesthesia of guinea pigs

The guinea pigs used in the study were placed under general anesthesia using dissociative agents. For this purpose, a combination of Xylazine Hcl (4 mg/kg, IM, Xylamax® 2% injectable, Royan, Iran) and Ketamin Hcl (60 mg/kg, IM, Ketasol® 10% injectable, Alfasan, Holland) was administered intramuscularly (Mitrović et al, 2023).

Computed tomography study

To obtain the CT images, each guinea pig was placed on the CT scanner in a *sternal* recumbent position with its hindlimbs fully extended toward the caudal side. The CT scan of the lumbosacral vertebrae was performed in the sagittal, transverse, and dorsal planes with a thickness and intervals of 1mm from the cranial part of the first lumbar vertebra to the caudal extremity of the sacrum. A helical scanner (Toshiba Multi-slice CT Scanner Asteion Premium 4, Model: TSX-021B, Japan) was employed for the CT imaging. Appropriate windows were selected to examine both soft and bone tissues. The technical factors of the CT scanner included gantry rotation time (400 ms), slice thickness (1 mm), reconstruction distance (0.5–1 mm), pitch ratio (1), kVp (120), mAs (22), physical detector collimation (32 × 0.6 mm), final section collimation (64 × 0.6 mm), resolution (512 × 512 pixels), and resolution range (0.92 × 0.92), Kernel (10 H), and increment (0.5 mm) (Ohlerth and Scharf, 2007; Badea, 2018). Imaging was performed based on the above-mentioned factors, and the obtained images were saved in DICOM format (Witkowska et al, 2014; Bouxsein et al, 2010).

Three-dimensional reconstruction

Following the saving of the obtained images in DICOM format, they were transferred to a computer equipped with 3D modeling software (Onis CT software, Multi-Modality Workplace: VE 2.5A) (Wilhite and Wölfel, 2019). These images were analyzed using the bone (WW: 4000 HU; WL: 550 HU) and soft tissue (WW: 450 HU; WL: 80 HU) settings. The electronic caliper of this software was used for the morphometric measurements.

Morphometric study

The morphometric measurements of different parts of lumbosacral vertebrae were performed and their means were recorded. The measurements were conducted once by the same individual. The NAV (Nomina Anatomica Veterinaria) was used as the obtained scientific term (Veterinaria, 2017; Özkadif et al, 2015). The investigated parameters are listed in Table 1 (Boonsri et al, 2021; De Silva et al, 2022).

Statistical analysis

The SPSS software version 21 was utilized in this trial. Statistical analysis of Student *t*-test was conducted, and the results were presented as Mean±SD. The Confidence Interval (CI) index was employed to calculate the normal ranges of the indices of the lumbosacral vertebrae in the adult guinea pigs. The significance level was set at 0.05.

Table 1: Description of the investigated parameters

Parameter	Abbreviation	Description
Body length	BL	Distance between the cranial articular surfaces to caudal articular surface in sagittal plan.
Articular processes distance	APD	Distance between the cranial articular processes to caudal articular process in sagittal plan.
Arch borders distance	AD	Distance between the cranial and caudal borders of vertebral in sagittal plan.
Total height	TH	Distance between the ventral crest to distal extremity of spinous process of vertebral in sagittal plan.
Body height	BH	Distance between the ventral crest to vertebral foramen in transverse plan.
Spinous process height	SPH	Distance between proximal and distal extremity of spinous in transverse plan.
Vertical diameter of cranial foramen	VDCrF	Distance between proximal extremities of cranial foramen to distal extremity of cranial foramen in transverse plan.
Horizontal diameter of cranial foramen	HDCrF	Distance between left extremities of cranial foramen to right extremity of cranial foramen in transverse plan.
Vertical diameter of caudal foramen	VDCaF	Distance between proximal extremities of caudal foramen to distal extremity of caudal foramen in transverse plan.
Horizontal diameter of caudal foramen	HDCaF	Distance between left extremities of caudal foramen to right extremity of caudal foramen in transverse plan.
Body cranial width	BCrW	Distance between left extremities of cranial body to right extremity of cranial body in transverse plan.
Body caudal width	BCaW	Distance between left extremities of caudal body to right extremity of caudal body in transverse plan.
Lateral sacral crest length	LSL	The craniocaudal length of lateral sacral crest in transverse plan.
Medial sacral crest length	MSL	The craniocaudal length of medial sacral crest in transverse plan.

Results

Based on the results of this study, all guinea pigs under study had 6 lumbar vertebrae with an irregular trapezoidal shape. Each lumbar vertebra was composed of an arch, body, cranial and caudal articular processes, mammillary processes, spinous process and cranial and caudal vertebral notches. The details of joints between transverse and mammillary processes, intervertebral discs and cranial and caudal articular processes could be evaluated in the CT images. All parts of the lumbosacral vertebrae and intervertebral joints of guinea pig (*Cavia porcellus*) can be observed and evaluated in the computed tomography images. The spinous process of the lumbar vertebrae in the sagittal plane and the cranial and caudal articular processes in the sagittal and transverse reconstruction planes were more identifiable. The mammillary processes and the cranial and caudal vertebral notches were better observed in the dorsal plan. The costal facets were not observed in these vertebrae. The spinous processes of the lumbar vertebrae were short and inclined towards the cranial side. The height of the spinous processes from L_1 to L_6 vertebrae decreased gradually. The transverse processes of the lumbar vertebrae were short and flat, located craniolaterally and somewhat ventrally. These processes were articulated with mammillary processes from their proximal part. The L_1 and L_3 vertebrae had the shortest and longest transverse processes, respectively. The lengths of these processes were almost equal in other vertebrae. The articular surfaces of the cranial and caudal extremities of the lumbar vertebrae were smooth. The body length of the L_3 vertebra was longer than other lumbar vertebrae in male and female guinea pigs. The L_4

vertebra had the greatest height and depth of the spinal canal. At the junction of the pedicle with the vertebral body (in the L_6 caudal vertebral foramina), two lateral recesses were visible. The arches of the lumbar vertebrae provided a relatively wide canal for the spinal cord. The intervertebral foramina of the lumbar vertebrae were narrow, but the intervertebral foramen of the last lumbar and the first sacral vertebra were wide. The diameter of intervertebral notches L_1 and L_2 was narrow but it gradually increased up to the L_6 vertebra. The accessory processes (*Anapophysis*) were observed in all lumbar vertebrae, located between the transverse and caudal articular processes. The *anapophysis* of both the first and second lumbar vertebrae of the guinea pig was relatively prominent. The cranial articular processes were visible as two rounded facets on the sides of the vertebral arch, and the caudal articular processes were visible as two facets at the root of the spinous processes. The caudal articular processes facets of the sixth lumbar vertebra were completely separated (Figure 1).

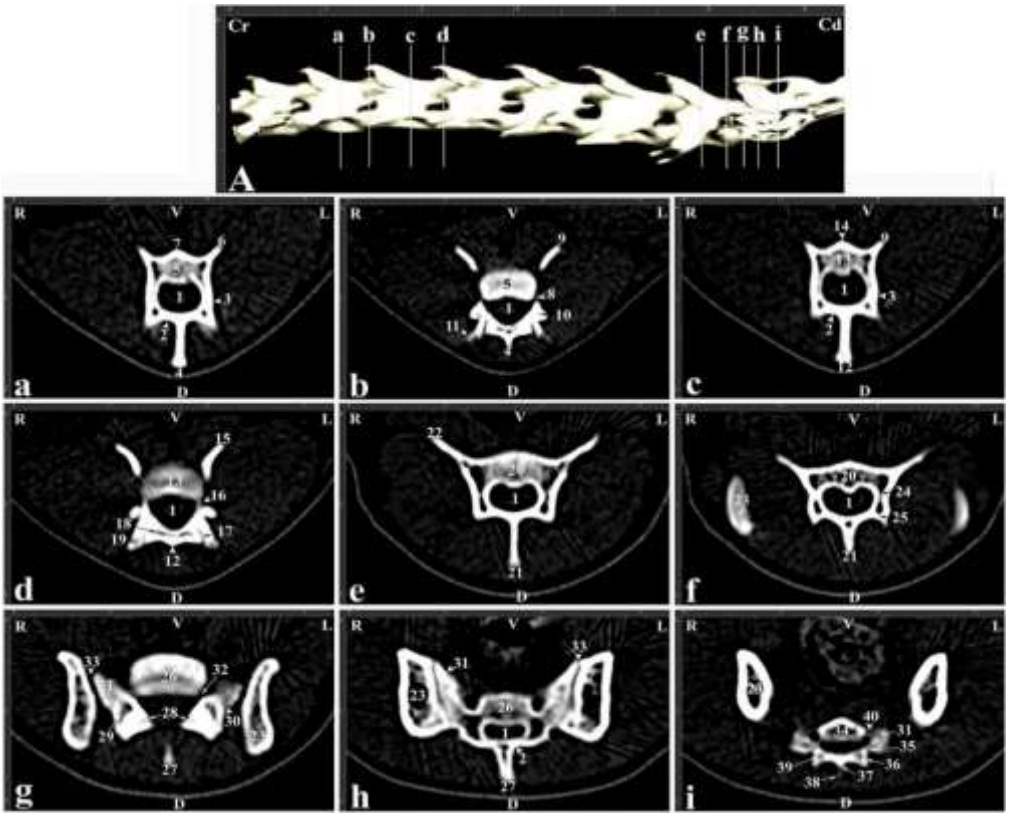

Figure 1: A. Three-dimensional reconstruction image of normal lumbosacral vertebrae of a one-year-old male guinea pig (*Cavia porcellus*) in the lateral plan. (a–d) Transverse computed tomography images related to L_1 to L_3 vertebrae and (e-i) Transverse CT images of L_6 to sacrum. (1) Vertebral foramen, (2) Vertebral lamina, (3) Vertebral pedicle, (4) Spinous process of L_1, (5) Body of L_1, (6) Transverse process of L_1, (7) Ventral crest of L_1 body, (8) Intervertebral notch of L_1, (9 Transverse process of L_2, (10) Caudal articular process of L_1, (11) Cranial articular process of L_2, (12) Spinous process of L_2, (13) Vertebral body of L_2, (14) Ventral crest of L_2 body, (15) Transverse process of L_3, (16) Intervertebral notch of L_2-L_3, (17) L_2-L_3 articular process joint, (18) Cranial articular process of L_3, (19), Caudal articular process of L_2 (20) Body of L_6, (21) Spinous process of L_6, (22) Transverse process of L_6, (23) Iliac wing, (24) Lateral recess, (25) Caudal articular process of L_6, (26) Body of S_1, (27) Spinous process of S_1, (28) Caudal articular process of L_6, (29) Cranial articular process of S_1, (30) L_6-S_1 articular process joint, (31) Sacral wing, (32) L_6-S_1 intervertebral foramen, (33) Sacroiliac joint, (34) Spinous process of L_4, (35) Body of L_4, (36) Transverse process of L_5, (37) Intervertebral notch of L_4-L_5, (38) Caudal articular process of L_4, (39) Cranial articular process of L_5, (40) L_4-L_5 articular process joint. L: Left, R: Right, V: Ventral, D: Dorsal, Cr: Cranial, Ca: Caudal. The scale of the figures is in centimeters.

According to our observations, the sacrum of all guinea pigs had a triangular shape and was composed of the fusion of four vertebrae (Figure 2). The sacrum bone included a body, two wings, base and apex. The wings had an articular facet in their lateral part to articulate with the ilium. The inner surface of the wings (or a*lae*) was narrow. The bodies of the sacrum vertebrae were seen as elongated segments. The long axis of the body of each sacrum vertebra was flat and had dorsal and ventral surfaces. The dorsal surface of the body was wide on the cranial part and narrow on the caudal part. The ventral surface of the sacral body was smooth and had three transverse lines. In fact, these three transverse lines were a sign of the fusion of the four sacral vertebrae. Two transverse lines were seen on the ventral surface of the sacral body of a female guinea pig that had three vertebrae. The promontory could be recognized at the body base of the first sacral vertebra. The spinous processes of the sacrum formed of a bony plate in both males and females. The height of the spinous process of the sacrum decreased from the cranial to the caudal side. The narrow transverse processes were located on the sides of the sacrum, with a small protrusion in their caudal part. Two transverse processes were seen as small projections originating from the body of the last sacral vertebra. Therefore, the integration of the transverse processes of the sacrum did not form a plate-shaped structure in guinea pigs. There were two and three foramina on the ventral and dorsal surfaces of the sacrum, respectively. A middle and two lateral crests were visible on the dorsal surface of the sacrum. The lateral sacral crests were thin and located on the sides of the middle crest. Two cranial articular processes were distinctly recognizable, articulating with the caudal articular processes of the last lumbar vertebra. The cranial opening of the sacral spinal canal was triangular, while the caudal opening was aperture-shaped. Fusion of the sacrum bone with the last lumbar vertebra and the first coccygeal vertebra was not observed in any of the studied guinea pigs study (Figure 3).

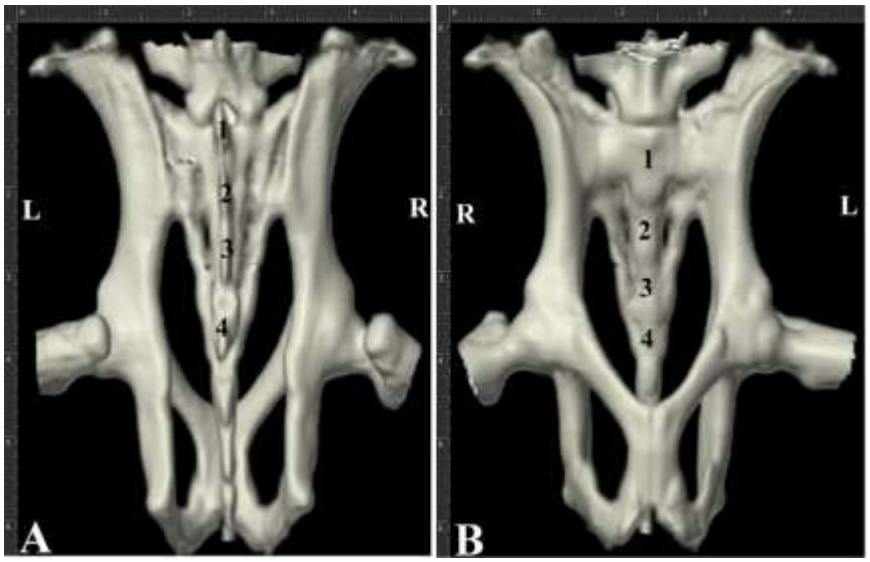

Figure 2: Dorsal (A) and ventral (B) views of the sacrum of a 12-month-old male guinea pig pig (*Cavia porcellus*). The sacrum is triangular in shape and is formed by the fusion of four vertebrae. The scale of the figures is in centimeters.

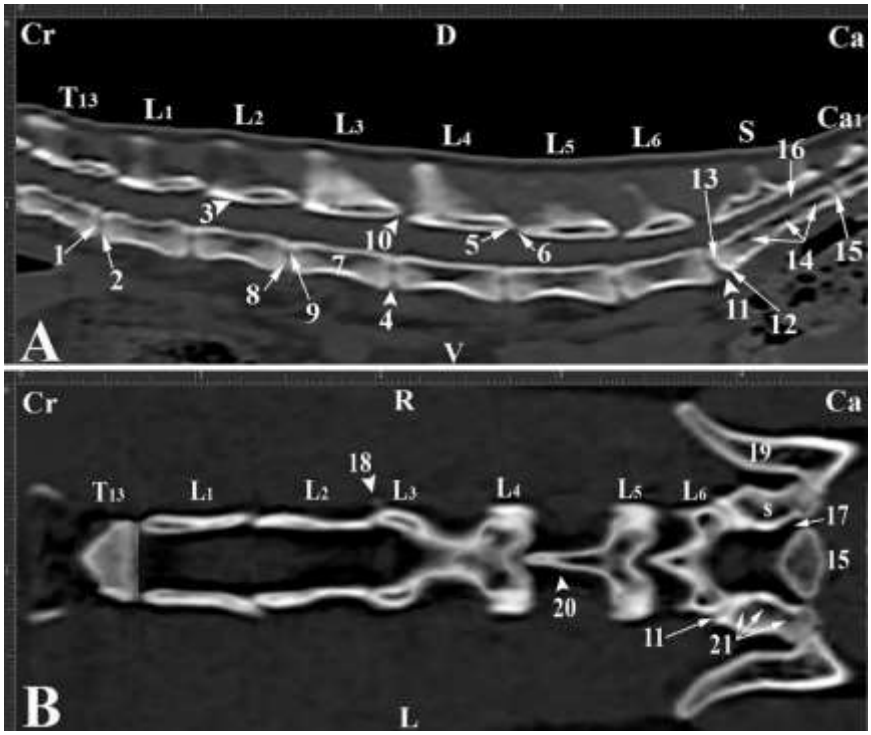

Figure 3: Sagittal (A) and dorsal (B) computed tomography reconstruction images of the lumbosacral vertebrae of a one-year-old female guinea pig (*Cavia porcellus*). (1) Caudal extremity of body T_{13}, (2) Cranial extremity of body L_1, (3) Vertebral arch, (4) Intervertebral disc of L_3 and L_4, (5) Cranial articular process of L_4, (6) Caudal articular process of L_5, (7) Body of L_3, (8) Annulus Fibrosus, (9) Nucleus Pulposus, (10) Joints of articular processes of L_3 and L_4, (11) Lumbosacral joint, (12) Promontory, (13) Base of sacrum, (14) Transverse lines, (15) Apex of sacrum, (16) Sacral canal, (17) Lumbosacral joint, (18) Mammillary process, (19) Iliac wing, (20) Spinous process of L_5, (21) Pelvic and dorsal sacral foramina of T_{13}. T_{13}: 13th thoracic vertebra, L_1: First lumbar vertebra, L_2: 2d lumbar vertebra, L_3: 3th lumbar vertebra, L_4: 4th lumbar vertebra, L_5: 5th lumbar vertebra, L_6: 6th lumbar vertebra S: Sacrum, Ca_1: The first caudal vertebra, L: Left, R: Right, V: Ventral, D: Dorsal, Cr: Cranial, Ca: Caudal. The scale of the figures is in centimeters.

In this study, morphometric measurements of different parts of the lumbosacral vertebrae were performed and subjected to statistical analysis (Tables 2 and 3). The average height of the sacrum body in male and female guinea pigs was 2.75±0.13 mm and 2.60±0.18 mm, respectively. The apex of the female guinea pigs sacrum was wider than that of males (Table 3). The BL and SPH parameters of the lumbar vertebrae were higher in males than in females, and this difference was statistically significant ($P \leq 0.05$). Other parameters of the lumbar vertebrae were greater in males than in females, but no statistically significant difference was observed between them ($P \geq 0.05$) (Table 2). The BL parameter of the sacrum bone was higher in males than in females, and the BCrW parameter was greater in females than in males, with these differences being statistically significant ($P \leq 0.05$). Other parameters of the sacrum bone were greater in males than in females, but no statistically significant difference was observed between them ($P \geq 0.05$) (Table 3).

Table 2: Statistical evaluation (Mean ± standard deviation) of variable values of Lumbar vertebrae (mm) in female and male guinea pigs (*Cavia porcellus*)

Parameters		L_1	L_2	L_3	L_4	L_5	L_6
BL	Female	7.23±0.22	8.11±0.17	8.68±0.25	8.98±0.17	8.41±0.27	7.73±0.22
	Male	7.95±0.22*	8.78±0.33*	9.46±0.23*	9.75±0.30*	9.23±0.26*	8.48±0.20*
APD	Female	9.88±0.27	10.30±0.32	10.95±0.30	11.28±0.27	11.43±0.27	11.13±0.25
	Male	10.13±0.35	10.56±0.27	11.21±0.30	11.71±0.22	11.88±0.11	11.45±0.22
AD	Female	6.48±0.22	7.35±0.32	7.26±0.27	8.11±0.30	8.35±0.30	6.51±0.30
	Male	6.63±0.30	7.48±0.20	7.45±0.20	8.30±0.32	8.53±0.35	6.66±0.27
TH	Female	6.01±0.27	6.46±0.27	8.51±0.30	9.53±0.35	8.70±0.25	9.63±0.32
	Male	6.21±0.20	6.60±0.25	8.70±0.32	9.75±0.30	8.90±0.25	9.81±0.32
BH	Female	2.53±0.12	2.18±0.17	2.61±0.20	3.10±0.15	3.23±0.17	2.70±0.18
	Male	2.63±0.07	2.31±.017	2.76±0.20	3.18±0.15	3.36±0.20	2.83±0.20
SPH	Female	1.00±0.15	1.21±0.12	1.10±0.10	1.06±0.10	1.01±0.12	0.81±0.07
	Male	1.28±0.07*	1.45±0.08*	1.40±0.05*	1.31±0.06*	1.31±0.08*	1.06±0.07*
VDCrF	Female	2.98±0.17	2.38±0.17	3.46±0.12	3.41±0.22	3.60±0.25	2.31±0.17
	Male	3.08±0.12	2.51±0.20	3.58±0.07	3.51±0.22	3.70±0.20	2.36±0.20
HDCrF	Female	4.51±0.22	4.60±0.13	4.38±0.22	4.13±0.22	4.21±0.22	4.13±0.22
	Male	4.63±0.15	4.70±0.05	4.53±0.12	4.25±0.08	4.30±0.10	4.25±0.15
VDCaF	Female	3.40±0.25	2.90±0.27	2.98±0.17	3.21±0.22	3.30±0.25	2.91±0.30
	Male	3.51±0.28	3.00±0.25	3.11±0.18	3.33±0.27	3.40±0.30	3.03±0.20
HDCaF	Female	4.61±0.32	3.51±0.25	3.13±0.22	4.41±0.30	4.40±0.25	4.10±0.25
	Male	4.71±0.32	3.63±0.20	3.26±0.22	4.53±0.30	4.50±0.25	4.18±0.25

Body length (BL), Articular processes distance (APD), Arch borders distance (AD), Total height (TH), Body height (BL), Spinous process height (SPH), Vertical diameter of cranial foramen (VDCrF), Horizontal diameter of cranial foramen (HDCrF), Vertical diameter of caudal foramen (VDCaF) and Horizontal diameter of caudal foramen (HDCaF). The significance level was set at 0.05 and the confidence limit was 95%. The significant parameters are marked with an asterisk (*).

Table 3: Statistical evaluation (Mean ± standard deviation) of variable values of Sacrum bone (in mm) in female and male guinea pigs (*Cavia porcellus*)

Parameters	Sacrum	
	Female	Male
BL	17.77±0.32*	19.91±0.25*
APD	19.25±0.62	19.53±0.55
AD	13.50±0.62	13.75±0.65
TH	9.80±0.32	9.93±0.35
BH	2.60±0.18	2.75±0.13
SPH	0.71±0.12	0.80±0.15
VDCrF	2.51±0.22	2.61±0.25
HDCrF	3.56±0.22	3.70±0.22
VDCaF	2.30±0.25	2.40±0.27
HDCaF	2.73±0.27	2.81±0.25
BCrW	16.75±0.28*	15.87±0.42*
BCaW	8.53±0.32	8.36±0.62
LSL	14.92±0.44	15.15±0.37
MSL	11.35±0.37	11.51±0.35

Body length (BL), Articular processes distance (APD), Arch borders distance (AD), Total height (TH), Body height (BH), Spinous process height (SPH), Vertical diameter of cranial foramen (VDCrF), Horizontal diameter of cranial foramen (HDCrF), Vertical diameter of caudal foramen (VDCaF), Horizontal diameter of caudal foramen (HDCaF), Body cranial width (BCrW), Body caudal width (BCaW), Lateral sacral crest length (LSL) and Medial sacral crest length (MSL). The significance level was set at 0.05. The significant parameters are marked with an asterisk (*).

Discussion

In veterinary science, different methods are used to diagnose diseases in animals (Muhamediyeva et al., 2023). Diagnostic imaging is one of these advanced methods that play an important role in the diagnosis and control of diseases. The CT is one of the

most practical and accurate diagnostic imaging methods used to investigate diseases in various animals (Zhou and Liu, 2024).

Recent studies have increasingly focused on the use of the CT in animals, with most research concentrating on diagnosing skeletal diseases (Kim et al., 2024). To accurately diagnose various diseases and complications in animals using the CT scan images, it is essential to first understand the images of the normal anatomical structures of these animals. However, there are limited reports on the normal anatomy of animals based on the CT findings (Gontijo et al, 2020).

In this study, the morphological and morphometric characteristics of normal lumbosacral vertebrae of guinea pig (*Cavia porcellus*) were investigated using the CT scan images. The findings presented in this study contribute to the existing body of knowledge by providing detailed information on the normal anatomical structures of guinea pigs, which is crucial for accurate diagnosis and treatment planning.

In a retrospective study, Grosso (2019) reported the normal skeletal anatomy of exotic animals based on the CT findings. Similarly, Lauber et al, (2017) described the CT as an accurate diagnostic method for evaluating skeletal diseases and spine complications in both exotic and laboratory animals. Mitrović et al, (2023), by examining the morphology of the lumbar vertebrae in guinea pig, reported that the greatest pressure is on the L_4 vertebra. This finding is consistent with our study, which showed that the L_4 vertebra had the greatest height and depth of the spinal canal. In contrast, in humans, the greatest pressure is typically on the last lumbar vertebrae due to the axial load of the spine.

Dayan et al, (2019) presented the 3D computed tomography anatomy of the cranial and caudal limb bones of the guinea pigs in a software format. In another study, De Silva (2022) provided a comparative anatomical atlas of various guinea pig tissues based on the CT images, intended for use by researchers and veterinary clinicians. Additionally, Del Chicca et al, (2023) stated that the CT anatomy of guinea pigs serves as a diagnostic reference and plays a role in identifying different species of this animal in biometric research.

The present study investigated the CT features of the lumbosacral vertebrae in adult guinea pigs. To do this, fourteen morphometric indices of lumbosacral vertebrae were described and their various parts were identified and analyzed statistically. According to the findings, all guinea pigs had six lumbar and four sacrum vertebrae. Notably, the sacrum of a female guinea pig consisted of three fused vertebrae. These results align with Martinez-Pereira's (2021) study, which reported that the number of sacral vertebrae is not constant in most rodents.

Green (2021) by conducting an anatomical study, reported that the guinea pig sacrum consists of four fused vertebrae and features a notched crest on its spinous process. This finding contradicts our study, as our observations indicated that the spinous processes of the sacrum in both male and female guinea pigs were in the form of a bony plate without any notched crest. Additionally, Shomer et al, (2015) reported the presence of spherical accessory processes on the caudal part of the pedicles of the first to third lumbar vertebrae in guinea pigs. This observation is also inconsistent with our findings.

Our findings indicate that in both male and female guinea pigs, two lateral recesses were visible in the caudal vertebral foramina of L_6 at the junction of the pedicle and the vertebral body. This anatomical feature of guinea pig lumbar vertebrae has not been previously described in any anatomical texts and is reported for the first time in this study. Some Studies on other rodents, such as mice and rabbits, have shown that these lateral processes contain nerve roots and spinal ganglia (Börü et al,

2024). It appears that the same might be true for guinea pigs; however, specific studies are recommended to confirm this. Furthermore, the CT images obtained in the present study reveal that the range of ligaments in the lumbar region of guinea pigs can be identified as a thin soft tissue in the central part of some intervertebral discs.

Considering that magnetic resonance imaging (MRI) is preferable to CT for examining the soft tissues (Nahas et al, 2024), it is recommended to use MRI or micro CT when examining these ligaments. The transverse processes of the lumbar vertebrae in both sexes of guinea pigs were short and flat, located craniolaterally and somewhat ventrally. These findings were consistent with the study of Usha et al, (2020) but in contrast with the report by Skinner et al, (2021), which stated that the length of transverse processes is uniform across all lumbar vertebrae in rodents such as guinea pigs and rats. In our observations, L_1 and L_3 vertebrae of guinea pigs had the shortest and longest transverse processes, respectively.

Sánchez-Macías et al, (2016) reported that the length of the lumbar vertebrae body of guinea pigs is 1.5 times the length of the thoracic vertebrae body, and the length of the second thoracic vertebra body is equal to the sixth lumbar vertebra body. The first part of this report does not align with our findings, as our observations indicated that the L3 vertebra body length was longer than that of the other lumbar vertebrae. Additionally, the L_4 vertebra had the greatest height and depth of the spinal canal.

The present study found that the diameter of the intervertebral notches L_1 and L_2 was narrow, but it gradually increased up to the L_6 vertebra. This observation is consistent with the study by Sui et al, (2022). Knowledge of this anatomical feature of guinea pig lumbar vertebrae can be crucial for diagnosing inflammation and swelling of the lumbar spinal cord.

Jerome et al, (2018) through extensive anatomical studies on various rodents, reported that the articular surfaces at the extremiti es of the lumbar vertebrae bodies in guinea pigs are narrow and uneven. However, this claim contradicts the findings of the present study. Our observations revealed that the articular surfaces of the cranial and caudal extremities of the lumbar vertebrae bodies in guinea pigs were wide and smooth.

By examining the spinal formula of 240 guinea pigs in a retrospective study, Proks et al, (2015) reported that 80.4% of them had four sacral vertebrae and 82% of six lumbar vertebrae. Additionally, a lumbosacral transitional vertebra was observed in one guinea pig. These findings are not consistent with our study. According to the results, all guinea pigs under study had six lumbar vertebrae, and the sacrum was composed of four fused vertebrae. No congenital vertebral anomalies such as lumbosacral transitional vertebra, were observed in any of these animals.

Our research was conducted on guinea pigs of the *Cavia porcellus* breed, while the study by Proks et al. did not mention the breed or species of these pigs, suggesting that this discrepancy might be due to breed variations In our present study, we used 10 guinea pigs (Cavia porcellus) to examine different parts of their normal lumbosacral vertebrae based on CT scan images. It is recommended that future research include a larger sample size of guinea pigs (Cavia porcellus) to provide more detailed information on their spinal formula.

In another retrospective study, Sasai et al, (2015) reported that micro-CT has a greater diagnostic value than radiography in diagnosing pelvic fractures in rabbits. This is due to the phenomenon of tissue overlap and superimposition in radiography, whereas CT images allow for separate examination of all tissues (Sasai et al, 2015). Additional advantages of CT include providing high-resolution and three-dimensional images of animal anatomy, better differentiation of similar tissues, more accurate identification and diagnosis

of cancers, and a reduced need for exploratory surgeries.

In a study, Chawla et al, (2021) reported that the spinous processes of the sacrum are triangular in guinea pigs. However, this finding is inconsistent with our observations. According to our CT images, the spinous processes of the sacrum in both sexes of guinea pigs were in the form of a bony plate and their height decreased from the cranial to the caudal side. Additionally, our observations indicated that there were two and three foramens on the ventral and dorsal surfaces of the sacrum on both sexes, respectively, which appear to be the exit sites of the dorsal and ventral sacral spinal nerves.

The anatomical differences observed in the lumbosacral vertebrae of guinea pigs, as detailed in this study, provide a basic reference for the clinicians and researchers working with this species. By establishing the normal morphology and morphometry of this region, our findings serve as a critical baseline for identifying deviations that may indicate pathological conditions, congenital anomalies, or trauma. The CT-anatomy plays a crucial role in the modern veterinary medicine, fundamentally changing how we diagnose and treat animals. It allows veterinarians to visualize the lumbosacral vertebrae of guinea pigs non-invasively, providing detailed insights into their anatomy and aiding in the diagnosis of a wide range of conditions.

In clinical practice, understanding these normal anatomical parameters allows veterinarians to more accurately interpret diagnostic imaging, such as radiographs CT scans or MRI, and differentiate between normal variations and potential abnormalities. For instance, variations in vertebral size, shape, or alignment could signal conditions like spinal deformities, degenerative diseases, or injuries. Early detection of such abnormalities can lead to more timely and targeted interventions, improving outcomes for affected animals. Furthermore, this knowledge is particularly valuable in comparative anatomy and translational research, where guinea pigs are often used as model organisms. By clarifying the normal anatomy of the lumbosacral region, our study enhances the accuracy of experimental designs and the interpretation of results in studies involving spinal health, biomechanics, or related fields. In summary, the anatomical data presented in this study directly supports the clinical interpretation and diagnosis by providing a clear standard for normal anatomy, enabling clinicians to recognize and address abnormal cases with greater confidence and precision.

One of the limitations of this research may include the small sample size of 10 healthy adult guinea pigs used in the study. While this sample size may be appropriate for a descriptive study, it may limit the generalizability of the findings to a larger population of guinea pigs. Additionally, the study focused on the normal lumbosacral vertebrae morphology and morphometrics, which may not fully capture the variability or abnormalities that could be present in diseased or injured guinea pigs.

Furthermore, the reliance of the study on the CT scan images for evaluation may have limitations in terms of resolution, image quality, and potential artifacts that could affect the accuracy of the measurements and observations made. Interpretation of the CT scan images, especially in exotic animals like guinea pigs, may also require specialized training and expertise, which could affect the reproducibility of the results. It is important for the future research in this area to consider these limitations and potentially address them through larger sample sizes, validation studies, and collaboration with veterinary professionals to ensure the clinical relevance and applicability of the findings.

This study was conducted on adult male and female guinea pigs (*Cavia porcellus*). It is hoped that the future studies will include more extensive research on immature

guinea pigs and other species of guinea pigs.

The findings of this study demonstrate that the CT can provide valuable clinical information about the anatomical structures of the lumbosacral vertebrae of the guinea pig (*Cavia porcellus*), information that is not attainable with conventional diagnostic imaging methods. Additionally, evaluating the tomographic characteristics of the lumbosacral vertebrae in this species can aid in identifying the anatomical features and assessing pathological complications. In this study, images with acceptable resolution of the details of different parts of the lumbosacral vertebrae of guinea pigs (*Cavia porcellus*) were obtained by employing the appropriate radiation factors and suitable windows.

The results of the current study allow the clinicians to detect abnormalities such as herniated discs, spinal tumors, lumbar spinal stenosis, fractures, and the other injuries that may not be visible on other imaging tests. These findings could also help screen guinea pigs with back and leg pain who do not have neurological deficits. It may determine the need for further studies, such as myelography. The results of this study revealed that the CT can easily detect disorders related to the lumbosacral vertebrae canal in guinea pigs. The CT allows for the investigation of articular process joints, sacroiliac joints, intervertebral foramina and dorsal sacral foramina without superimposition. According to our findings, the interarcuate spaces of guinea pig lumbar vertebrae were very narrow, but this space was wide and large between the L_6 and S_s vertebrae. Therefore, for epidural anesthesia, surgeons can perform cerebrospinal fluid puncture and administer anesthetic drugs at this location.

The results of this research can be applied in teaching the CT anatomy of lumbosacral vertebrae, interpretation of the CT scan images, as well as in clinical and treatment decisions of guinea pig (*Cavia porcellus*).

Ethical Consideration

Approval for the study was granted by the Ethical Review Committee at the Islamic Azad University of Urmia Branch, Iran (Approval Number: IR.IAU.URMIA.REC.1403.129), ensuring compliance with ethical codes for research on animals. The experiment was supervised by the Iranian Society for Prevention of Cruelty to Animals, underscoring the commitment to humane treatment throughout the study.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Evaluating the effect of oxytocin or carbetocin combined with flunixin meglumine administration and uterine lavage on the treatment of persistent-breeding induced endometritis in Dare-shuri mares

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Abstract

Persistent-breeding induced endometritis (PBIE) as the third most common disease in mares is a leading reason for infertility. Some mares do not respond effectively to traditional treatments including uterine lavage, and administration of antibiotics, anti-inflammatories, and ecbolic agents. This study explored the combined use of oxytocin and carbetocin with flunixin meglumine for treating PBIE in Dareh Shouri mares. The study involved 45 Dareh Shouri mares with PBIE. Treatment group1: mares were treated with oxytocin, flunixin meglumine, and uterine lavage with normal saline (15); treatment group2: mares were treated with carbetocin, flunixin meglumine, and uterine lavage with normal saline (15); and control, only uterine lavage with normal saline (15). Cytology samples were collected post-ovulation and pre-treatment to confirm endometritis. Pregnancy rates were assessed via ultrasonography 14 days post-ovulation. Results indicated a significantly higher pregnancy rate in the carbetocin-treated group (86%) and oxytocin-treated group (66%). A significant correlation was found between pregnancy outcomes and factors such as uterine edema, follicle size, and the interval between mating and ovulation. This study highlights the potential effectiveness of using oxytocin and carbetocin with flunixin meglumine for treating PBIE in Dareh Shouri mares, though further research is necessary for definitive conclusions.

Key words: Persistent Breeding-Induced Endometritis, Carbetocin, Oxytocin, Flunixin meglumine, Dareh Shouri Mares

Introduction

Endometritis as a prevalent cause of infertility in mares has been reported to be the third health condition in the equine species (Talebkhan Garoussi et al, 2022; Traub-Dargatz et al, 1991). Endometritis occurs when the uterus exhibits a weakened

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ability to clear microorganisms (Morris et al, 2020). Also, predisposing factors of this health disorder encompass a history of dystocia or placental retention, reproductive tract anatomical defects, and inadequate uterine clearance after natural mating or artificial insemination (AI) (Troedsson, 2006; Troedsson and Woodward, 2016). These factors contribute to a decrease in horse fertility (Bucca et al, 2008).

There are three crucial types of endometritis in mares including genital endometritis, persistent-breeding-induced endometritis (PBIE), and chronic uterine infections. Persistent-mating-induced endometritis has been introduced as the predominant form of non-infectious endometritis (Troedsson, 1999; Troedsson and Woodward, 2016). Reports suggest that approximately 10 to 15 percent of mares are susceptible to PBIE (Canisso et al, 2020; LeBlanc, 2010).

Endometritis contributes to a decline in the fertility rate in mares by creating an unsuitable uterine environment for embryos 5 to 6 days after ovulation. It is noteworthy that the occurrence of endometritis after mating is a normal phenomenon in all mares (Zent et al, 1998). However, in most mares, this inflammatory process is transient, and within 24 to 48 hours, the uterus is cleared of inflammatory substances and cells (Pycock and Allen, 1990). In contrast, this inflammation persists in sensitive mares and causes persistent-breeding induced endometritis. Several factors predispose mares to persistent-breeding induced endometritis. Aging and poor perinatal structure are the most significant contributors (Brito and Barth, 2003). Older mares are more susceptible to persistent-mating endometritis than their younger counterparts.

Treatment options for endometritis are diverse and encompass various approaches. These may involve uterine lavage using solutions such as Ringer's lactate or buffered saline, anti-inflammatory treatment, mucolytic compounds, antibiotics and oxytocin (Christoffersen et al, 2012; Fedorka et al, 2018; MacAllister et al, 1993; Rogan et al, 2007).

Oxytocin is synthesized in the hypothalamus and stored in the posterior pituitary gland to be released in response to the related signals. Oxytocin stands out as the most widely recognized drug for enhancing uterine contraction and fluid outflow in mares (Holleboom et al, 2013; LeBLANC et al, 1994; Risco et al, 2009).

Carbetocin, a synthetic oxytocin analog, is a long-acting alternative with a half-life of around 2.5 times longer than oxytocin in horse. The half-life of oxytocin is relatively short (6.8 minutes in horse). The extended duration of action of carbetocin may offer advantages in certain therapeutic contexts compared to the transient effects of oxytocin (de Amorim et al, 2023; Holleboom et al, 2013; Schramme et al, 2008).

According to studies, repeated doses of oxytocin are employed to treat endometritis after mating. Considering the extended half-life of carbetocin and therefore, the possibility of using it as a single dose for treatment persistent-breeding induced endometritis, the present study was designed to evaluate the effect of using exogenous administration of oxytocin or carbetocin along with flunixin meglumine and uterine lavage on the treatment of persistent-breeding-induced endometritis in Dare-shuri mares.

Materials and Methods

Animals and Experimental Design

This study was conducted on 45 Dereh-Shuri mares with PBIE between the ages of 8 and 24 years, from June 2022 to November 2023. The studied mares were divided in to three groups. Treatment group 1: mares were treated with oxytocin along with flunixin meglumine and uterine lavage with 2-3 liters saline solution (15). Treatmet group 2: mares were treated with carbetocin along with flunixin meglumine and uterine lavage with 2-3 liters of saline solution (15),

control: only uterine lavage with 2-3 liters saline solution. The Mean±SE time intervals between mares' parturition and entering the study were 35.6±4.99, 29.4±4.34 and 35.2±4.58 in control, treatment group 1 and treatment group 2 respectively. The Mean±SE of mares' parity was 8.13±0.46, 6.06±0.44 and 7.26±0.40 in control, treatment group 1 and treatment group 2 respectively. All mares included in this study were kept in stables that provide adequate and appropriate nutrition for the mares.

Evaluation and preparation of mares for mating or AI

Prior to the clinical trial and the commencement of the study, the mares under investigation underwent rectal ultrasound examinations (Emp V9, China) at 24-hour intervals during the estrous period to determine the precise mating time. These mares were evaluated for signs of ovulation (such as changes in follicle shape, reduction in uterine edema, and constancy of follicle size). Subsequently, 25 micrograms of Vetaroline (Aburaihan, Tehran, Iran) were administered via intramuscular injection 24 and 12 hours before mating to induce ovulation. The uterus was also evaluated by ultrasound examination. The mares showed more than 1 cm of non-echogenic fluid or any amount of echogenic fluid accumulation in the uterus during estrus, were excluded from the study. The uterine edema and follicular size were measured and recorded at the last ultrasonography before mating or insemination.

Twelve stallions were used for mating or artificial insemination in this study and all of the stallions had normal fertility based on their history. Based on the indicators of the nearness of ovulation mentioned above, the approximate time of ovulation was determined. At this point, natural mating or artificial insemination was performed using a 65 cm pipette (Mini Tube, Germany), which was inserted into the posterior part of the uterine body. The total volume of semen inseminated to obtain normal fertility was 15-20 ml. This volume was based on the motility and concentration of sperm in the sample.

Evaluation for diagnosis of endometritis and uterine cytology preparation

Between 24 to 48 hours following mating or insemination with fresh sperm, mares underwent another ultrasound examination to confirm ovulation. In some of the studied mares and because of the field conditions, ultrasound examination for detection of ovulation was preformed less than 24 hours after insemination. If ovulation was detected, uterine fluid cytology was examined to confirm the diagnosis of endometritis before initiating treatment. The cytology samples were collected from the mares that showed more than 1 cm of non-echogenic fluid or any amount of echogenic fluid accumulation in the uterus. The study continued until 45 mares with PBIE were gathered for the study.

The mares' tails were first thoroughly closed to conduct the cytology, and the vulva was meticulously cleaned with betadine scrub. Subsequently, the sample was gently collected using a sampling brush to prepare a smear. Using the index finger, sampling brush was put into the body of uterus through cervix. In the uterine body, its cap was removed by moving it forward. The brush was held in the endometrial cavity for 10 to 15 sec. The collected sample was used for smear preparation.

After fixation of smear with 95% methanol for 30 seconds, prepared smears were stained using Giemsa 10% for 20 minutes. Subsequently, all slides were rinsed with water. Following drying, the slides were examined using a light microscope with 1000x magnification and immersion oil, and interpreted as follows: between 0-2 neutrophils in each field were considered normal. Between 3-5 neutrophils in each field were deemed indicative of moderate inflammation. The

presence of >5 neutrophils in each field was indicative of severe inflammation (McKinnon et al, 2011). Fourteen days after ovulation, the mares under study underwent rectal ultrasound (Emp V9, China) examination to assess pregnancy.

Just one mating or AI was done for each mare and if the mare did not ovulate during 48 h after mating or AI, that mare was excluded from the study. Treatment procedures in all groups were done once and the result of pregnancy was evaluated and considered for the present study after this treatment. Non-pregnant mares were treated based on their uterine and ovarian condition in the next estrus cycles.

Treatment procedures for mares with PBIE

The first treatment group comprises mares subjected to uterine lavage with 2 to 3 liters saline solution, along with intramuscular injection of 20 units of oxytocin (Rooyan, Iran) and flunixin meglumine (Razak, Iran) that administered intravenously at a 1.1 mg/kg dosage, one hour after oxytocin injection and uterine lavage, and upon diagnosis of the complication (within 24 to 48 hours after mating). The second treatment group comprised mares that received uterine lavage with 2 to 3 liters saline solution, along with intramuscular injection of 0.175 mg of carbetocin (Hanlim Pharm, South Korea) and flunixin meglumine was administered intravenously at a 1.1 mg/kg dosage, one hour after the injection of carbetocin. The control group included mares that underwent uterine lavage with 2 to 3 liters saline solution at the time of diagnosis, which occurred between 24 to 48 hours after mating. All treatments were started within 24 to 48 hours after mating for all groups. Endometritis detection was done at the time of ovulation detection in this study (within 24 to 48 hours after mating).

Statistical analysis

The data were analyzed using IBM SPSS23. Categorical data were analyzed by Kruskal-Wallis Test. The correlation between data were analyzed using Spearman's correlation. The differences at the level of 5% ($P<0.05$) were considered significant.

Results

Age of the mares

The age of the mares in this study, among the groups receiving oxytocin, carbetocin, and the control, was between 8-24 years, 14 years on average. The mares range and average of the mares are shown in Table 1. The results indicated no significant difference in the average age of mares between the groups ($P>0.05$).

Table 1: The range and mean±SE of the age of mares in experimental groups

Groups	Range (year)	Mean±SE (year)
Oxytocin Group	11-20	13.93±2
Carbetocin Group	8-24	13.06±3
Control Group	12-22	15.92±2

Pregnancy rates in mares

In total, 32 mares breed by natural mating and 13 mares breed by AI in this study. In the control group, out of a total of 15 treated mares, 6 mares (40%) became pregnant (Figure 1). In the group receiving oxytocin, out of 15 treated mares, 10 mares (66%) became pregnant which was statistically different compared to the control group ($P<0.05$) (Figure 1). In the group receiving carbetocin, out of 15 treated mares, 13 mares (86%) became pregnant which was different compared to the control group ($P<0.05$) (Figure 1). The pregnancy rate of the oxytocin group was not significantly different in comparison with the carbetocin group ($P>0.05$).

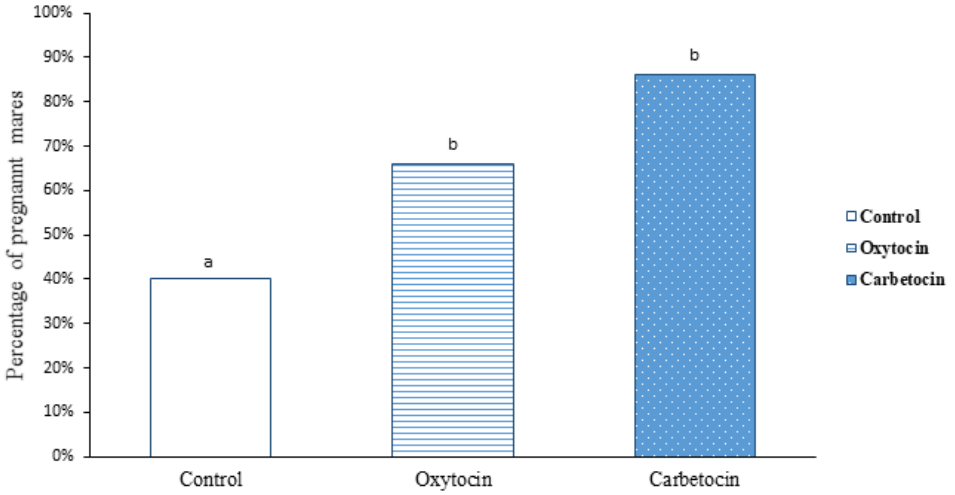

Figure 1: The pregnancy rate of mares among experimental groups. Different letters show significant differences between groups.

Uterine cytology

The results related to evaluation of uterine cytology between 24 to 48 hours following mating or insemination and immediately before treatment in the groups receiving oxytocin, carbetocin, and the control are shown in Figure 2. No significant difference in the uterine cytology status was observed among the groups (P>0.05).

Uterine edema

The results of comparing uterine edema degree among the oxytocin, carbetocin, and the control groups, showed no significant difference (P>0.05) (Figure 3).

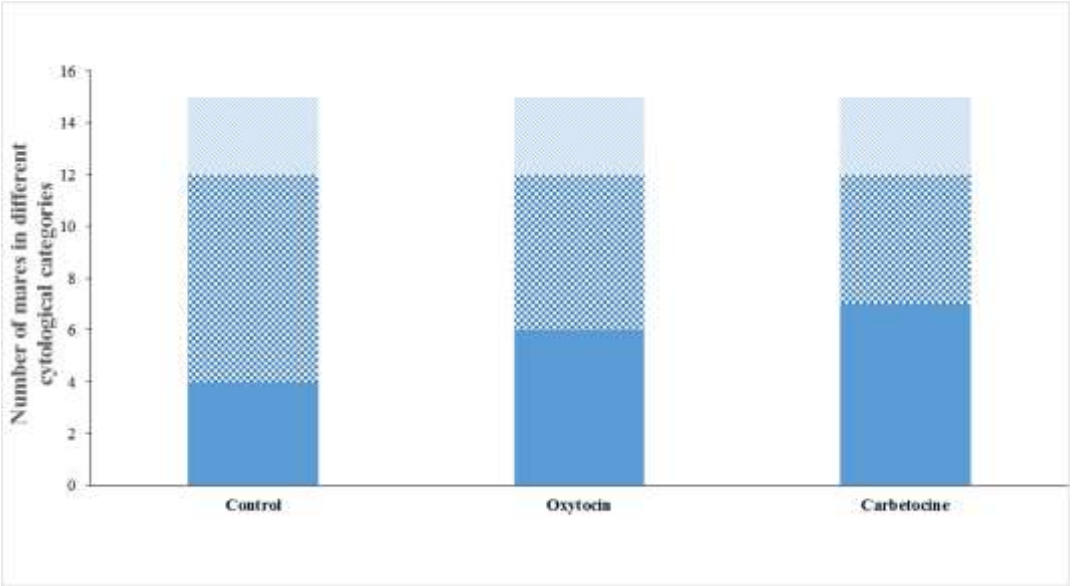

Figure 2: Frequency of mares based on cytology category among experimental groups. : Number of 0-2 neutrophils in 5 microscopic sections with X1000 magnification; : Number of 3-5 neutrophils in 5 microscopic sections with X1000 magnification; : Number of <5 neutrophils in 5 microscopic sections with X1000 magnification

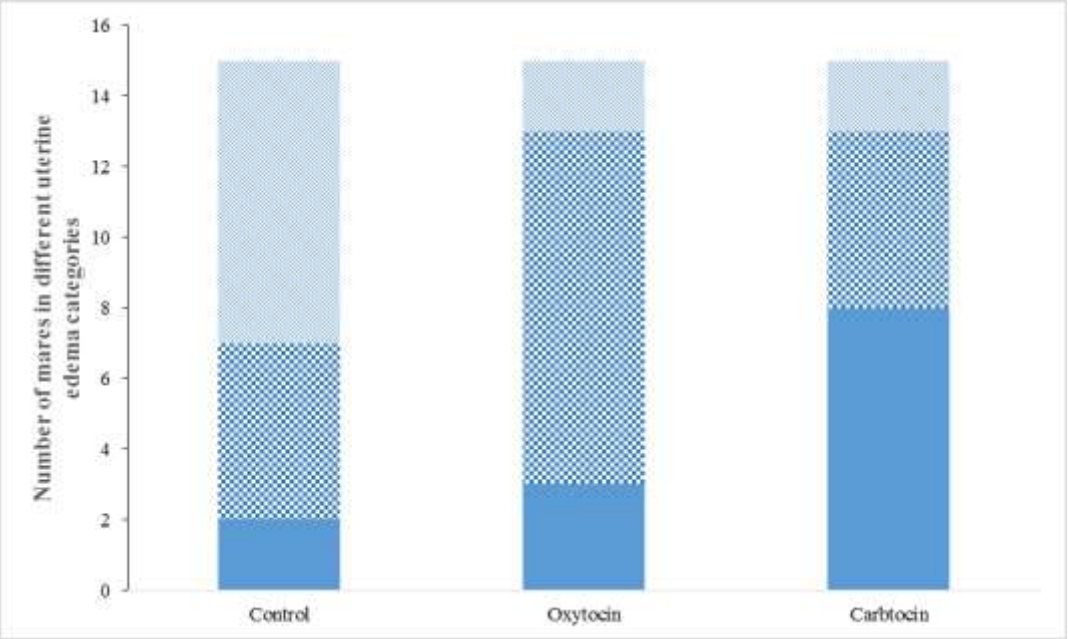

Figure 3: Frequency of mares based on uterine edema category among experimental groups. : edema grade 1; : edema grade 2; : edema grade 3.

Preovulatory follicle diameter

Preovulatory follicle diameter in the studied mares ranged from 3.9 cm to 4.5 cm, with an average of 4.4 cm. The range and average diameter of preovulation follicles in the experimental group are shown in Table 2. A statistically significant correlation was observed between pregnancy in mares with Preovulatory follicle diameter before mating (P<0.003).

Table 2: The range and mean±SE of preovulatory follicle diameter of mares in experimental groups

Groups	Range (cm)	Mean±SE (cm)
Oxytocin Group	3.7-4.6	4.3±1
Carbetocin Group	3.9-5.6	4.3±2
Control Group	4.1-4.2	4.2±1.5

Mating to ovulation interval

The interval between mating to ovulation in the studied mares varied from 18 to 48 hours, with an average of 38 hours. The range and average of the mating interval to ovulation in experimental groups are shown in Table 3. The correlation between pregnancy rate in mares and mating to ovulation interval was not significant (P<0.068).

Table 3: Mating to ovulation interval in mares

Groups	Range (h)	Mean±SE (h)
Oxytocin Group	48-18	34.9±4
Carbetocin Group	48-18	36.9±3
Control Group	39-48	42±2

Correlation between evaluated variables

There were significant correlations between pregnancy and uterine cytology 48 hours after mating and immediately before treatment, pregnancy and dominant follicle size before mating and uterine cytology and dominant follicle size before mating (P<0.05). The correlations between other evaluated variables were not significant (P>0.05) (Table 4).

Table 4: Correlation between evaluated variables

	Pregnancy	Cytology	Uterine edema	Uterine fluid	dominant follicle size	Age	Mating to ovulation interval	GnRH injection to ovulation interval
Pregnancy	1	r= 0.603 p= 0.001	r= -0.340 p= 0.24	r= 0.103 p= 0.252	r= 0.443 p= 0.003	r= -0.038 p= 0.807	r= -0.278 p= 0.068	r= -0.018 p= 0.228
Cytology		1	r= 0.336 p= 0.14	r= -0.083 p= 0.297	r= -0.436 p= 0.003	r= 0.205 p= 0.182	-	-
Uterine edema			1	r= 0.179 p= 0.244	r= -0.219 p= 0.153	-	-	-
Uterine fluid				1	r= 0.116 p= 0.455	r= 0.27 p= 0.860	-	-
dominant follicle size					1	-	-	-
Age						1	-	-
Mating to ovulation interval							1	r= -0.185 p= 0.228
GnRH injection to ovulation interval								1

Discussion

Post-breeding-induced endometritis (PBIE) as the third common disease in adult mares accounts for the main cause of infertility, affecting approximately 15% of mares (Hurtgen, 2006; LeBlanc and Causey, 2009). Traditionally, endometritis is treated with the administration of drugs such as anti-inflammatory substances, antibiotics, and ecbolics concomitant with lavage of the uterus. Unfortunately, some mares do not respond to these treatments (Liu and Troedsson, 2008). In addition to the ecbolic agents, administration of cloprostenol can be used for uterine clearance in the mare and it is mentioned that this prostaglandin analogue has a longer activity duration compared to oxytocin (Combs et al, 1996). However, it is shown that when this drug is administrated during the periovulatory period until two days post-ovulation, it has a negative effect on progesterone levels during the early days after ovulation (Nie et al, 2003).

These treatments are sometimes not effective enough, and the prevalence of antibiotic-resistant microorganisms has increased, which necessitates the development of alternative medications for mares with persistent-breeding endometritis

(Buczkowska et al, 2015; Scoggin, 2016). The present research was aimed to investigate the effect of using exogenous administration of oxytocin or carbetocin along with flunixin meglumine and uterine lavage on the persistent-breeding induced endometritis treatment in Dare-Shuri mares. The results of some studies show that oxytocin administration stimulates a pulse of PGFM (a PGF2a metabolite) that mimics a natural PGFM pulse during luteolysis in mare. In the study of Santos et al, (2016), single injection of oxytocin with each of doses (1–10 IU/mare) stimulated a burst of PGFM that was maximum in 4 minutes, but this burst was unlike a natural pulse. In this study, oxytocin infusion during 2 hours with doses of 1.25, 2.5, or 5 IU/100 kg induced a PGFM pulse similar natural pulse and the peak of an induced seemed similar to reported natural peaks. Percentage decrease in progesterone within 8 hours was significantly greater for this group (oxytocin infusion during 2 hours) than saline injected group. Considering that, oxytocin injection was 1 or 2 days after ovulation in our study and to prevent possible adverse effect of oxytocin on Cl development and progesterone concentration, only one injection of oxytocin was considered in this study. In the study by Rasch et al., the effect of oxytocin on the rate of uterine clearance and pregnancy in mares showed that intravenous oxytocin administration in mares suffering from the accumulation of intrauterine fluid increased fertility (Rasch et al, 1996). According to Guthjahr et al, oxytocin treatment should begin in the preovulatory period because the response of the uterus to oxytocin is greater when progesterone levels are low and estrogen levels are high. If oxytocin treatment is used after ovulation, the dose should be increased to achieve a better effect. However, caution is necessary, as administering more than 25 IU of oxytocin can cause persistent contractions, spasms, and tetany, leading to uterine fluid retention (Gutjahr et al, 2000). In the present study, the pregnancy rates in mares of the control and oxytocin groups were 40%, and 66% respectively, which was significantly different between the oxytocin-treated and the control groups. Therefore, according to the obtained results, oxytocin increases the pregnancy rate in mares.

Carbetocin, with a half-life of 17.2 minutes, can be used in mares as an alternative to oxytocin, which has a half-life of 6.8 minutes (Schramme et al, 2008). One of the disadvantages of oxytocin is the need for repeated doses, making carbetocin more practical. However, to date and to the extent that the authors have investigated, no studies have compared the effect of oxytocin and carbetocin to accelerate uterine clearance (de Amorim et al, 2023; Steckler et al, 2012). In the present study, the pregnancy rate examined in mares was 40% in the control group compared to 86% in carbetocin group. A significant difference was observed between the carbetocin group and the control group. In the study by Khan et al., preovulatory administration of ecbolics in mares resistant to endometrial infection did not affect the clearance of the uterus or luteal development. Moreover, carbetocin did not have the expected effect on uterine clearance but this treatment had no side effect on the corpus luteum until day 14. Therefore, pre- and post- ovulation treatment with carbetocin, twice-daily, is safe in inseminated mares (Khan et al, 2024).

In this study, flunixin meglumine was used in treatment groups alongside oxytocin and carbetocin. In the study by Donnelly et al., the administration of flunixin meglumine did not affect the time interval between the detection of a follicle ≥ 30 mm to ovulation. The pregnancy rate per cycle was not significantly different in flunixin meglumine-treated (83% of matings) compared to the control group (68% of matings). Flunixin meglumine had no significant effect on the behavioral symptoms of estrus, uterine edema, or

serum progesterone concentration (Donnelly et al, 2019).

In our study, there was no significant difference in cytology grade, among the groups that received oxytocin or carbetocin or no treatment, at 48 hours after mating and immediately before treatment. In the study by Riddle et al. (2007), the pregnancy rate of mares with positive cytology or bacterial culture was lower than those with negative culture ($P<0.01$). The mares with severe endometrial inflammation had a lower pregnancy rate than those with moderate inflammation (21% vs 48%). In this study, uterine cytology was used to confirm endometritis in all studied mares, and the lack of a significant difference in the present study indicates similar conditions of the studied mares, consistent with other studies.

On the other hand, to create uniform conditions and reduce the impact of physiological factors on fertility, a statistical comparison was made for the age of the different groups. The results of this comparison showed no significant difference in age among the groups receiving oxytocin, carbetocin, and the control. Nazem et al, (2023), reported a significant difference in the age of mares with endometritis after mating. The absence of significant difference in age between the groups in our study helped to clarify the effect of treatments among groups.

To ensure the uniformity of study conditions in mares, the average diameter of the follicles before ovulation and the average mating interval until ovulation was investigated across different groups. No statistically significant difference was observed. These results are important in confirming the presence of the normal estrus cycle and similar physiological conditions among the mares.

The results for uterine edema in mares receiving oxytocin, carbetocin, and the control showed no statistically significant difference. Inflammatory changes in the uterus of mares are typically indicated by the presence of uterine fluid or a significant increase in endometrial edema. During the normal estrous cycle, endometrial edema is used as a diagnostic factor and correlates with estrogen production. Typically, the degree of edema near ovulation is low or absent. When edema is detected in mares that are in diestrus or pregnant, it is a sign of inflammation. In the present study, there was a direct but not significant correlation between pregnancy in mares and uterine edema before mating (Del Prete, Montano, et al, 2024; Del Prete, Nocera, et al, 2024).

Mateu-Sánchez et al, reported that the pregnancy rate was positively correlated with the duration of estrus and endometrial edema in both natural and prostaglandin-induced cycles. Additionally, a positive and significant correlation was found between the lenght of endometrial edema and both the interval between ovulation and the interval from PG treatment to ovulation (Mateu-Sánchez et al, 2016). In the study by Grabowska and Kozdrowski, there was a significant relationship between the level of progesterone at 14 days post ovulation and the presence of endometrial edema observed at estrus. Sometimes, lack of sufficient endometrial edema at estrus has been related to low progesterone concentration 14 days post-ovulation (Grabowska and Kozdrowski, 2022).

Upon our results, a positive and significant correlation was detected between pregnancy and the size of the dominant follicle before mating. The diameter and growth rate of the follicle before ovulation at the beginning of estrus can affect the duration of estrus until ovulation in mares. It has been demonstrated that the size of the dominant follicle significantly affects the intensity of estrus in mares (Grabowska and Kozdrowski, 2022).

The results of the present study support the effectiveness of using a combination of flunixin meglumine and carbetocin to treat persistent-mating endometritis in mares. Considering that, carbetocin has a longer duration of action than oxytocin and does

not have the side effects mentioned in previous the studies, it is probably a suitable option for persistent-breeding-induced endometritis treatment in mares. However, more studies especially on more severe or complicated endometritis cases which do not respond to routine oxytocin treatments, probably may indicate the advantages of using carbetocin in treatment of endometritis in mares.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Expression profiles of pro-inflammatory cytokine genes in milk somatic cells at different stages of the first lactation in Holstein dairy cattle

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Abstract

Milk somatic cells produce numerous soluble proteins like cytokines that play important roles in the immunity of the mammary gland. This study aimed to investigate the expression profiles of bovine pro-inflammatory cytokine genes including IL-2, IL-6, IL-8, TNF-α, IFN-γ, and GM-CSF in the somatic cells of milk in healthy Holstein cows at different lactation stages in their first lactation cycle. For this purpose, milk samples were collected from eighteen dairy cows at the early, middle, and late lactation stages. Total RNA was extracted from the somatic cells of milk and then the first strand of cDNA was synthesized. Real-time PCR was performed for the bovine pro-inflammatory cytokine genes. As reference genes, the β-actin and GAPDH genes were used to normalize the data. The real-time PCR data were analyzed with the REST and SAS programs. According to the results, the six-cytokine genes were expressed in the milk somatic cells of healthy cows in different lactation stages. The results showed that the expressions of almost all cytokine genes (except for the TNF-α gene) were significantly higher in animals at the middle compare to the early lactation stage. However, the expression of cytokine genes also showed a trend to be higher at the late lactation stage compared to early lactation. Still, these differences were only significant for mRNA levels of TNF-α and GM-CFS genes. Furthermore, the expression differences of cytokine genes were not significant in cows at the late relative to animals at the middle lactation stage. In the entire lactation cycle, the mRNA transcription levels of IL-6 and IL-2 were observed at high and low concentrations compare to other cytokine genes, respectively. The highest stability was shown for IL-6 throughout the three lactation stages, while the lowest stability was found for the expression of TNF-α. The correlation between the gene expression levels was almost not significant for most of the studied genes in different stages of lactation, however, a significant correlation was found between IL-8 and GM-CSF in the entire, early and late stages of lactation.

Key words: Gene expression, Cytokine, Cattle, Lactation stage

Introduction

The breeding strategy continues to increase its focus on health traits in dairy cattle (Koeck et al, 2012; Parker et al, 2014). It has been recommended to include immune response traits in breeding indices to improve the inheritance of disease resistance in dairy cattle (Abdel-Azim et al, 2005; Mallard et al, 2011). For example, it is suggested to use somatic cell score (SCS) as an indicator trait to obtain genetic

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improvement in mastitis resistance, and the somatic cell count (SCC) is widely used as a selection criterion for the genetic improvement of mastitis resistance in dairy cattle (Odegard et al, 2003). Since variations in milk SCC depend mainly on the leukocyte recruitment in response to an inflammatory reaction, SCC is usually used to distinguish between healthy and infected mammary glands and, thus, to monitor udder health (Schukken et al, 2003). The somatic cells are also normally present in milk at low levels. They consist of different cell types such as lymphocytes, polymorphonuclear leukocytes (PMN), macrophages, and a smaller number of epithelial cells. The number and composition of milk somatic cells depend on many factors such as animal health, milk productivity, genetics, and environmental factors (Riollet et al, 2002; Alhussein, 2018). In addition, variations are observed in the composition of milk somatic cells including the lymphocyte population in the lactating healthy gland during lactation stages and cycles (Alluwami, 2002; Park et al, 1992). Furthermore, an increase in SCC is associated with a decrease in daily milk production, which depends on the lactation stages and parity, and it was very extensive in late lactation stage regardless of parity (Hagnestam-Nielsen et al, 2009). Decreased milk production due to increased SCC in multiparous cows was more than primiparous cows (Bennedsgaard et al, 2003; Hagnestam-Nielsen et al, 2009). Generally, total milk SCC is less than 10^5/ml in the healthy lactating mammary gland (Leutenegger et al, 2000). However, the high milk production of the Holstein breed creates a constant state of stress in lactating animals, and therefore a small amount of tissue damage and possibly the presence of low-grade bacteria are common. This assumption is important to understand the expression of cytokines that indicate a certain level of inflammation in the healthy state of lactating cows (Leutenegger et al, 2000). Bacteria in infected mammary glands release their metabolites and cell walls, chemoattractants for leukocytes (Kehrli et al, 1994). Then, larger amounts of various soluble factors are secreted by somatic cells in the mammary gland, which leads to the massive recruitment of additional leukocytes into the gland. These factors involve cytokines, complement components, leukotrienes, prostaglandins, serotonin, and histamine (Giri et al, 1984; Harati et al, 2022; Rose et al, 1989; Shuster et al, 1993; Zia et al, 1987). Cytokines are small proteins that transmit intercellular signals for inflammation, immunity, hematopoiesis, stress, mammary gland development, and tissue repair (Belardelli and Ferrantini, 2002; Brenmoehl et al, 2018; Rouveix, 1997; Wood and Rothel, 1997). The use of bovine cytokines in mastitis immunotherapy also indicates their important role in the defense regulation of the mammary glands (Godson et al, 1997). Accordingly, the cytokine genes have considered as strong candidate markers for mastitis resistance selection in dairy cattle (Sarikaya et al, 2006; Oviedo-Boyso et al, 2007; Curone et al, 2018). Understanding the cytokines activity at different stages of the lactation cycle is important to monitor the well-being of the mammary gland. Therefore, the objective of the present study was to investigate the mRNA levels of normal transcription of cytokines, IL-2, IL-6, IL-8, IFN-γ, TNF-α, and GM-CSF in the milk cells of the bovine mammary gland at the early, mid, and late stages of lactation period in the first lactation of Holstein dairy cows.

Materials and methods

Eighteen healthy dairy Holstein cows in their first lactation were grouped according to their lactation stages (6 at 7-10, 6 at 140-150, and 6 at 290-295 days after parturition). The criterion for selecting animals at the early stage was the SCC< 350,000, while this criterion was SCC<100,000 for the two middle and late

lactation stages. One liter of milk sample representing all four quarters was collected in sterile tubes. Then the milk sample was centrifuged for 20 min at 1500 g at 4 °C. The obtained cell pellet was washed in PBS pH 7.4 twice and centrifuged for 20 min at 4 °C and 220g according to Liebe (1996). The pellets were lysed with 500 µl PBS-EDTA and, then, total RNA was isolated using a Denazist kit according to the manufacturer's protocol. The extracted RNA samples were treated with *DNase* I to remove DNA contamination. RNA quality and quantity were assessed by agarose gel electrophoresis and spectrophotometric readings. Synthesis of first strand cDNA was performed with *AccuPower® RocketScriptTM RT PreMix* kit (Bioneer) and random hexamer primers (Takapozist) according to the manufacturer's instructions. The final volume was adjusted to 50 µl with RNase-free water. The amplified cDNA samples were then stored at -20 °C until use in real-time PCR. The primers for the gene expression evaluation were used as described by Lee et al, (2006) and the β-actin and GAPDH genes were used as endogenous references for the calculation of dCP (Table 1). Real-time PCR was performed using CFX96 (Bio-Rad, USA) and *Hot Taq Eva Green qPCR* kit (Cinnagen) according to the manufacturer's instructions. All reactions were carried out in duplicate. Amplification conditions were an initial step of 95 °C for 15 min, followed by 50 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C (depending on the product length, 5 s per 100 bp) in a 10-µl reaction volume. After amplification, all samples were submitted to analysis of the dissociation curve to confirm the absence of nonspecific products and primer dimmers (melting curve by 95 °C for 5 s, 65 °C for 15 s, and 95 °C for 0 s). In each reaction of real-time PCR, the cycle number at which the fluorescence rises appreciably above the background fluorescence is determined as the crossing point (CP). Descriptive statistics are calculated using the derived CPs for each cytokine gene by the SAS program. The real-time PCR results were analyzed with the REST program to compare differences in gene expression across groups (Pfaffl et al, 2002).

Table 1: Primers sequences of bovine cytokines and references genes in real-time PCR.

Gene	Primer	Sequence (5'-3')	Length	Accession
Il-2	IL-2.107f IL-2.271r	5'-GGATTTACAGTTGCTTTTGGAGAAA-3' 5'-GCACTTCCTCTAGAAGTTTGAGTTCTT-3'	165	M12791
IL-6	IL-6.f209 IL-6.r313	5'-TCATTAAGCGCATGGTCGACAAA-3' 5'-TCAGCTTATTTTCTGCCAGTGTCT-3'	105	NM173923
IL-8	IL-8.f251 IL-8.r355	5'-CACTGTGAAAATTCAGAAATCATTGTTA-3' 5'-CTTCACAAATACCTGCACAACCTTC-3'	105	NM173925
IFN-γ	IFN-γ.f296 IFN-γ.f480	5'-TCATTAAGCGCATGGTCGACAAA-3' 5'-TCAGCTTATTTTCTGCCAGTGTCT-3'	185	M29867
TNF-α	TNF-α.f2377 TNF-α.r2794	5'-TCTTCTCAAGCCTCAAGTAACAAGC-3' 5'-CCATGAGGGCATTGGCATAC-3'	418	AF011926
GM-CFS	GM-CFS.f170 GM-CFS.r250	5'-AGTAATGACACAGAAGTCGTCTCTG-3' 5'-GCCGTTCTTGTACAGCTTCAGG-3'	87	U22385
β-actin	β-actin.f38 β-actin.r428	5'-CCTTTTACAACGAGCTGCGTGTG-3' 5'-ACGTAGCAGAGCTTCTCCTTGATG-3'	391	AH00130
GAPDH	GADPH.463f GADPH582r	5'-GGCGTGAACCACGAGAAGTATAA-3' 5'-CCCTCCACGATGCCAAAGT-3'	120	AF022183

Results

The form of data observations is the raw CP or threshold cycles (Ct) that are generated by a real-time PCR. Usually, the raw CPs or Ct are normally distributed and seem to be the best estimators of the gene expression levels, thus, a parametric test can be performed. In real-time PCR, the amount of amplicon is inversely related to the numerical value of the Ct (i.e., the greater the amount of amplicon, the lower the value of the Ct). Six cytokine genes were expressed in somatic cells of all milk samples at the early, middle, and late stages of lactation (Fig 1). Although the Ct average in all cytokines, except for IL-6 with an average of 25.8, was higher than 30, which indicates the low expression of studied genes in the milk somatic cells of healthy cows. The lowest expression level was detected for IL-2 (average Ct= 34.1) in the entire lactation cycle (Figure 1a). As shown in Fig 1b, the mRNA level of IL-6 was also higher in each lactation stage compared to the other cytokines. Among all cytokine genes, the expression of IL-2 was lower in the early (mean Ct= 35.1) and late (mean Ct= 34.3) lactation stages, while a lower expression level was observed for TNF-α (mean Ct=36.3) in the middle lactation stage (Figure 1b).

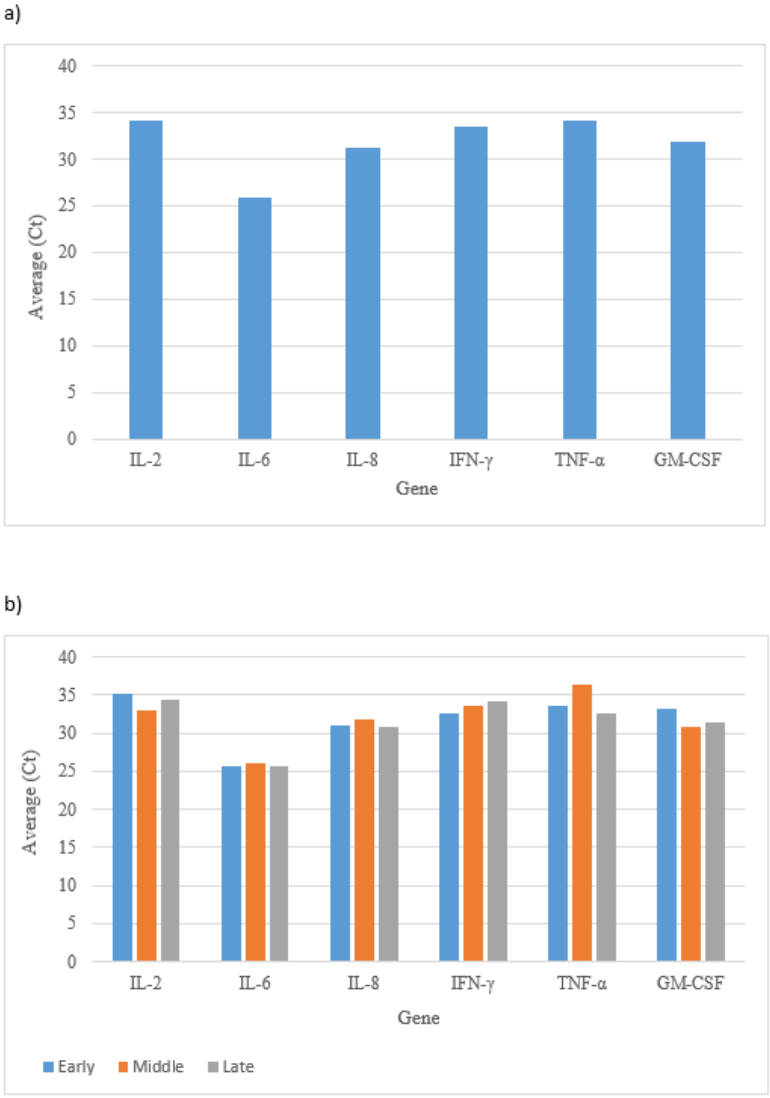

Figure 1: Average of Ct values for cytokine genes in the entire lactation (a) and different lactation stages (b)

Based on the descriptive results of Ct values for cytokine genes, the IL-6 and TNF-α, with standard deviations of 0.54 and 3.21, showed the highest and lowest expression stability during the entire lactation cycle, respectively (Figure 2a). In the early stage of lactation, as in the entire lactation period, the IL-6 and TNF-α revealed the highest and lowest expression stability, with a standard deviation of 0.44 and 3.94, respectively (Figure 2b). The IL-6 gene showed maximum expression stability in the middle (SD Ct=0.70) and late (SD Ct=0.43) lactation stages (Figure 2b). The highest expression variations were observed for IL-8 (SD Ct= 2.26) and IFN-γ (SD Ct=3.29) genes in the middle and late lactation stages, respectively (Figure 2b).

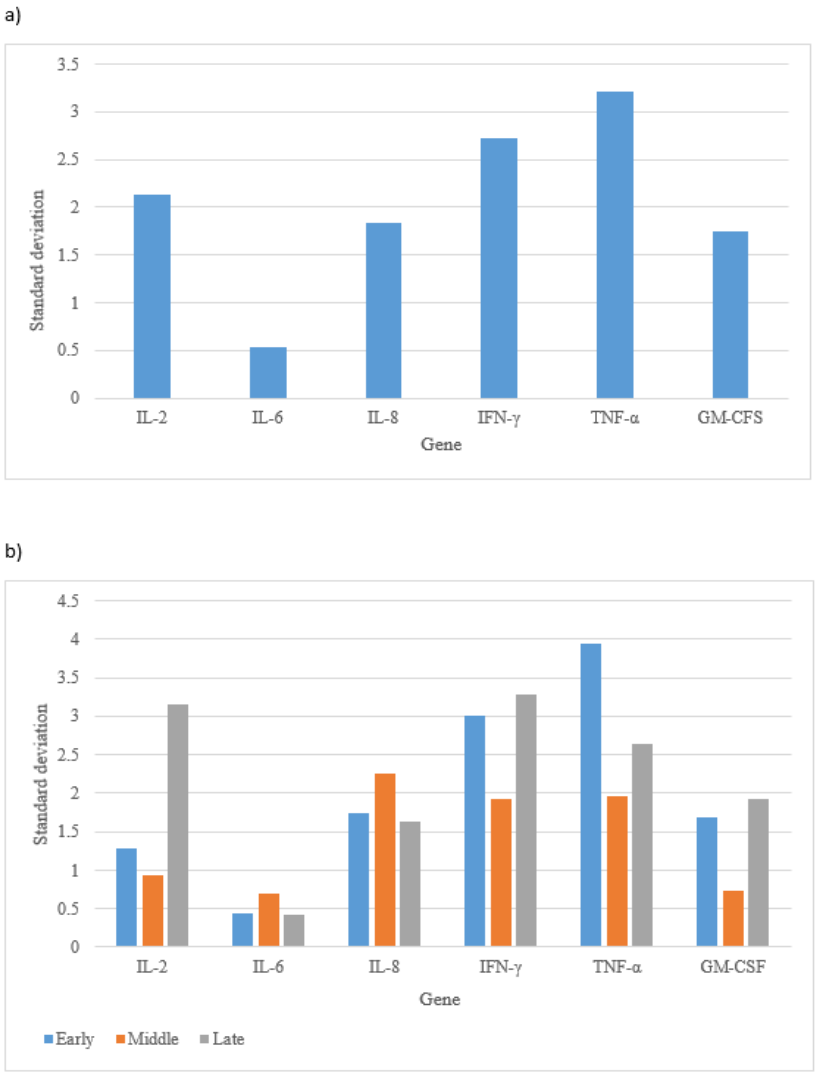

Figure 2: The standard deviations of Ct values for cytokine genes in the entire lactation (a) and different lactation stages (b)

The comparison of the relative expression of cytokine genes at different lactation stages is given in Table 2. The comparison of mRNA levels in cytokine genes was represented as folds' induction, and large variations were shown among the studied genes in terms of magnitude (Table 2). The results showed no significant difference in the expressions of the TNF-α gene in cows at the middle lactation stage

compared to animals at the early lactation stage. However, in cows at middle lactation, the expression of the TNF-α was 3.05 times higher than in cows at early lactation, but this difference was not significant (p=0.24). The expressions of other studied cytokine genes were significantly higher in cows at middle lactation compared to the early lactation stage (P<0.05).

Table 2: Relative gene expression in animals at different lactation stages (E: Early lactation stage, M: middle lactation stage, and L: late lactation stage).

Gene	Expression rate			Results		
	M vs E	L vs E	L vs M	M vs E	L vs E	L vs M
IL-2 *p*-value	92.46** 0.002	9.34 0.19	0.10 0.12	Up	–	–
IL-6 *p*-value	15.43* 0.01	5.44 0.19	0.35 0.31	Up	–	–
IL-8 *p*-value	15.52* 0.04	6.23 0.25	0.30 0.36	Up	–	–
–γIFN *p*-value	12.23* 0.03	1.81 0.71	0.148 0.19	Up	–	–
TNF-α *p*-value	3.05 0.24	11.70* 0.03	3.830 0.24	–	Up	–
GM-CSF *p*-value	100.45** 0.001	16.97* 0.02	0.169 0.10	Up	Up	–

* Statistically significant (p < 0.05)
** Statistically significant (p < 0.01)

The striking differences were found in the mRNA levels of GM-CSF and IL-2 between cows in the early and middle lactation stages. The cows in the middle lactation stage showed 100.45 and 92.46-fold increases in GM-CSF and IL-2 transcripts compared with animals in the stage of early lactation, respectively. In addition, significant differences were observed in the expression of TNF-α and GM-CFS genes at late relative to early lactation stage (p<0.05); however, the expression of other cytokine genes also showed a trend to be higher at the late lactation stage relative to early lactation, but these differences were not significant. No significant differences were observed in the expression of cytokine genes in cows at the late lactation stage relative to cows at the middle lactation stage (Table 2).

Significant correlations were found between the expressions of cytokine genes at different lactation stages (Table 3). In the early stage of lactation, the correlation between the expression of IL-8 and GM-CSF genes was significant (p<0.05). Whereas, only the correlation between expression of IL-6 and IFN-γ genes was significant in the middle stage of lactation (p<0.05). In the late lactation stage, a significant correlation was observed in the expression of IL-2 and IL-6 (p<0.01). In addition, the correlation between IL-8 and GM-CSF was significant in the late lactation stage (p<0.05). In the entire lactation cycle, correlations between IL-8 and GM-CSF, and also, IL-8 and TNF-α were significant (p<0.05).

Table 3: Correlations between the transcriptional activity of cytokine genes in the different lactation stages and the entire lactation cycle

		IL-2	IL-6	IL-8	IFN-γ	TNF-α
IL-6	Early	0.39				
	Middle	-0.65				
	Late	0.98**				
	Entire	0.14				
IL-8	Early	-0.10	-0.05			
	Middle	0.48	-0.19			
	Late	0.25	0.28			
	Entire	0.11	0.03			
IFN-γ	Early	0.66	0.39	-0.77		
	Middle	0.56	-0.89*	0.05		
	Late	0.31	0.34	-0.34		
	Entire	0.31	-0.01	-0.41		
TNF-α	Early	0.48	0.64	-0.28	0.63	
	Middle	-0.68	0.25	-0.55	-0.09	
	Late	0.57	0.50	0.25	0.64	
	Entire	0.05	0.52	-0.07	0.40	
GM-CSF	Early	0.13	0.17	-0.89*	0.78	0.54
	Middle	-0.36	-0.04	0.47	-0.13	0.27
	Late	0.001	0.001	-0.82*	0.35	-0.23
	Entire	0.23	-0.12	-0.52*	0.28	0.01

* Statistically significant ($p < 0.05$)
** Statistically significant ($p < 0.01$)

Discussion

The somatic cells of milk play an important role in the innate immune defense of the bovine mammary gland. Macrophages are the most abundant cell type (54–83%) in milk from healthy mammary glands, whereas neutrophils become the predominant cell type (>95%) in milk during mastitis. Milk somatic cells rapidly increase to reach 10^6 cells/ml or higher in the acute phase of mastitis (Lee et al, 1980; Sordillo et al, 1997). Gene expression of milk somatic cells mainly depends on mammary gland conditions (healthy or infected), lactation stages and cycle, and pathogens types (Alluwami, 2002; Boulanger et al, 2003; Lee et al, 2006; Pisoni et al, 2010). Expression of immunity-related genes represents only 1% of the total expressed genes in the milk somatic cells of the caprine mammary gland under normal conditions, while a significant transcriptomic disruption occurred in milk somatic cells after 24 h in experimentally infected mammary glands by *S. aureus* (Pisoni et al, 2010). The most abundant genes expressed in somatic cells of infected goat mammary glands included pro-inflammatory cytokines, chemokines, and their receptors (Pisoni et al, 2010). The cytokines are produced in various types of milk somatic cells such as leukocytes, especially T-cells, macrophages, and mammary epithelial cells (Smith and Goldman, 1968). They are known as not only pro- and/or anti-inflammatory agents to neutralize harmful pathogens but also act as growth stimulants by affecting the immune system maturation and development (Brenmoehl et al, 2018). Furthermore, the cytokines are sensitive tools to study the immune response of the bovine mammary gland and serve as a suitable marker to monitor udder health. The regulatory role of cytokines in mobilizing innate and adaptive immunity of bovine mammary glands is well-documented (Sordillo et al, 1997). In this study, transcriptions of pro-inflammatory cytokines (IL-2, IL-6, IL-8, IFN-γ, TNF-α, and GM-CSF genes) were detected in milk somatic cells of all healthy cows at the early, middle, and late stages of lactation.

Leutenegger et al, (2000) also reported high levels of transcription for TNF-a, GM-CSF, IL-12 p40, and IFN-γ in milk somatic cells of healthy Holstein cattle at middle lactation. However, mRNA expressions of IL-12 p40 and IL-8 genes were significantly lower than the TNF-a, GM-CSF, and IFN-γ (Leutenegger et al, 2000). IFN-γ and IL-6 mRNAs were also identified in the milk somatic cells of all healthy cows, but no transcribed mRNA was detected for TNF-α in any samples (Taylor et al, 1997). Bovine GM-CSF was found in normal bovine mammary glands at middle and late lactation with a significant elevation in its transcriptional activity in late lactation (Alluwaimi and Cullor, 2002). The high transcriptional level of TNF-α was also observed at the late lactation stage (Rewinski and Yang, 1994; Sordillo et al, 1995; Allowiami and Cullor, 2002). A comparison of healthy animals in two cattle breeds (Gyr with Black and White Holstein cattle) showed a significantly lower expression of IL-2 and IFN-γ genes in Gyr cows, whereas no significant difference was observed in mRNA levels between Gyr and BW Holstein cows for IL-4, IL-6, IL-8, IL-10, and TNF-α genes (Fonseca et al, 2009). In dairy cows and heifers, the expressions of IFN-γ and TNF-α displayed in two groups; however, the mRNA level of IFN-γ was significantly higher in cows 2 weeks after parturition (Jonsoon et al, 2013). The highest expressions of TNF-α and COX-2 were also found in the milk somatic cells of Holstein and Brown Swiss dairy cows (Pfaffl et al, 2003). The serum concentrations of pro-inflammatory cytokines (i.e. TNF-α, IL-1β, and IL-6) increased during middle and late gestation in the murine model (Orsi et al, 2006). In our study, among cytokine genes, higher expression was observed for IL-6 in the entire and three lactation stages, while IL-2 showed lower mRNA transcription levels in the entire, early, and late stages of lactation. However, higher expression was found for the TNF-α gene in the middle lactation stage. IL-2 plays an important role in stimulating the immunological memory of T cells and is necessary for the proliferation and activation of specific cytotoxic T cells (Smith, 1988). In this study, the lower levels of IL-2 expression with low somatic cells may indicate a lower probability of mammary glands' exposure to foreign antigens.

Based on the observed variability, the studied cytokine genes can be ordered from the most stably expressed, exhibiting the lowest variation, to the least stable one, exhibiting the highest variation. In this study, the expression profile of IL-6 showed the highest stability in the entire stages of lactation. This means that there is low variation in mRNA levels of IL-6 among samples compared to other genes. In contrast, high variability was observed for TNF-α, IL-8, and IFN-γ for the early, middle, and late lactation stages, respectively. Johnson et al, (2013) found higher variability in the expression of cytokine genes between heifers and dairy cows. A relationship was found between the expression of IL-2 and the lactation numbers, in which the variability in heifers was lower than in cows (Jonsoon et al, 2013). The mRNA expression of blood cells for IFN-γ, TNF-α, IL-17, and IL-10 greatly varied between individual healthy dairy cows during the first week after calving (Heiser et al, 2015). Genetic variations in immunity-related genes can cause variations in the inflammatory response to pathogens in different inflammatory conditions. Additionally, single nucleotide polymorphisms (SNPs) in promoter regions of cytokine genes may alter the transcription rate. Numerous studies have confirmed the association of genetic variations in bovine cytokine genes with susceptibility or resistance to mastitis disease in dairy cattle (Khan et al, 2023; Usman et al, 2015). Therefore, genetic polymorphisms in bovine cytokine genes could play an important role in differences between animals in their cytokine

expressions and immune responses or resistance to mastitis pathogens.

Also, no significant correlations were found between transcriptional activities of the most studied cytokine genes in the entire and different lactation stages in animals at first lactation cycle. Expression of IL-2 positively correlated with the IL-6 in the late lactation. In the middle of lactation, mRNA levels of IL-6 negatively correlated with the IFN-γ. In addition, positive significant correlations were observed between the expression of IL-8 and GM-CSF in the entire, early and late stages of lactation. In an earlier study, a significant correlation was reported between GM-CSF and TNF-α in the late and middle lactation period in the second-and third-lactating Holstein cows (Alluwaimi and Cullor, 2002). Furthermore, the correlation analysis indicated that the transcriptional activity of GM-CSF and IFN-γ significantly correlated to TNF-α at the middle lactation stage (Alluwaimi and Cullor, 2002). Bhatt et al, (2012) reported a significant and positive correlation among IFN-γ, GM-CSF, and TNF-α in crossbred cows but no significant correlation was found among these cytokine genes in Gir and Kankrej cattle breeds. IL-1β concentration was significantly correlated with IL-6 during the first month after calving in multiparous Holstein dairy cows (Trevisi et al, 2015). In general, the production of pro-inflammatory cytokines occurs due to the stimulation of NF-kB and MAPK cell signaling pathways by Pathogen Recognition Receptors (PRRs), namely Toll-like receptors (TLRs) (Guo et al, 2017). NF-kB is a key transcription factor that plays an important role in various physiological and pathological processes such as cell growth, metastatic functions, and inflammation (Kulms et al, 2006). The NF-κB activation has been demonstrated in regulated patterns during the various stages of mammary gland development (Connelly et al, 2010). In addition, the effect of NF-κB could be observed in the obvious symptoms of many infectious diseases, including inflammation of the mammary glands, and mastitis (Naugler and Karin, 2008). The bacterial lipopolysaccharides (LPS) start their pathogenesis using NF-κB cell signaling to cause mastitis after binding with relevant TLRs on epithelial cells of mammary glands (Khan et al, 2020). The IL-8 and GM-CSF are NF-κB-dependent cytokines that are involved in initiating and perpetuating neutrophilic inflammation. The level of NF-κB activity was extremely correlated with the expression levels of IL-8 and GM-CSF in milk cells of mastitis-affected cows (Boulanger et al, 2003).

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Conflict of Interest

There was not any conflict of interest in this research.

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Comparison of intraperitoneal medetomidine and paraincisional bupivacaine on post-operative pain management of ovariohysterectomy in dogs

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Abstract

Providing effective pain relief after surgery in veterinary medicine is a crucial aspect of ethical and clinical care, particularly during procedures like spaying. Various analgesic drugs have been used for this purpose, but the management of analgesia in different animals during and after surgery requires different strategies. In this study, the effect of medetomidine and bupivacaine with different methods of administration in ovariohysterectomy surgery was investigated in bitches. Twenty-Five native breed bitches (1-4 years, 15-25 kg) were divided into 5 groups of 5 based on the type of drug and the method of administration: control group, medetomidine IM, medetomidine IP, medetomidine IP and bupivacaine SC, and bupivacaine SC alone. In all groups, defined drugs for each group were administered at three stages: prior to the skin incision, simultaneously with ligation of the first ovarian pedicle, and before suturing the *linea alba*. Before, during and after surgery, sedation quality, pain quality, anesthesia depth, recovery quality, vital signs (body temperature, respiratory rates, cardiovascular parameters), and biochemical, parameters were measured at predetermined times. After surgery, analgesia was measured by Simple Descriptive Scores (SDS), Visual Analogue Scale (VAS), University of Melbourne Pain Scale (UMPS), and Glasgow Composite Measure Pain Scale-Short Form (CMPS-SF) tests. The obtained data were analyzed with SPSS software and appropriate statistical tests. The results indicated that administering the same dose of intramuscular medetomidine compared to intraperitoneal medetomidine resulted in significant differences in pain (measured by CMPS-SF and VAS test), heart rate, and cortisol levels at specific times after surgery. Administering bupivacaine alone significantly reduced surgical pain and decreased recovery time compared to administering medetomidine alone or in combination with bupivacaine. Animals receiving intraperitoneal medetomidine required a rescue dose, while no rescue dose was needed in other groups. The doses used in all groups did not disrupt the animals' physiological functions, and cardiovascular, respiratory, and rectal temperature parameters remained within the normal range. The activity of serum enzymes related to general tissue integrity also stayed within the normal range. Evaluation of pain using VAS, UMPS, and CMPS-SF methods did not show a preference for the effectiveness of administering the same dose of intraperitoneal medetomidine over intramuscular medetomidine (alone or with bupivacaine at the surgical incision) for managing pain, recovery after surgery, and physiological parameters of cardiovascular, respiratory, and rectal temperature.

Key words: Pain management, Ovariohysterectomy, Female dogs, Medetomidine, Bupivacaine

Introduction

Sneddon (2009) defines pain in animals as follows: "The perception and aversive sensory experience of a noxious stimulus associated with potential or actual injury"

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(Walters, 2018). Pain is classified into physiological and pathological types. Physiological pain arises without tissue damage, alerting the animal to potential harm. Pathological pain occurs following tissue and nerve damage; its intensity may vary depending on the severity and speed of tissue injury (Kania et al, 2021). Following tissue destruction, a series of local and systemic responses such as inflammation are created in the organism. Inflammation occurs following a chain of organized cellular and vascular responses that include the secretion of a group of inflammatory mediators and signaling molecules (such as histamine, prostaglandin, and leukotriene, free radicals derived from oxygen, nitrogen, and serotonin) by immune defense cells (Abdulkhaleq et al, 2018). Analgesics are essential for managing pain in veterinary medicine, most commonly for postoperative pain and also for various other painful conditions. If the administration of analgesics is ignored in animals, an increase in stress and its related consequences, i.e. an increase in norepinephrine, epinephrine, cortisol, vasopressin, renin, angiotensin II, aldosterone, ACTH, glucose, and a decrease in insulin and testosterone levels occur (Kania et al, 2021). Alpha-2 receptor agonist drugs, such as medetomidine, in the cortical layer of the brain, brain stem, and spinal cord are used as sedatives and hypnotics in pre-anesthesia protocols. The combination of medetomidine with some drugs like alfaxalone has been used to induce sedation or pre-anesthesia in dogs and cats (Kamohara et al, 2022). Medetomidine is associated with side effects such as hyperglycemia, pulmonary edema, bradycardia, and decreased blood pressure, cardiac output, body temperature and increased cardiac afterload (Raekallio et al, 2017). Among the reasons for surgery and the need for anesthesia in animals is ovariohysterectomy to control the population, prevent diseases of the reproductive system, and weaken unpleasant behaviors related to sex hormones. The mild to moderate pain experienced by the animal in this surgery requires appropriate analgesics. Therefore, it is necessary to determine the preference for using the analgesics from an economic and ethical point of view (Rezaeipour et al, 2022).

Intraperitoneal administration of local anesthetics is a valuable and validated method for pain control after abdominal surgery. Due to capillary structures in the peritoneum and wide surfaces for drug exchange between the peritoneum and plasma, this part is used in medicine and veterinary medicine along with intravenous, intramuscular, and subcutaneous methods. In this method, before suturing the *linea alba*, a local anesthetic or analgesic is splashed on the viscera. Intraperitoneal administration of lidocaine or bupivacaine has been shown to provide adequate analgesia after ovariohysterectomy surgery in dogs (Chilkoti et al, 2019). In a study, medetomidine was used as an intraperitoneal infusion to control pain after laparotomy in pregnant sheep, and its effects in creating a continuous plasma concentration within 10 hours; also, controlling appropriate pain after surgery without other analgesic compounds have been proven (Murdoch et al, 2013). In human medicine, intraperitoneal administration of dexmedetomidine (an active isomer of medetomidine) along with local anesthetics such as ropivacaine and bupivacaine has been used for analgesia after laparoscopy, and its effectiveness was reported (Kandi et al, 2022).

Since ovariohysterectomy is one of the most common surgeries in spaying a bitch and pain management is important in this type of surgery, this study aims to investigate the effectiveness of intraperitoneal and/or intramuscular administration of medetomidine at the same time as the administration of bupivacaine at the surgical site for pain management after ovariohysterectomy.

Materials and methods

Animals

Twenty-five adult female dogs of native breed (aged 1-4 years and weighing 15-25 kg) were selected and examined by a small animal veterinary specialist for signs of inflammation or infection. A blood test (Complete Blood Count (CBC), total protein) was done to confirm their health status. A wet diet included canned dog food and clean water available to all dogs. The dogs were acclimated to the new environment and people involved in the study for seven days. Subsequently, they were randomly divided into five groups of 5 based on the type of drug and the method of administration.

1. Control Group: After aseptic preparation of the surgical site and prior to the skin incision, saline (0.2 ml/kg, SC) was injected into the subcutaneous tissue around the incision site; saline (0.9%, IM) was injected simultaneously with ligation of the first ovarian pedicle; saline (0.2 ml/kg, IP) was splashed into the peritoneal cavity before suturing the *linea alba*.

2. Medetomidine IM Group: After aseptic preparation of the surgical site and prior to the skin incision, saline (0.2 ml/kg, SC) was injected into the subcutaneous tissue around the incision site; Medetomidine (20 µg/kg, IM) was injected simultaneously with ligation of the first ovarian pedicle; saline (0.2 ml/kg, IP) was splashed into the peritoneal cavity before suturing the *linea alba*.

3. Medetomidine IP Group: Saline (0.2 ml/kg, SC) was injected into the surgical incision sites; saline (0.9%, IM) was injected simultaneously with ligation of the first ovarian pedicle; Medetomidine (20 µg/kg, IP) was splashed into the peritoneal cavity before suturing the *linea alba*.

4. Medetomidine IP and Bupivacaine SC Group: Bupivacaine (1mg/kg, SC) was injected into the surgical incision sites; saline (0.9%, IM) was injected simultaneously with ligation of the first ovarian pedicle; Medetomidine (20 µg/kg, IP) was splashed into the peritoneal cavity before suturing the *linea alba*.

5. Bupivacaine SC Group: Bupivacaine (1mg/kg, SC) was injected into the surgical incision sites; saline (0.9%, IM) was injected simultaneously with ligation of the base of the first ovarian pedicle; saline (0.2 ml/kg, IP) was splashed into the peritoneal cavity before suturing the *linea alba*.

Preparation of animal before surgery

Access to the food and water was restricted 12 and 2 hours before surgery, respectively. The hair over the cephalic veins in both forelimbs was shaved,–and a peripheral venous catheter (20 gauges) was inserted in both cephalic veins for administration of the fluids and blood sampling. Sedation was induced with Acepromazine (0.05mg/kg, IM) and Morphine (0.5mg/kg, IM) (Lambertini et al, 2018).

The skin from the xiphoid to the pubis and laterally to 10 cm on either side of the ventral midline was clipped. Cefazolin (22 mg/kg, IV) was administered as prophylaxis. Five minutes before induction of general anesthesia, oxygen was provided via a flow-by system. Anesthesia was induced with propofol (6 mg/kg, IV) and titrated until the jaw was easily opened and intubation was possible. Dogs were positioned in dorsal recumbency and the monitoring leads were attached. Anesthesia was maintained with an inhalation anesthesia machine equipped with an isoflurane vaporizer (concentration 1.5%) and an oxygen flow of 1.5 liters. Ringer's lactate solution (5 ml/kg/hour) was infused intravenously throughout the surgery. The surgical area was prepared aseptically with betadine scrub (7.5%) and chlorhexidine (2%).

Surgical Technique

All the procedures of this study were performed by a fixed three-member team, one surgeon, and two non-scrubbed assistant (blinded to the allocation) for blood sampling and data recording during and after surgery. The evaluations were carried out in specific timings that started 5 minutes before the induction of anesthesia

(T0) and ended (T11) 6 hours after the completion of the surgery. Based on this, the time of skin incision (T1), *linea alba* incision (T2), ligature of the first ovarian pedicle (T3), skin suture (T4), and 30, 60, 120, 180, 240, 300, 360 minutes (T5-T11) were defined after the completion of surgery. Blood samples were collected at baseline (T0), skin incision (T1), skin closure (T4), and at 2, 6, and 24 hours post-surgery (T7, T11, and T24, respectively) in separate tubes. During the ovariohysterectomy surgery, the required parameters were obtained by the monitoring device and recorded in specific forms.

Ovariohysterectomy

The main surgical procedure was performed from the midline approach. First, using a scalpel blade (number 15), an incision of about 10 cm was made on the lower skin surface of the umbilical scar. The linea alba was exposed with Metzenbaum scissors and then incised. The peritoneal cavity was subsequently accessed (Bencharif et al, 2010).

Using a spay hook, the left ovary was searched and revealed, then the suspensory ligament was transected. After creating a window in the broad ligament of the uterus (close to the ovarian pedicle), the hemostat forceps were placed on the ovarian pedicle. The vessels were ligated with the polyglycolic acid synthetic absorbable sutures (number 0) and transected with scissors. Following confirmation of the hemostasis, the ovarian pedicle was released into the abdominal cavity. These steps were repeated for the right ovary. Lastly, the uterine body (caudal to the uterus horns) was double ligated and excised cranial to the cervix. After ensuring that there was no bleeding, it was released into the abdominal cavity.

The surgical incision was closed in three layers:

Linea alba: simple continuous suture with 0 PGA suture material

Subcutaneous tissue: simple continuous suture with 3-0 PGA suture material

Skin: cruciate pattern suture with 3-0 non-absorbable nylon suture material

The time of the final suture was recorded, and the surgical site was dressed.

Evaluations of some parameters during and after surgery

Evaluation of the quality of sedation: After 30 minutes of intramuscular injection of sedative drugs, the quality of sedation was scored from 0 to 3. Score 0: no sedation. Score 1: mild, Score 2: moderate sedation. Score 3: deep sedation (Mair et al, 2009).

Assessment of the pain quality and depth of anesthesia: The following method was used to evaluate the rate of painful stimuli after anesthesia induction and tracheal intubation. A Rochester-Pean hemostatic forceps was used to apply pressure on the second toe joint of the left hind foot for 30 seconds or until the animal withdrew the foot. The degree of response to stimulation was scored based on the following grading: 0: No response; 1: Weak and low movement; 2: Retraction of limbs; 3: Limb retraction accompanied by head elevation (Muir et al, 2009).

Recovery Quality Evaluation: This parameter was evaluated from stopping the anesthetic to standing the animal completely and scored as follows, 1: Lying on the sternum with low or no struggle, standing and walking without a problem or with the tiniest trouble. 2: Low struggle needs help to lay on the sternum and stand, respond to external stimuli, and silence the animal after lying on the sternum. 3: Struggle for a long time, difficulty on the sternal positioning and standing, enhance the feet and taps for a long time when helping the animal (Muir et al, 2009).

Duration of surgery*:* This parameter was recorded from the start of the skin incision to the last skin suture.

Duration of anesthesia: It was recorded from the time of the tracheal intubation to the time the patient was standing.

Vital signs: Rectal temperature; Respiratory rate, percentage of hemoglobin oxygen saturation (SpO_2), End-tidal CO_2 ($EtCO_2$); Cardiovascular indicators including systolic

blood pressure, diastolic blood pressure, mean arterial blood pressure, heart rate and capillary refill time (CRT). The parameters were measured after tracheal intubation by a multi-parameter monitoring device, and a capnograph machine (Drager Company, Lubeck, Germany). The CRT was assessed by applying pressure on the gingival mucosa.

In order to evaluate the cardiovascular effects of medetomidine, heart rate, CRT, SpO_2, systolic, diastolic, and mean arterial blood pressure were measured at 5, 10, 15, and 20 minutes after the intramuscular or peritoneal administration of medetomidine.

Blood Tests: Serum glucose, and cortisol levels, along with lactate dehydrogenase (LDH), Aspartate aminotransferase (AST), and Creatine kinase (Bussen et al.) activity were measured. at T0, T1, T4, and T7, T11, and T24.

To measure the level of glucose and the activity of CK, LDH, and AST enzymes, a commercial kit (BiorexFars, Fars, Iran) and an autoanalyzer (BT-1500 biochemical analyzer, Italy) were applied. The competitive ELISA method was used to measure cortisol concentration according to the manufacturer's instructions for the commercial kit (Monobind Company, USA).

Analysis of analgesia after surgery: We used different methods to investigate the postoperative analgesia; simple descriptive scores (SDS), Visual Analogue Scale (VAS), University of Melbourne Pain Scale (UMPS), and Glasgow Composite Measure Pain Scale-Short Form (CMPS-SF) at 0, 15, and 30 minutes and then at 1, 2, 3, 4, 5, and 6 hours after the surgery (Baniadam et al, 2021; Lambertini et al, 2018).

statistical analysis

The SPSS software version 24 (IBM Corporation, NY, USA) was used to analyze the data. One-way ANOVA (analysis of variance) with Tukey's post hoc test was utilized to compare the data obtained from the heart rate, respiration rate, arterial blood pressure, rectal temperature, plasma glucose, and cortisol levels among the groups. Repeated measures ANOVA was employed to evaluate these parameters within the groups over time. The Kruskal-Wallis test was used to compare the data obtained from pain assessment among the groups. Friedman's test assessed differences within the groups for these parameters. Results were presented as mean ± standard error. For qualitative parameters, results were presented as median (minimum-maximum), with $p<0.05$ values considered significant in all statistical analyses.

Results

Weight, Length of surgical incision, Duration of surgery and Duration of anesthesia

Among the parameters of mean weight, length of surgical incision, duration of surgery, and duration of anesthesia in each of the studied groups, only the duration of anesthesia showed a statistically significant difference between groups (P=0.005). However, there was no statistically significant difference in other parameters (Table 1).

Table 1: Mean ± Standard deviation of weight, length of surgical incision, duration of surgery and duration of anesthesia in different groups (n=5)

Group	Weight (kg)	Surgery duration (min)	Anesthesia duration (min)	Length incision (cm)
Control	23.10±6	31.33± 6	88.7±41	6.94±0.65
Med-IM	23.17±5	31.45±5	195.98±53 [a]	6.93± 0.54
Med-IP	23.45±2.3	29.67±2	205.64±52 [a]	6.87± 0.44
Med-Bup	24.64±3	27.69±5	190.12±66 [a]	6.17± 0.44
Bup-SC	22.76±4.5	29.86±2	113.13±30	6.18± 0.27

Med: Medetomidine; IM: Intramuscular; IP: Intraperitoneal; SC: Subcutaneous; Bup: Bupivacaine.
[a] Significant difference with the control group (P=0.005)

Anesthesia Parameters

Among the parameters of anesthesia, there was a statistically significant difference in terms of recovery quality in each of the studied groups. The quality of recovery in the medetomidine groups was poorer compared with the Bup-SC group and the control group (P<0.05) (Table 2).

Table 2: Mean ± standard deviation of sedation, depth of anesthesia and recovery quality in different groups (n=5)

Groups	Sedation quality	Depth of anesthesia	Recovery quality
Control	2.4±0.5	0.8±0.5	1.6±0.5
Med-IM	2.4±0.5	0.8±0.4	2.8±0.4[a]
Med-IP	2.4±0.5	1.4±0.5	3±0[a]
Med-Bup	2.4±0.9	0.6±0.5	3±0[a]
Bup-SC	2.4±0.5	0.4±0.3	1.4±0.5

Med: Medetomidine; IM: Intramuscular; IP: Intraperitoneal; SC: Subcutaneous; Bup: Bupivacaine.
[a]Significant difference with the control group (P<0.05)

Vital Parameters

Cardiovascular Parameters

The data analysis did not reveal a statistically significant difference in the *Cardiovascular Parameters* between the groups (Table 3). Changes in *Cardiovascular Parameters* at different time points among the groups were also not significant [F(6,36)=0.6, P=0.72].

There was a significant increase in diastolic blood pressure in the Med-IM and Med-Bup groups at T4 compared to the control group. Additionally, in the Med-Bup group at this time, there was a significant increase in diastolic pressure compared to the Med-IP group (Table 3). The mean diastolic blood pressure in the groups receiving medetomidine after five-minute intervals showed a statistically significant difference. In the Med-Bup group, at 15 minutes after the administration of medetomidine, there was a significant increase in diastolic blood pressure compared to the Med-IP group (P=0.028) (Table 5).

A significant increase in the mean arterial blood pressure was observed in the Med-IM (P=0.019) and Med-Bup (P=0.045) groups at T4 compared to the control group (Table 3).

A significant decrease in the mean heart rate was observed in the Med-IM (P=0.014), Med-IP (P=0.005), and Med-Bup (P=0.001) groups at T4, as well as in these same groups at T5 (P=0.001) and T7 [Med-IM (P=0.003), Med-IP (0.038), Med-Bup (P=0.026)] compared to the control group. Additionally, at T3, the Med-IP and Med-Bup groups showed a significant decrease (P=0.022) compared to the Med-IM group (Table 3). The mean heart rate at different time points in various groups exhibited statistically significant changes [F (22,110) = 3.03, P<0.0001]. Fluctuation in the mean heart rate in the groups receiving medetomidine at five-minute intervals after administration showed a significant decrease in the Med-IP and Med-Bup groups (P=0.022) compared to the Med-IM group (Table 5).

Capillary refill time (CRT) was within the normal range in all groups. The results showed statistically significant differences in this factor at T2 and T3 times among the groups. The CRT was lower in the medetomidine-administered groups compared to the control group (Table 3). Changes in the CRT over time in different groups were not significant [F (12, 63) =1.2, P=0.30].

Respiratory Parameters and Rectal Temperature

The percentage of hemoglobin saturation with oxygen (SpO_2) was within the normal range in all groups, but there were differences observed in some groups. At T6, there was a significant decrease in the Med-IM group compared to the control group (P=0.018). At T10, the percentage of SpO_2 was significantly decreased (P=0.044) in the Med-Bup and Bup-SC groups compared

to the control group (Table 4). The mean fluctuations of SpO2 over time in different groups were not statistically significant [F(14,70)=1.07, P=0.39].

There was no statistically significant difference between groups in EtCO2 (Table 4). Fluctuations in EtCO2 over time in different groups also showed no statistically significant difference [F(7.5,37)=0.55, P=0.79].

In all groups, the mean breathing rate was within the normal range, but differences were observed in some groups. At T6, there was a statistically significant decrease in breathing rate in the Med-IP group compared to Bup-SC (P=0.039) (Table 4). Fluctuations in the number of breaths per minute overtime in different groups were not significant [F(22,114)=0.95, P=0.53].

The mean rectal temperature in the Med-Bup and Med-IP groups at T4 (P=0.019) and T6 (P=0.03) increased significantly compared to the control group (Table 4). However, changes in body temperature at different times across various groups did not show any statistically significant differences [F (27, 138)=1.5, P=0.069].

Pain Assessment

Pain evaluation using the SDS method at different times in different groups did not show significant differences among the groups (Figure 1). However, there was a significant variation in pain scores over time within each group at the specified intervals [$\chi 2$= 35; P<0.0001]. The highest median pain score was recorded at T7 in the Med-IM and Med-IP groups.

When evaluating the pain by using the VAS method, there was a statistically significant difference in pain ratings among the groups (P<0.05). At T6, median pain score in the Med-IM group was significantly lower than control, Med-IP and Bup-SC; but difference between Med-IM and Med-bup was not significant (P>0.05). At T7 and T8, the pain rating in Bup-SC group was lower compared to the control, Med-IM and Med-IP groups (P<0.05) (Figure 2). No statistically significant difference was observed between groups at T9-T11. There was also a statistically significant difference in pain rating fluctuations over time among the groups [$\chi 2$=91; P<0.0001].

Furthermore, pain evaluation using the UMPS method, a statistically significant difference was observed in pain ratings among the groups (P<0.05). Specifically, at T6, the pain rating in the Med-IM, Med-Bup and Bup-SC groups was significantly lower than the control group. The difference between Med-IP and control group was not significant at this time (P>0.05). Additionally, the median pain score at T7 in the Med-Bup and Bup-SC groups was lower than the control group, but this difference was not statistically significant (P>0.05) (Figure 3). No statistically significant difference was observed between groups at T8-T11. Similar to the VAS method, a statistically significant difference was observed in pain rating fluctuations over time among the groups [$\chi 2$= 93; P<0.0001].

There was a statistically significant difference in pain ratings among the groups in the CMPS-SF (P < 0.05). At T6, median pain score in the Med-IM group was significantly lower than other groups (P<0.05). At T7, no statistically significant difference was observed between groups. At T8, the median pain score in the Bup-SC group was significantly lower than control, Med-IM and Med-IP; but, at this time, median pain score in the Med-Bup group was lower than Bup-SC significantly (P < 0.05) (Figure 4). No statistically significant difference was observed between groups at T9-T11. A statistically significant difference was observed in the changes in pain ratings over time in different groups [$\chi 2$ = 108; P < 0.0001].

During the experiment, three dogs in the control group and two dogs in the Med-IP group received a dose of morphine (0.5 mg/kg, IM) as a rescue dose after surgery. None of the groups receiving analgesia showed significant changes in the physiological parameters investigated in this study.

Table 3: Mean ± standard deviation of cardiovascular parameters in different groups at different times (n=5)

Variable	Groups	T0 (a)	T1 (b)	T2 (c)	T3 (d)	T4 (d)	T5 (e)	T6 (f)	T7 (g)	T8 (h)	T9 (i)	T10 (j)	T11 (k)
Systolic arterial pressure (mmHg)	Control (A)	119 ±11	89 ±14	96 ±9	102 ±14	110 ±23	121 ±15	134 ±26	126 ±15	140 ±18	132 ±8	140 ±17	141 ±13
	Med-IM (B)	129 ±6	106 ±23	105 ±15	120 ±14	123 ±10	112 ±21	113 ±16	122 ±25	126 ±21	143 ±18	132 ±13	139 ±18
	Med-IP (C)	118 ±13	99 ±10	97 ±13	104 ±22	109 ±9	108 ±10	122 ±14	129 ±12	120 ± 12	130 ±27	143 ±19	131 ±7
	Med-Bup (D)	129 ±19	102 ±5	98 ±13	119 ±9	113 ±9	108 ±22	110 ±11	128 ±7	132 ±16	131 ±22	129 ±10	132 ±14
	Bup-SC (E)	118 ±20	96 ±16	100 ±18	120 ±10	101 ±10	127 ±9	125 ±15	136 ±22	133 ±10	134 ±14	134 ±7	134 ±28
Diastolic arterial pressure (mmHg)	Control (A)	63 ± 8	43 ±5	46 ±3	64 ±23	55± 14 B,D	77± 11	80 ±16	69 ±15	82 ±22	85 ±19	84 ±16	87 ±11
	Med-IM (B)	69 ± 12	47 ±18	47 ±15	68 ±23	79± 10A	80± 16	79 ±24	87 ±17	90 ±10	97 ±21	83 ±12	92 ±12
	Med-IP (C)	70 ± 20	44 ±11	45 ±8	58 ±14	61± 6D	74± 11	79 ±15	87 ±7	85 ±4	79 ±15	83 ±11	74 ±12
	Med-Bup (D)	76 ± 16	45 ±8	47 ±5	67 ±14	79± 6A,C	73± 18	76 ±18	86 ±7	79 ±6	79 ±18	77 ±11	78 ±25
	Bup-SC (E)	66 ±7	40 ±6	45 ±11	69 ±3	42±8	69± 23	80 ±17	91 ±9	76 ±14	87 ±8	90 ±11	92 ±13
Mean arterial pressure (mmHg)	Control (A)	88 ±7	60 ±19	67 ±9	79 ±21	69±13 B,D	90 ±9	93 ±19	89 ±10	97 ±23	99 ±19	99 ±15	98 ±17
	Med-IM (B)	89 ±14	62 ±19	63 ±15	84 ±20	91± 15A	90± 14	93 ±14	99 ±19	98 ±13	108 ±13	96 ±10	106 ±11
	Med-IP (C)	89 ±14	62 ±9	62 ±3	72 ±17	76±2	85 ±9	89 ±13	102 ±5	96 ±6	96 ±18	101 ±18	89 ±9
	Med-Bup (D)	94 ±13	61 ±1	57 ±10	84 ±11	89±2 A	87± 17	87 ±14	98 ±6	91 ±9	92 ±17	93 ±9	94 ±19
	Bup-SC (E)	84 ±8	58 ±8	63 ±14	87 ±8	62±8	93± 12	94 ±15	107 ±14	90 ±12	99 ±8	107 ±2	104 ±17
Heart rate (beats per minute)	Control (A)	109 ±26	91 ±55	104 ±27	95 ±21	126± 29 B,C,D	146 ±62 B,C,D	128 ±64	114 ±34 B,C,D	105 ±43	99 ±31	100 ±31	99 ±25
	Med-IM (B)	104 ±9	97 ±6	101 ±3	86± 6C,D	81± 11A	52± 16 A	51 ±14	53± 12A	62 ±9	82 ±16	85 ±11	93 ±9
	Med-IP (C)	96 ±21	80 ±17	77 ±27	72 ±7B	75± 13A	54± 14A	63 ±20	69± 23A	75 ±17	85 ±24	89 ±22	88 ±10
	Med-Bup (D)	96 ±13	76 ±15	77 ±16	69± 12B	66± 24A	55± 8A	62 ±13	67± 12A	82 ±12	78 ±13	80 ±14	82 ±7
	Bup-SC (E)	104 ±22	100 ±24	92 ±18	93 ±17	102 ±14	95± 20	108 ±51	92± 23	97 ±26	108 ±20	107 ±21	107 ± 24
Capillary refill time (second)	Control (A)	1.8 ±0.44	1.4± 0.54	1.6± 0.54 B,C,D	1.6± 0.54 B,C,D	1.4± 0.54	1.4± 0.54	1.4± 0.54	1.4± 0.54	1.2± 0.44	1±0	1±0	1±0
	Med-IM (B)	1±0	1±0	1± 0A	1± 0A	1±0	1±0	1±0	1±0	1±0	1±0	1±0	1±0
	Med-IP (C)	1.2± 0.44	1±0	1± 0A	1± 0A	1±0	1±0	1±0	1±0	1±0	1±0	1±0	1±0
	Med-Bup (D)	1.4± 0.54	1.2± 0.44	1± 0A	1± 0A	1±0	1±0	1±0	1±0	1±0	1±0	1±0	1±0
	Bup-SC (E)	1.4± 0.54	1.2± 0.44	1.2± 0.44	1.2± 0.44	1.2± 0.44	1.2± 0.44	1±0	1±0	1±0	1±0	1±0	1±0

Before induction of anesthesia (T0), Time of skin incision (T1), *Linea alba* incision (T2), Ligation of the first ovarian pedicle (T3), Skin suture (T4), and 30, 60, 120, 180, 240, 300, 360 minutes after the completion of surgery (T5-T11).

Table 4: Mean ± standard deviation of respiratory parameters and rectal temperature in different groups at different times (n=5)

Variables	Groups	T0 (a)	T1 (b)	T2 (c)	T3 (d)	T4 (d)	T5 (e)	T6 (f)	T7 (g)	T8 (h)	T9 (i)	T10 (j)	T11 (k)
SPO_2 (%)	Control (A)	96.8 ±1	97.4 ±2	96.8 ±2	96.2 ±3	95.4 ±3	94.4 ±2	97.4 ±1B	95.8 ±1	96.6 ±1	97.2 ±1	97.2± 1D,E	97.6 ±1
	Med-IM (B)	97.8 ±0 E	94.4 ±5	95.4 ±5	93.4 ±5	93.4 ±2	90.2 ±2	92.4 ±2 A	94±2	95.6 ±1	96.8 ±1	97.4 ±0	97±0
	Med-IP (C)	95.6 ±2	95.8 ±2	96.2 ±1	94.6 ±2	97±2	93.4 ±4	94.4 ±2	94.6 ±1	95.4 ±1	95.8 ±0	96±1	96.6 ±1
	Med-Bup (D)	96.2 ±1	96.2 ±3	97±2	92.4 ±6	95.4 ±3	91.8 ±2	94.4 ±2	97±1	97.2 ±1	95.2 ±2	94.4 ±2 A	96.4 ±1
	Bup-SC (E)	94.4 ±1 B	96.2 ±1	95.6 ±3	94.6 ±2	97±2	93.6 ±1	94.6 ±2	93.8 ±1	95.8 ±1	96.4 ±1	94.4 ±0 A	95.6 ±0
$EtCo_2$ (mmHg)	Control (A)	-	25 ±1	24.8 ±2	25.4 ±3	25.8 ±3	-	-	-	-	-	-	-
	Med-IM (B)	-	25.4 ±5	26.2 ±4	27 ±3	28.8 ±4	-	-	-	-	-	-	-
	Med-IP (C)	-	24.4 ±1	26.2 ±3	26.6 ±2	30.4 ±6	-	-	-	-	-	-	-
	Med-Bup (D)	-	23.2 ±8	26.2 ±3	25.4 ±3	29.4 ±4	-	-	-	-	-	-	-
	Bup-SC (E)	-	18 ±8	22.8 ±6	23±6	25.2 ±5	-	-	-	-	-	-	-
Respiratory Rate (breath per minute)	Control (A)	39± 14	34± 18	28± 19	23±4	19±8	24±5	24±4	24±4	24±7	25±6	24±5	24±7
	Med-IM (B)	39± 13	20 ±4	23± 11	21± 10	24±5	26±7	21±5	20±5	20±3	21±2	21±5	20±3
	Med-IP (C)	32±7	22 ±7	23±7	25±9	22±5	20±2	19± 3 E	20±2	20±2	22±4	22±2	21±3
	Med-Bup (D)	33±3	22 ±6	21±7	24± 10	24±8	18± 10	21±4	23±3	24±3	25±4	27±5	30±8
	Bup-SC (E)	33±2	23 ±8	30± 14	20±3	20±8	27±6	28± 4 C	30± 15	29± 15	25±3	29±4	28±1
Rectal temperature (°C)	Control (A)	38.8 ±0.5	37.9 ±0.4	37.8 ±0.4	37.8 ±0.3	37.5± 0.4 C,D	37.0 ±0.7	36.7± 0.6C,D	37.4 ±0.4	37.9 ±0.5	38.3 ±0.3	38.4 ±0.6	38.4 ±0.2
	Med-IM (B)	38.8 ±0.3	38.4 ±0.5	38.4 ±0.5	38.3 ±0.6	38.3 ±0.6	37.3 ±0.8	37.4 ±0.7	37.6 ±0.6	37.8 ±0.8	37.9 ±0.4	38.3 ±0.2	38.4 ±0.2
	Med-IP (C)	38.9 ±0.6	38.5 ±0.1	38.3 ±0.4	38.3 ±0.4	38.5± 0.17 A	37.5 ±0.5	37.7± 0.4 A	37.7 ±0.7	38.1 ±0.5	38.2 ±0.4	38.4 ±0.3	38.5 ±0.08
	Med-Bup (D)	38.7 ±0.5	38.6 ±0.2	38.5 ±0.2	38.5 ±0.2	38.5± 0.17 A	37.8 ±0.4	37.9± 0.2A	38.0 ±0.3	38.2 ±0.2	38.2 ±0.2	38.4 ±0.2	38.5 ±0.2
	Bup-SC (E)	38.8 ±0.4	38.1 ±0.5	38.0 ±0.4	37.9 ±0.5	37.8 ±0.6	37.3 ±0.3	37.6 ±0.3	37.6 ±0.5	37.8 ±0.6	38.0 ±0.5	38.2 ±0.4	38.2 ±0.4

Before induction of anesthesia (T0), Time of skin incision (T1), *Linea alba* incision (T2), Ligation of the first ovarian pedicle (T3), Skin suture (T4), and 30, 60, 120, 180, 240, 300, 360 minutes after the completion of surgery (T5-T11).

Table 5: Mean ± standard deviation of cardiovascular parameters in different groups at 5 minutes intervals after administration of Medetomidine

Variable	Groups	5min	10min	15min	20min
Systolic arterial pressure (mmHg)	Control (A)	*	*	*	*
	Med-IM (B)	125±13	121± 11	122± 10	110± 12
	Med-IP (C)	109±23	112± 20	107± 14	103± 10
	Med-Bup (D)	119±27	114±20	114±11	112±26
	Bup-SC (E)	*	*	*	*
Diastolic arterial pressure (mmHg)	Control (A)	*	*	*	*
	Med-IM (B)	78± 18	80± 9	73± 12	81± 12
	Med-IP (C)	69± 15	70± 12	62± 8 D	62± 9
	Med-Bup (D)	76± 11	73± 18	80± 9 C	72± 14
	Bup-SC (E)	*	*	*	*
Mean arterial pressure (mmHg)	Control (A)	*	*	*	*
	Med-IM (B)	93± 17	95± 9	89± 13	95± 13
	Med-IP (C)	79± 21	83± 12	75± 9	77± 4
	Med-Bup (D)	85± 14	89± 12	92± 6	80± 16
	Bup-SC (E)	*	*	*	*
Heart rate (beats per minute)	Control (A)	*	*	*	*
	Med-IM (B)	86±6 C,D	84± 11	87±7	75± 13
	Med-IP (C)	72± 7 B	74± 6	72± 6	69± 12
	Med-Bup (D)	69± 12 B	70± 25	71± 19	64± 20
	Bup-SC (E)	*	*	*	*
SPO_2 (%)	Control (A)	*	*	*	*
	Med-IM (B)	92.8± 5.1	93.6± 3.8	93.2± 3.2	93.4± 3
	Med-IP (C)	93.2± 3.1	94.8± 2.7	96± 4	96.4± 3.9
	Med-Bup (D)	94.2± 5.8	94.6± 6.4	94.2± 4.9	94.6± 3.3
	Bup-SC (E)	*	*	*	*

* Capital letters in each column indicate significant differences between groups ($p < 0.05$).

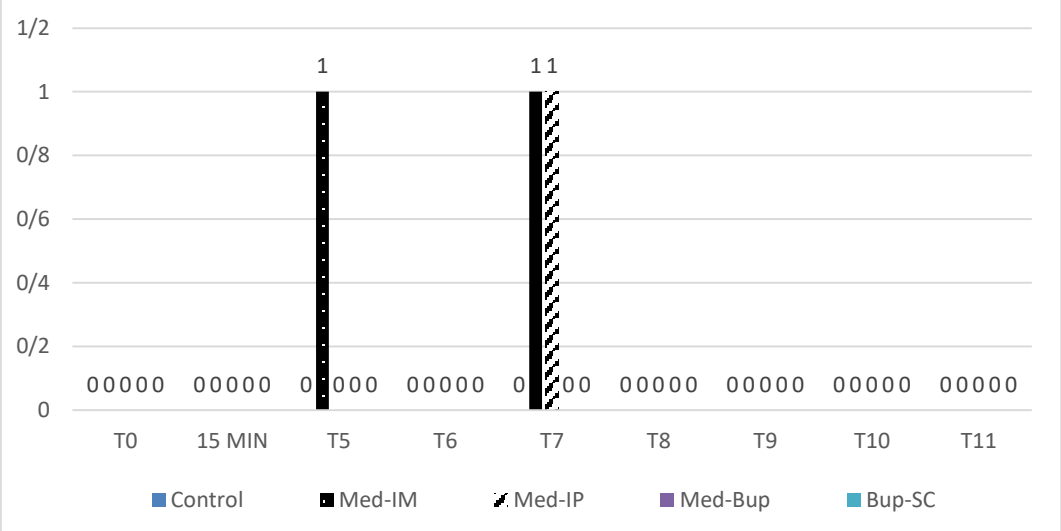

Figure 1: Median (Minimum-Maximum) Pain Assessment with SDS

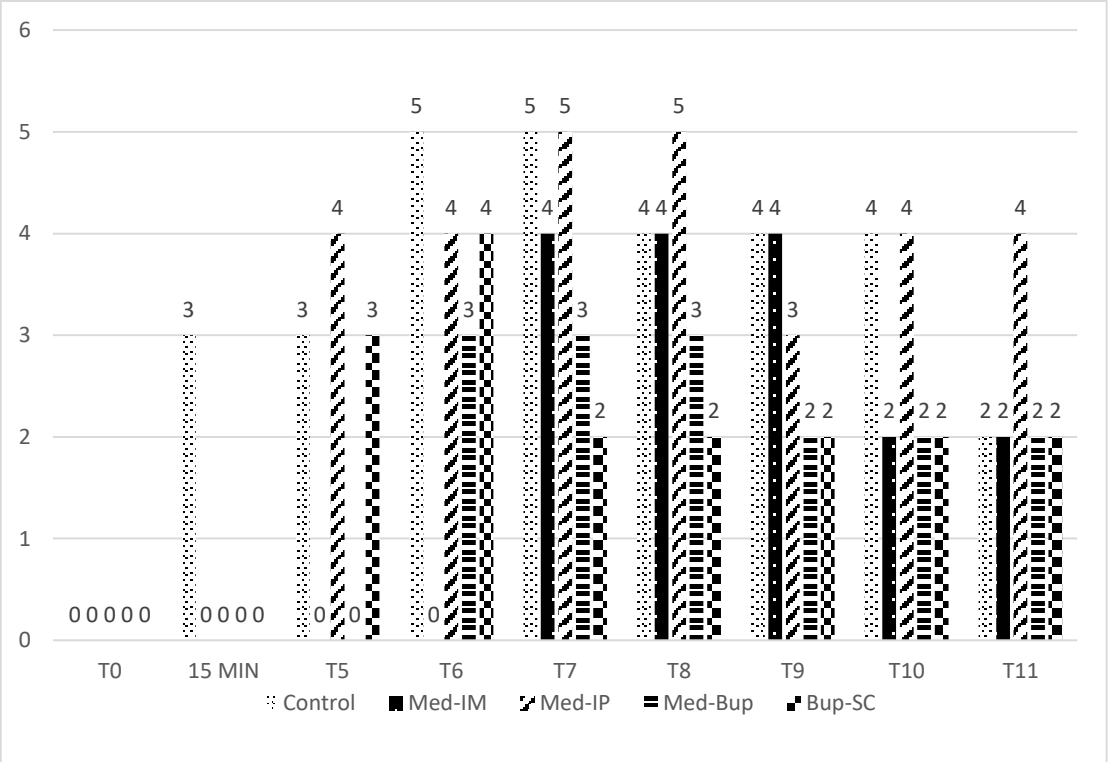

Figure 2: Median (Minimum-Maximum) Pain Assessment with VAS

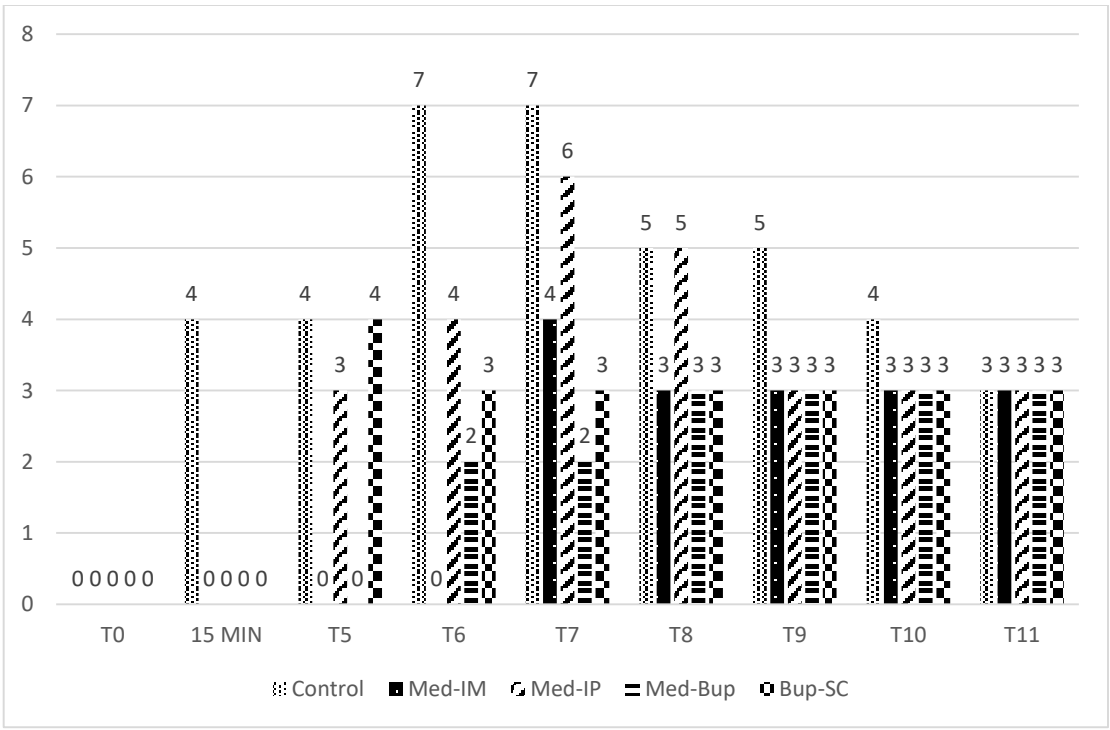

Figure 3: Median (Minimum-Maximum) Pain Assessment with UMPS

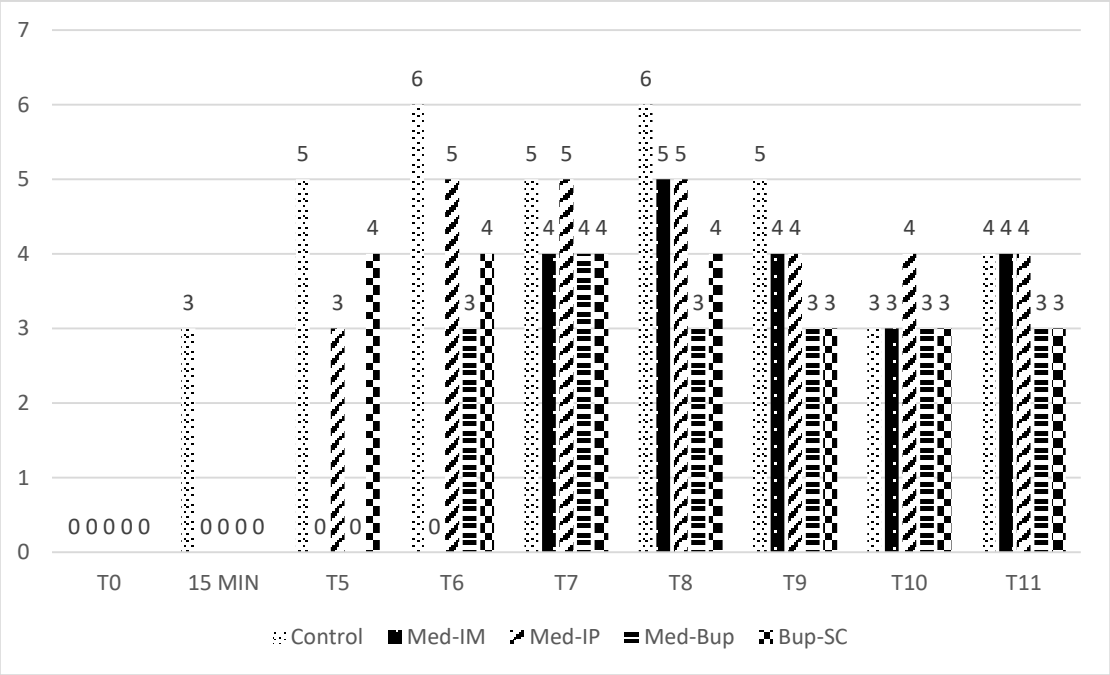

Figure 4: Median (Minimum-Maximum) Pain Assessment with CMPS-SF

Biochemical Parameters

When comparing the mean activity of lactate dehydrogenase, aspartate transaminase, and serum glucose levels in different groups, no statistically significant differences were observed among groups at different times (Table 6). However, fluctuations in the mean activity of the above enzymes and glucose levels at different times in the test groups were statistically significant.

By comparing the mean activity of CK in different groups, there was no statistically significant difference among the groups (Table 6). Furthermore, there were no statistically significant changes in the mean activity of CK at various time points in the test groups [F CK (12, 40)=1.92, P=0.06].

In the assessment of serum cortisol levels, there was a statistically significant difference among the groups. Cortisol levels in the groups that received the drug significantly decreased compared to the control group. At T1, the cortisol level in the Med-IM group increased significantly compared to the Med-Bup group ($P<0.05$). By T24, the cortisol level of the Med-IM group had increased to the same level as the control group, and this increase was significant compared to the other groups ($P<0.05$) (Table 6). The cortisol level of the Med-IM group was significantly higher than the Med-Bup in T1, and the Bup-SC group was higher than other test groups in T7. At this time, cortisol levels in all groups were lower than the control. By T24, the cortisol levels of Med-IM and control were higher than the other groups. Fluctuations in the mean serum cortisol level over time showed significant differences. In the control group, the cortisol level at T4 was lower than at other times [F cortisol (7, 37) = 2.82, P=0.017].

Table 6: Mean ± standard deviation of serum biochemical parameters in different groups at different times

Variable	Group	T0 (a)	T1 (b)	T4 (c)	T7 (d)	T11 (e)	T24 (f)
Creatine kinase (U/L)	Control (A)	130±5.5	133±13	243±9.5	293±6.5	378±11.5	244±11.5
	Med-IM (B)	133±13	180±10	220±6.7	292±10.7	337±34.6	245±7.8
	Med-IP (C)	168±10	208±8	276±8.6	309±6.5	396±23	245±38
	Med-Bup (D)	134±23	207±27	259±17.5	301±8.3	383±17.3	244±30.6
	Bup-SC (E)	172±45	222±47	262±47	337±40	412±53	249±15.7
Lactate dehydrogenase (U/L)	Control (A)	54.6±3	79±9	79.2±23	92.6±33	105.8±36	145.8±37
	Med-IM (B)	70.2±16	78.8±24	90.8±23	101.2±25	110.2±43	149.8±31
	Med-IP (C)	77.4±8	116.2±36	131±46	140±39	144.2±37	185.8±38
	Med-Bup (D)	67.2±21	84.8±25	85.4±34	109.8±42	125.8±30	139.8±24
	Bup-SC (E)	50.75±14	69.5±25	90.5±32	104.25±35	128.25±37	155.25±40
Aspartate transaminase (U/L)	Control (A)	13.2±4.9	11.51±4.7	14.43±4	15.45±3.7	20.12±4.7	18.14±8.2
	Med-IM (B)	18.12±3.5	19.34±4.3	17.37±4	19.29±5.7	24.98±5.5	18.64±3.8
	Med-IP (C)	20.34±6.9	16.87±13.1	16.76±11	25.25±12.3	30.33±7.9	22.32±6.4
	Med-Bup (D)	17.45±3.3	17±12	14.78±8.8	13.54±7.9	19.34±4.6	20.54±6.4
	Bup-SC (E)	18.65±6.9	23.33±5.5	18.76±3.1	18.34±6.1	23.43±11	20.54±10
Glucose (mg/dL)	Control (A)	94.73±10	107±38	131±62	139.79±27	112.32±23	79.29±42
	Med-IM (B)	117.7±20	134.65±31	183±97	241.51±103	132.54±43	116.36±14
	Med-IP (C)	130±24	127.27±25	129±19	164.64±51	130.3±33	113±13
	Med-Bup (D)	109.98±29	116.36±32	122.32±36	189.79±68	137.43±19	122±19
	Bup-SC (E)	111.98±32	141±51	135.65±48	135.35±41	122.2±18	116±16
Cortisol (µg/dL)	Control (A)	1.671±0.05 C,D,E,b,d, c,e,f	1.633±0.015 B,C,D,E, a,d	1.601±0.012 a,b,d,e,f	1.63±0.006 B,C,D,E,a,c,e	1.644±0.021 B,C,D,E,a,c	1.623±0.01 C,D,E,a,c
	Med-IM (B)	1.624±0.01 4 b,c,d	1.607±0.006 A,E,a,f	1.605±0.004 a,f	1.605±0.002 A,E,a,f	1.613±0.003 A	1.614±0.005 C,D,E,b,c,d
	Med-IP (C)	1.614±0.02 A,c,e,f	1.602±0.01 A	1.598±0.011 a	1.601±0.001 A,E	1.596±0.003 A,a	1.592±0.004 A,B,a
	Med-Bup (D)	1.604±0.01 7 A,b	1.589±0.009 A,a	1.594±0.002	1.601±0.001 A,E	1.602±0.004 A	1.602±0.0 A,B
	Bup-SC (E)	1.61±0.002 A	1.605±0.003 A,B	1.609±0.002	1.614±0.004 A,B,C,D,	1.608±0.003 A	1.602±0 A,B

Before induction of anesthesia (T0), Time of skin incision (T1), Skin suture (T4), 120 minutes after the completion of surgery (T7), 6 hours after surgery (T11); 24 hours after surgery (T24).

* Lowercase letters in each line indicate a significant difference between different times in each group ($p < 0.05$). ** Capital letters in each column indicate significant differences between groups ($p < 0.05$).

Discussions

We investigated the effectiveness of intraperitoneal administration of medetomidine alone and with bupivacaine at the surgical site for the pain and recovery management after ovariohysterectomy surgery in the female dogs. Pain assessment using VAS, UMPS, and CMPS-SF methods showed that medetomidine (IP) alone is not superior to intramuscular administration, in contrast to its simultaneous administration with bupivacaine, in pain management, recovery, and cardiovascular and respiratory physiological parameters.

On the other hand, administering bupivacaine alone may be more effective in reducing surgical pain and shortening recovery time compared to administering medetomidine alone or with bupivacaine. The onset time of pain reduction after surgery is faster in the bupivacaine-administered groups than in the groups that did not receive bupivacaine.

During the experiment, three dogs in the control group and two dogs in the Med-IP group received a rescue dose of morphine (0.5 mg/kg, IM) after surgery.

The anesthesia instructions in our study included the use of premedication (acepromazine and morphine), induction (propofol), and maintenance of anesthesia (isoflurane), which were administered in the same manner across all groups. These drugs, known for their sedative, anti-inflammatory, and anesthetic properties, along with the administration of medetomidine and bupivacaine, resulted in a synergistic effect in the groups studied. This synergy led to a reduction in pain scores following ovariohysterectomy in dogs. Considering the effect time and half-life of each drug during surgery and anesthesia with isoflurane, the animals experienced minimal pain and fewer side effects such as fluctuations in blood pressure, cortisol levels, glucose levels, breathing rate, and heart rate (Jiang et al, 2019).

In our study, medetomidine was administered at T3 (simultaneously with ligation of the first ovarian pedicle), and its analgesic effect was observed during the pain evaluation using CMPS-SF, VAS, and UMPS methods at T5 (half an hour post-surgery). This effect was evident until 6 hours post-surgery. Murdoch et al, (2013) investigated the analgesic effect of medetomidine. Within 10 hours post-surgery, the pain scores were significantly lower in the medetomidine group, and no animals required a rescue dose. They employed continuous infusion of medetomidine while we did not. The results in the medetomidine-IM group were consistent with Murdoch et al.'s study; but, there was a discrepancy in the medetomidine-IP group due to the differences in administration protocol (F. Murdoch et al, 2013).

The administration of morphine before ovariohysterectomy surgery to reduce pain after surgery, as outlined in our protocol, has also been supported by the previous studies. Kongara et al, (2012) suggested that giving a low dose of morphine before and tramadol after surgery had a greater impact on the duration of postoperative analgesia (Kongara et al, 2012). In a study by Karna *et al.* (2021), combining morphine (0.3 mg/kg) with dexmedetomidine or maropitant (a neurokinin-1 receptor antagonist) showed better analgesic effects compared to using morphine alone at a higher dose (0.6 mg/kg) (Karna et al, 2022). In our study, we administered morphine and acepromazine before anesthetizing female dogs to sedate them and reduce the pain during surgery. Due to the half-life of morphine, there was an analgesic effect during and after surgery.

In our study, three dogs in the control group and two dogs in the Med-IP group required a rescue dose two hours after surgery (T7). In this case, there was a significant statistical difference between the Med-IP group and the other groups (including the Med-IM group), which could be attributed to the method of medetomidine administration (Turner et al, 2011). Studies on the intraperitoneal (IP) administration method and its limitations have shown that one limitation is first-pass metabolism, similar to what is observed with orally administered medications because drugs absorbed from the peritoneal cavity end up in the portal vein and pass through the liver. Therefore, the pharmacokinetics of drugs administered IP are similar to oral administration in terms of metabolic fate and the first-pass metabolism, resulting in lower bioavailability compared to intramuscular or intravenous administration. Additionally, the time to reach the maximum plasma concentration (Cmax) with IP administration is longer than with IM administration (Al Shoyaib et al, 2019). As a result, the pain reduction time with IM medetomidine was faster than with the IP method. The difference in Cmax levels between the two administration methods contributed to more pain reduction with the IM method compared to the IP method.

In a study by Saponaro et al, (2013), the cardiovascular effects of medetomidine (2

µg/kg), acepromazine (20 µg/kg), and their combination were investigated intravenously in healthy dogs. The blood pressure and non-invasive echocardiography were measured at 0, 15, 50, and 80 minutes after drug administration. The results showed a decrease in the left ventricular afterload due to the acepromazine and an increase in the right ventricular afterload caused by medetomidine. However, the combination of these drugs reduced the mentioned effects and prevented the occurrence of atrioventricular block (Saponaro et al, 2013).

Our study focused on examining the physiological parameters of cardiovascular, respiratory, and rectal temperature. The protocol applied before, during, and after surgery successfully maintained these parameters within the physiological range.

One known side effect of medetomidine is a transient increase in blood pressure followed by a decrease (Sinclair, 2003). The timing of medetomidine administration may explain the significant increase in blood pressure at T4. Additionally, in the group that received intraperitoneal medetomidine, the time to reach Cmax and different bioavailability prevented similar effects seen with IM administration (Al Shoyaib et al, 2019).

The study by Ramesha et al, (2022) compared the pain management, quality of recovery, and physiological parameters during and after surgery using a combination of medetomidine and dexmedetomidine with propofol as an anesthesia inducer, versus using propofol alone without medetomidine or dexmedetomidine in dogs. Their results showed that in the groups where propofol was combined with one of these drugs, the anesthesia induction was faster, physiological parameters remained within normal range, and sedation, analgesia, and quality of recovery were not associated with adverse effects (Ramesha et al, 2022). In our study, propofol was an induction agent and sedation achieved in all groups when combined with medetomidine. When examining the quality of recovery, groups that received medetomidine scored higher degree than the other groups (i.e. poorer recovery quality with more ataxia and struggling to stand). One of the side effects of using medetomidine is its impact on blood flow to motor muscles, caused by an increase in deoxygenated hemoglobin concentration. This effect, combined with the decrease in blood pressure and bradycardia induced by the drug, can affect the animal's recovery time (Sinclair, 2003).

Bupivacaine is an aminoamide that blocks the action potential in nerve cells by blocking sodium channels and is used as a local anesthetic (Shafiei et al, 2018). In a prospective randomized clinical study, Campagnol et al, (2012) compared the effect of intraperitoneal or incisional bupivacaine on pain and the need to rescue dose after ovariohysterectomy in dogs. They suggested that intraperitoneal bupivacaine led to lower pain scores in the first hour after surgery and there was a trend towards reducing the need for rescue analgesia after ovariohysterectomy in dogs (Campagnol et al, 2012). Results of their study were consistent with the results of ours in terms of pain reduction and no need for a rescue dose in the groups receiving bupivacaine. In the study by Shankar *et al.* (2022), the analgesic effect of bupivacaine alone and in combination with dexmedetomidine was investigated in laparoscopic surgery. They assessed the quality of analgesia and the time to first request for a rescue dose. Their findings indicated that the co-administration of dexmedetomidine (1 µg/kg; IP) with bupivacaine (0.25%) resulted in decreased post-operative pain and reduced the need for a rescue dose compared to bupivacaine alone (Shankar et al., 2022). The results of these studies consistently demonstrated that combining bupivacaine with a α2 agonist leads to pain relief and eliminates the need for a rescue dose.

In the present study, there was no significant difference in blood glucose levels between different groups; however, the glucose levels fluctuated in the groups over time. At T7 (two hours after surgery), the highest level of glucose was observed in the groups receiving medetomidine, followed by the group that received only bupivacaine. In stress and pain conditions, cortisol levels elevate, and its effect on gluconeogenesis leads to increased serum glucose levels. The higher the cortisol level, the higher the glucose level (Hannibal and Bishop, 2014). The results of our study showed a correlation between glucose and cortisol fluctuations over time, but the cortisol levels in all test groups had decreased compared to the control group. One potential reason for the increased glucose levels in the medetomidine groups compared to other test groups is the effect of medetomidine as an α2 agonist on the pancreatic islets. This results in the suppression of insulin secretion in response to sympathetic stimulation, leading to increased blood sugar (Hampton et al, 2022).

Due to the liver metabolism and cardiac effects of the medicine used in the protocol of this study, biochemical parameters associated with heart and liver function were investigated to determine the adverse effects of the doses used. The activity of lactate dehydrogenase, and aspartate aminotransferase were all within the normal range, and no significant differences were observed in the test groups. The activity of the creatine kinase enzyme was within the normal range in all groups. Creatine kinase activity increases in conditions such as coronary artery bypass surgery, heart transplantation, myocarditis, and pulmonary embolism after surgery. Creatine kinase activity rises within 12 hours after muscle injury, peaks at 24 to 36 hours, and returns to normal after 48 to 72 hours (Aujla and Patel, 2019). In our study, the highest increase in the activity of this enzyme was observed 8 hours after surgery (T11) in all groups, which was likely caused by the surgical incision.

There were limitations in our study. One limitation was in assessing the pain after surgery using various tests. We applied a multimodal analgesia approach in all groups; so, the results of pain assessment could not definitively be attributed to our study target drugs (medetomidine and bupivacaine) due to the synergistic impact of these drugs. Morphine was used in our protocol as a sedative and pre-emptive analgesia, with duration of action of about 3-8 hours. According to our procedure, its effect may last at least 7 hours after surgery (T10) and affect the pain evaluation results. Additionally, the combination of acepromazine, morphine, propofol, and isoflurane reduces the blood pressure, which can affect the effect of target drugs in this study on blood pressure during and after surgery (depending on the half-life).

We compared IM and IP medetomidine administration methods in dogs undergoing ovariohysterectomy surgery. Due to differences in bioavailability, Cmax, and time to reach Cmax between the two administration methods, using the same dose of medetomidine can impact the conclusion of which administration method is preferable. The future research should consider this issue and use appropriate doses for each administration method.

The sample size in this study was small, although it was sufficient to achieve our study goals regarding the effect of medetomidine and bupivacaine in inducing analgesia during and after the surgery. However, a larger sample size is needed to draw more definitive conclusions about the analgesic and sedation effects of medetomidine. Despite the multimodal analgesia approach, consistent analgesia was not observed in all animals, underscoring the importance of individualized pain assessment.

When evaluating the pain using VAS, UMPS, and CMPS-SF methods, there was no evidence to suggest that the effectiveness

of administering the same dose of intraperitoneal medetomidine alone was superior to intramuscular administration of medetomidine alone or in combination with bupivacaine at the surgical site for pain management, quality of recovery after Ovariohysterectomy surgery, and cardiovascular parameters in the female dogs.

Conversely, administering bupivacaine alone significantly reduced surgical pain and recovery time compared to administering medetomidine alone or in combination with bupivacaine. Animals receiving intraperitoneal medetomidine required a rescue dose, while no rescue dose was needed in the other groups. Doses of all medications used in each group did not interfere with the animals' physiological functions, and cardiovascular, respiratory, and rectal temperature results remained within normal ranges. Additionally, levels of serum enzymes related to liver and heart tissue function stayed within normal ranges.

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Conflict of Interest

The authors declare that they have no known conflict of interest.

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Improving immune system and antioxidant status in Japanese quails through biochar supplementation

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Abstract

The study evaluated the effects of pistachio by-products biochar (PBB) on performance, blood metabolites, immune response, antioxidant status, and ammonia gas emissions in Japanese quails. A total of 500 one-day-old Japanese quails were assigned to a completely randomized design with five dietary treatments and five replicates for 35 days. The experimental diets included: (1) a basal feed without additives (control), (2) a basal feed with 0.05% flumequine 10% (positive control), (3) a basal feed with 0.35% PBB, (4) a basal feed with 0.65% PBB, and (5) a basal feed with 1% PBB. The results showed that weight gain significantly increased in birds fed 0.65% biochar compared to the control and flumequine groups, without any effect on feed intake. A trend towards a lower feed conversion ratio was observed in birds fed 0.65% biochar compared with the control. Quails fed 1% biochar had significantly lower cholesterol and LDL levels, while the control group exhibited the highest levels. The highest lymphocyte percentage was observed in quails fed 1% biochar, and increasing biochar levels in the diet significantly reduced the heterophil/lymphocyte ratio. However, biochar supplementation had no significant effect on immunoglobulin (IgG, IgM, IgY, and IgT) levels. Antioxidant markers, including total antioxidant capacity, glutathione peroxidase, and superoxide dismutase, were highest in birds receiving 1% PBB, with no significant difference between the 0.65% and 1% levels. Additionally, biochar supplementation significantly reduced ammonia gas emissions. Overall, incorporating at least 0.65% PBB in meat quail diets improved growth performance, blood parameters, antioxidant enzyme activity, and immune function, offering an eco-friendly alternative to antibiotics.

Key words: biochar, pistachio by-products, blood metabolites, immune response, ammonia emission

Introduction

Ensuring food security for a growing global population requires optimizing livestock production through sustainable resource utilization. Poultry production plays a crucial role in global food supply by providing meat and eggs. However, the industry faces challenges, including maintaining food safety, reducing environmental impact, and ensuring economic sustainability (Schmidt et al, 2019; Nair et al, 2023). Contaminated poultry products pose health risks, highlighting the need for safe and high-quality production systems (Vimal et al, 2022).

To achieve sustainability, utilizing locally available natural resources to enhance poultry performance and reduce production costs is essential (Man et al, 2021; Al-Khalaifah and Al-Nasser, 2023).

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Antibiotics have historically been used to control pathogens and improve productivity, but concerns over antimicrobial resistance led to their ban as growth promoters in the European Union in 2006 (Saleh et al, 2018). International organizations such as the FAO and WHO have since 2016 pushed for stricter regulations (FAO, 2016; WHO, 2019). Consequently, the poultry industry must identify alternative strategies to support animal health and maintain profitability while reducing antibiotic dependence (Abdel-Moneim et al, 2022).

Biochar, a carbon-rich material produced from the pyrolysis of plant residues and organic waste, has emerged as a promising natural alternative. Its properties vary based on feedstock type and pyrolysis conditions, with lignin-rich materials yielding higher biochar production (Ahmed and Hameed, 2020; Man et al, 2021). As a feed additive, biochar has demonstrated benefits in improving growth, feed efficiency, and nutrient utilization across various livestock species, including poultry, cattle, and fish (Vimal et al, 2022; Nair et al, 2023).

One of biochar's key advantages is its potential to mitigate environmental pollutants. While widely recognized for reducing methane emissions in ruminants, biochar may also decrease ammonia (NH_3) emissions in poultry production. Ammonia poses risks to farm workers, bird health, and air quality, making emission control a priority for farmers and regulatory agencies (Nowak et al, 2016; Kalus et al, 2019). Additionally, biochar supplementation has been associated with enhanced immune function, reduced mycotoxin toxicity, improved antioxidant status, and better liver enzyme activity in poultry (Jandosov et al, 2017; Rajput et al, 2017).

While most biochar research has focused on conventional biomass sources such as wood and bamboo, agricultural by-products like pistachio by-products (PB) offer a sustainable and cost-effective alternative. PB, generated during the de-hulling process, include soft outer shells, twigs, leaves, hard shells, and green kernels (Mokhtarpour et al, 2014). The effectiveness of biochar varies based on feedstock type and production techniques (Al-Khalaifah and Al-Nasser, 2023), and while excessive biochar inclusion may interfere with nutrient availability, moderate supplementation levels (0.5–1%) have shown beneficial effects (Schmidt et al, 2019). However, the impact of PB-derived biochar on poultry performance, particularly in Japanese quail, remains largely unexplored. Given its unique structural properties and potential bioactive effects (Mirheidari et al, 2020), we hypothesize that PB-derived biochar may influence bird health. Therefore, this study aimed to evaluate the effects of PB-derived biochar on hematological parameters, immune response, antioxidant status, and ammonia emissions in Japanese quail. By identifying an effective biochar source from agricultural waste, this research contributes to sustainable poultry production by improving bird health, reducing environmental pollutants, and providing an eco-friendly alternative to antibiotic growth promoters.

Materials and Methods

Biochar Preparation and Experimental Design

Pistachio by-products were obtained from a pistachio de-hulling factory in Kashmar, Khorasan Razavi Province, Iran. The material was sun-dried and ground using a 2-mm mesh screen before undergoing pyrolysis according to the method described by Mirheidari et al. (2020). The total carbon (C), hydrogen (H), and nitrogen (N) content in PB biochar were 55.3%, 2.1%, and 1.6%, respectively (CHNS analyzer, Thermo Finnigan, Flash EA 1112 Series).

Five hundred day-old Japanese quails were assigned to five experimental diets with five replications (20 quails per replicate) in a completely randomized design for 35 days.

Temperature and management conditions were maintained according to standard breeding guidelines (Du Sert et al, 2020). The experimental diets (Table 1) formulated based on the nutritional requirements recommended by the NRC (1994) included: 1) basal diet without additives (control), 2) basal diet supplemented with 0.05% flumequine (10%) (positive control), 3) basal diet supplemented with 0.35% PBB, 4) basal diet supplemented with 0.65% BPP, 5) basal diet supplemented with 1% PBB. All birds had ad libitum access to feed and water.

Table 1: Ingredients and composition of basal diet

Ingredient	Amount (%)
Corn	49.25
Soybean meal (46%)	46.00
Soybean oil	1.00
Di-calcium phosphate	1.00
Limestone	0.80
L-Lysine	0.67
DL-Methionine	0.63
NaCl	0.32
Mineral premix*	0.15
Vitamin premix†	0.15
Nutrient composition	
AME (Kcal/kg)	2950
CP (%)	24.6
Total Lysine (%)	1.86
Total Methionine (%)	1.00
Total Methionine + Cysteine (%)	1.38
Total Tryptophan (%)	0.35
Calcium (%)	0.75
Available Phosphorus (%)	0.25

*Mineral premix provided per kilogram of diet: Mn (from MnSO4·H2O), 65 mg; Zn (from ZnO), 55 mg; Fe (from FeSO4·7H2O), 50 mg; Cu (fromCuSO4·5H2O), 8 mg; I (from Ca (IO3)2·H2O), 1.8 mg; Se, 0.30 mg; Co (from Co2O3), 0.20 mg; Mo, 0.16 mg.

†Vitamin premix provided per kilogram of diet: vitamin A (from vitamin A acetate), 11,500 IU; cholecalciferol, 2100 IU; vitamin E (from DL-α-tocopherylacetate), 22 IU; vitamin B12, 0.60 mg; riboflavin, 4.4 mg; nicotinamide, 40 mg; calcium pantothenate, 35 mg; menadione (from menadione dimethyl-pyrimidinol), 1.50 mg; folic acid, 0.80 mg; thiamine, 3 mg; pyridoxine, 10 mg; biotin, 1 mg; choline chloride, 560 mg; ethoxyquin, 125 mg.

Measurements

Daily feed intake was determined by measuring the amount of feed offered and the refusals. Body weights were recorded weekly to track weight gain, and the feed conversion ratio (FCR) was calculated by dividing the total feed consumed by the corresponding weight gain for each treatment group.

On day 35, two quails were randomly selected from each replicate (n = 10 per treatment), and blood samples were collected from the jugular vein. The samples were maintained at 4°C for 10 minutes and then centrifuged at 3000 rpm to separate serum. The serum samples were stored at -80°C for further analysis.

Biochemical parameters, including total cholesterol, triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL), glucose, and total protein, were determined using an autoanalyzer (BT3000, Roma, Italy). Oxidative stress status was assessed by measuring plasma malondialdehyde (MDA) levels (Pilz et al, 2000). Intracellular antioxidant markers were evaluated by determining the activities of superoxide dismutase (SOD) and glutathione peroxidase via spectrophotometry. All measurements were performed in duplicate.

To evaluate the humoral immune response, 0.5 mL of a 7% suspension of sheep red blood cells (SRBC), prepared in phosphate-buffered saline (PBS), was injected into the right subwing vein of birds at 28 days of age. Seven days post-injection (day 35), blood samples were collected from the left wing vein to assess antibody titers. After clotting, the serum was separated and incubated at 56°C for 30 minutes to inactivate complement. The total anti-SRBC antibody titer, as well as immunoglobulin M (IgM) and immunoglobulin Y (IgY) levels, were determined using serial dilution. The titers were expressed as $\log_2$ values, with the highest dilution exhibiting complete agglutination. IgM levels were measured by treating serum samples with 0.01 M 2-mercaptoethanol, which selectively inactivates IgM. The difference between total anti-SRBC and IgY titers was used to estimate IgM concentrations.

Hematological Analysis

At the end of the experiment, blood samples were also collected to determine the heterophil-to-lymphocyte (H:L) ratio. Blood smears were prepared and stained with Wright's stain following the method of Lucas and Jamroz (1961). A total of 100 white blood cells were counted per sample, and the H:L ratio was calculated.

Ammonia Emission Measurements

Ammonia (NH_3) emissions from manure were assessed in the final week of the experiment following the method described by Kalus et al. (2020) with some modifications. Approximately 200 g of excreta was collected from each cage and stored in plastic zipper bags. These bags were then placed in a plastic container with a lid, which had two holes; one sealed with a membrane filter and the other used for ammonia measurement. The samples were allowed to ferment at room temperature, and ammonia levels were recorded throughout the process with time zero indicating the start of the ammonia emission monitoring. A gas-sampling pump (AP-20, Gastec Corp., Kitagawa, Japan) fitted with a detector tube (3LA, 3M) was used to measure ammonia emissions at 0 and 24 hours. The NH_3 concentration was reported as ppm per 100 mL.

Statistical Analysis

Data were analyzed using a completely randomized design. The GLM procedure in SAS (2001, version 9.1) was used for statistical analysis. Differences among treatment means were compared using Duncan's multiple range test at a significance level of $P<0.05$.

Results

Effect of pistachio by-products biochar and antibiotic on growth performance of Japanese quails are showed in Table 2. The effects of PBB and antibiotic supplementation on selected blood parameters in Japanese quails are presented in Table 3. Blood glucose, total protein, and HDL levels were not significantly affected by dietary treatments ($P>0.05$). However, cholesterol levels were significantly lower in quails fed 1% PBB (116 mg/dL) compared to the control group, which exhibited the highest cholesterol level (165 mg/dL, $P<0.05$). Similarly, LDL levels were significantly reduced in birds receiving 0.65% and 1% biochar, showing the lowest values among all experimental groups ($P<0.05$).

Table 2: Effect of pistachio by-products biochar and antibiotic on growth performance of Japanese quails

Item	Control	Treatment				SEM	P value
		Flumequine	0.35% Biochar	0.65% Biochar	1% Biochar		
Feed intake (g)	719	740	743	735	749	5.11	0.41
BW gain (g)	236 c	250 b	255 ab	263 a	256 ab	2.29	0.001
FCR	3.05	2.96	2.91	2.78	2.93	0.031	0.08

[a, b, c] Different superscripts within the same row indicate significant differences ($P<0.05$).

Table 3: Effect of pistachio by-products biochar and antibiotic on blood biochemical parameters of Japanese quails (mg/dl)

Item	Control	Treatment				SEM	P value
		Flumequine	0.35% Biochar	0.65% Biochar	1% Biochar		
Glucose	184	189	191	206	210	3.61	0.06
Protein	3.45	3.48	3.52	3.55	3.62	0.033	0.61
Triglycerides	225	218	213	205	201	4.46	0.49
Cholesterol	165 a	137 ab	141 ab	129 b	116 b	5.51	0.04
HDL	63.8	59.2	69.9	67.6	71.5	1.79	0.17
LDL	80.1 a	64.5 ab	71.0 a	52.8 b	55.4 b	3.32	0.02

[a, b] Different superscripts within the same row indicate significant differences ($P<0.05$).

Table 4 presents the differential leukocyte counts, heterophil-to-lymphocyte (H:L) ratio, and humoral immune response. The addition of PBB to the diet significantly influenced lymphocyte percentage and the H:L ratio (P<0.05). The highest lymphocyte percentage was observed in quails receiving 1% biochar, whereas the control and antibiotic-treated groups exhibited the lowest levels. Moreover, increasing dietary biochar inclusion led to a significant reduction in the H:L ratio (P<0.05), with the lowest ratio recorded in the 1% biochar group, while the control group displayed the highest ratio. However, heterophil percentages did not differ significantly among treatments (P>0.05). Assessment of humoral immunity, based on the levels of IgG, IgM, IgY, and IgT, revealed no significant effects of biochar supplementation (P>0.05). Nonetheless, a numerical increase in IgG, IgY, and IgT levels was observed in biochar-fed groups.

Table 4. Effect of pistachio by-products biochar and antibiotic on immune system parameters of Japanese quails

Item	Control	Treatment				SEM	P value
		Flumequine	0.35% Biochar	0.65% Biochar	1% Biochar		
Heterophils (%)	36.6	34.2	35.8	33.5	32.7	0.72	0.45
Lymphocytes (%)	56.9 c	57.2 c	60.7 bc	65.1 ab	67.8 a	1.31	0.003
Heterophil/ Lymphocyte Ratio	0.64 a	0.60 ab	0.59 ab	0.51 bc	0.48 c	0.019	0.009
IgG (mg/mL)	4.34	4.36	4.41	4.65	4.81	0.070	0.11
IgM (mg/mL)	1.27	1.31	1.30	1.42	1.36	0.034	0.81
IgY (mg/mL)	1.96	2.02	2.19	2.26	2.22	0.045	0.12
IgT (mg/mL)	7.56	7.69	7.90	8.31	8.38	0.122	0.10

[a, b, c] Different superscripts within the same row indicate significant differences (P<0.05).

The effects of biochar and antibiotics on antioxidant indices are summarized in Table 5. Serum malondialdehyde (MDA) levels were not significantly influenced by dietary treatments (P>0.05). However, total antioxidant capacity and red blood cell antioxidant enzyme activities were significantly improved by dietary supplementation (P<0.05). The highest values for total antioxidant capacity, glutathione peroxidase, and superoxide dismutase were observed in the 1% biochar group (P < 0.05), though no significant difference was detected between the 0.65% and 1% biochar levels. The lowest antioxidant enzyme activities were recorded in the control group.

Table 5: Effect of pistachio by-products biochar and antibiotic on antioxidant parameters of Japanese quails

Item	Control	Treatment				SEM	P value
		Flumequine	0.35% Biochar	0.65% Biochar	1% Biochar		
Malondialdehyde (nmol/mL)	2.97	2.88	2.91	2.69	2.72	0.043	0.13
Total Antioxidant Capacity (mmol/L)	1.02 b	1.09 ab	1.18 ab	1.21 ab	1.28 a	0.032	0.07
Glutathione Peroxidase (mmol/L)	3.10 b	3.21 b	3.63 ab	4.09 a	4.28 a	0.016	0.02
Superoxide Dismutase (U/L)	178 c	182 bc	196 abc	228 ab	240 a	8.64	0.04

[a, b, c] Different superscripts within the same row indicate significant differences (P < 0.05).

Table 6 presents the effects of dietary treatments on ammonia (NH_3) gas emissions from quail litter. Ammonia release at 0 and 24 hours post-excretion significantly decreased as biochar levels increased ($P<0.05$). The most pronounced reduction in ammonia emissions was observed in quails fed 0.65% and 1% biochar, demonstrating its effectiveness in reducing environmental nitrogen losses.

Table 6. Effect of pistachio by-products biochar and antibiotic on ammonia gas emission from Japanese quail litter (ppm)

Item	Control	Treatment				SEM	P value
		Flumequine	0.35% Biochar	0.65% Biochar	1% Biochar		
Hour_0	98 a	100 a	65 ab	53 b	41 b	7.67	0.01
Hour_24	126 a	119 ab	84 bc	77 c	59 c	7.99	0.007

[a, b, c] Different superscripts within the same row indicate significant differences ($P<0.05$).

Discussion

The PB biochar was included at 0.35%, 0.65%, and 1% to assess dose-dependent effects, following prior studies and preliminary data (Schmidt et al, 2017). The previous findings on biochar's effects in poultry diets have been inconsistent, even with similar inclusion levels. For example, Evans et al. (2016) reported that 2% poultry litter biochar (PLB) increased the feed conversion ratio (FCR) without affecting feed intake, while 4% PLB reduced weight gain. In contrast, Al-Jumaily et al. (2022) observed improved growth performance and feed efficiency at both 2% and 4% PLB, whereas other studies found no significant impact on feed intake, weight gain, or FCR at similar inclusion rates. These conflicting results highlight the influence of biochar's source and composition on its effectiveness. In the present study, biochar supplementation had no adverse effect on feed intake, indicating that palatability remained intact. However, the observed improvement in weight gain led to a more efficient FCR in biochar-fed groups, enhancing productivity without increasing feed costs. This aligns with Kana et al, (2011), who reported enhanced weight gain and FCR in broilers fed 0.2%–0.6% maize cob and Canarium charcoal, though higher levels reduced feed intake and growth. The optimal FCR in their study was achieved at 0.6% inclusion, similar to the 0.65% identified as optimal in the current research. The improved performance in biochar-fed quails may be attributed to the antimicrobial properties of PBB, which likely reduced E. coli counts (Reaggi et al, 2023) and increased *Lactobacillus* populations (Choi et al, 2009) leading to better digestion, nutrient absorption, and energy utilization. This supports the findings of Gerlach and Schmidt (2012), who noted biochar's role in enhancing digestion and feed efficiency. Nao Takeuchi-Storm et al, (2025) reported that both 2% biochar and the probiotic-like product (0.125% *Saccharomyces cerevisiae* fermentate) improved growth performance and gut health markers compared to the control group. Notably, biochar supplementation led to a significant reduction in *Campylobacter* in broilers highlighting its potential as an alternative or complementary strategy to probiotics in poultry nutrition. Similarly, Islam et al, (2014) concluded that 1% Sea tangle charcoal can be used as a potential alternative to antibiotic (0.01% Chlortetracycline) in duck production.

In this study, biochar supplementation at levels of 0.65% and 1% significantly reduced serum cholesterol and LDL levels compared to the control group. These findings are consistent with the previous research that demonstrated biochar's ability to modulate lipid metabolism, likely through its adsorption properties, which may alter lipid absorption in the

gastrointestinal tract (Boonanunatasarn et al, 2014). The reduction in cholesterol levels is particularly relevant, as elevated cholesterol is a risk factor for cardiovascular disease, even in poultry (Dim et al., 2018). Biochar may reduce serum cholesterol by binding bile acids, thereby disrupting the enterohepatic circulation and enhancing hepatic cholesterol catabolism (Neuvonen et al, 1989). This process reduces bile acid reabsorption, promoting increased conversion of cholesterol to bile acids and lowering blood cholesterol levels (Elghalid et al, 2022; Kramer and Glombic, 2006). Thus, the observed lipid-lowering effects of PB biochar highlight its potential as a functional feed additive for improving metabolic health in poultry.

Interestingly, while the antioxidant status of quails improved with biochar supplementation, the effects on glucose and protein levels were minimal. The slight increase in total protein levels in biochar-fed groups could be attributed to biochar's adsorption of mycotoxins, which may otherwise impair liver function and protein synthesis (Elghalid, 2022). While further research is warranted, this trend suggests that biochar may contribute to improved protein metabolism through its detoxifying action, promoting liver health and overall physiological stability.

Biochar supplementation significantly altered the differential leukocyte counts, particularly increasing the percentage of lymphocytes and reducing the heterophil-to-lymphocyte (H:L) ratio. These findings align with research indicating that biochar can enhance immune function by modulating the stress response (Salah et al, 2015). The reduction in the H:L ratio, in particular, may indicate a less stressful environment for the birds, as a lower H:L ratio is often associated with reduced stress and better immune competence (Minias, 2019). The elevated lymphocyte percentage suggests a stronger adaptive immune response, likely due to biochar's role in improving overall health and possibly reducing subclinical infections. However, it is noteworthy that biochar supplementation did not significantly affect immunoglobulin levels (IgG, IgM, IgY, and IgT). This could be attributed to the relatively short experimental duration, and therefore future studies should explore the long-term effects of biochar on humoral immunity, particularly in terms of antibody production.

The numerical increase (P=0.10) in IgT levels in biochar-fed groups suggests that biochar may have immunomodulatory properties, although the lack of statistical significance warrants further investigation. Biochar's ability to reduce mycotoxin toxicity (through adsorption) could indirectly improve immune function, as mycotoxins are known to impair cellular immunity (Rajput et al, 2017). Therefore, the observed trends in immunoglobulin levels could reflect biochar's potential to alleviate the immunosuppressive effects of mycotoxins and other environmental stressors.

The beneficial effects of biochar supplementation were also demonstrated in broilers fed 0.5% biochar derived from rice husk, which resulted in lower mortality rates, enhanced growth performance, and improved immune function (Nair et al, 2023). These improvements were linked to the mitigation of aflatoxin-related immunosuppressive effects, as aflatoxin-contaminated feed is known to impair cellular immunity and reduce antibody production in response to sheep red blood cells (Bagherzadeh Kasmani et al, 2012). The immunosuppressive effects of aflatoxins are primarily attributed to the inhibition of protein synthesis, including key immunoglobulins such as IgG and IgA (Rajput et al, 2017), along with a reduction in complement hemolytic activity (Chen et al, 2014) and a decline in lymphocyte counts.

Biochar exhibits significant electron transfer capabilities that may help mitigate

oxidative stress in animals. It can activate hydrogen peroxide (H_2O_2), generating hydroxyl radicals (•OH) through interactions with persistent free radicals in its structure (Fang, 2014). This process facilitates electron transfer, promoting the degradation of harmful compounds. The graphitic structure of biochar enhances this function by lowering the energy barrier for electron movement, increasing its efficiency (Dou, 2023). Additionally, biochar can act as an electron mediator between bacteria and minerals, further supporting its role in biological electron transfer processes (Kappler, 2014).

Moreover, biochar's potential to enhance the antioxidant defense system may involve modulation of key endogenous enzymes such as glutathione peroxidase (GSH-Px), catalase, and superoxide dismutase. Glutathione peroxidase (GSH-Px), in particular, eliminates hydrogen peroxide produced during lipid oxidation (Almeina et al., 2012). Biochar is proposed to exert antioxidant effects by stabilizing lipid membranes and inhibiting free radical-induced lipid peroxidation. Malondialdehyde (MDA), a marker of polyunsaturated fatty acid oxidation, reflects oxidative stress, with elevated levels indicating lipid damage (Gawel et al, 2004). These properties suggest that biochar may help mitigate oxidative stress by neutralizing free radicals and enhancing electron transfer.

One of the notable environmental benefits of biochar supplementation in poultry diets is its potential to reduce ammonia (NH_3) emissions from manure (Sha et al, 2019). In the present study, biochar inclusion at 0.65% and 1% significantly reduced ammonia emissions at both 0 and 24 hours post-excretion. This result aligns with the previous studies showing that biochar, with its high surface area and porosity, can adsorb and sequester ammonia (Prasai et al, 2018). The reduction in ammonia emissions not only mitigates air pollution in poultry houses but also improves animal welfare by decreasing exposure to high concentrations of ammonia, which can lead to respiratory issues and stress in birds (Kalus et al, 2019). The mechanisms behind biochar's ability to reduce ammonia emissions are multifaceted. Biochar may increase the pH of manure, which could lead to the conversion of ammonium (NH_4^+) to ammonia gas (NH_3), which is then adsorbed by biochar particles (Agyarko-Mintah et al, 2017). Moreover, biochar's porous structure provides a vast surface area for nitrogen retention, further decreasing ammonia volatility and odor. The results from this study highlight the dual benefit of PBB in poultry diets: improving bird health while simultaneously reducing environmental pollutants.

While moderate levels of biochar inclusion—such as the 0.65% used in this study—have demonstrated positive outcomes, it is essential to recognize that higher dosages may introduce safety risks. At elevated concentrations, the inherently adsorptive properties of biochar may interfere with nutrient availability, potentially binding vital components like amino acids and vitamins (Al-Khalaifah and Al-Nasser, 2023). Although the analysis of 112 scientific papers on biochar feed supplements has shown that no significant negative effects on animal health were found in any of the reviewed publications (Schmidt et al, 2019), comprehensive toxicological assessments, encompassing extended feeding trials and residue analyses, remain crucial to validating the safety of biochar as a feed additive.

Dietary inclusion of PB biochar at 0.65% significantly improved weight gain, feed efficiency, and antioxidant enzyme activity in Japanese quails. Additionally, PB biochar enhanced immune function by increasing lymphocyte counts and decreasing the heterophil-to-lymphocyte ratio, without negatively affecting feed intake or immunoglobulin levels. Both

0.65% and 1% PBB effectively reduced ammonia emissions from manure, indicating environmental benefits. The findings suggest that PB biochar can be incorporated into quail diets at levels as low as 0.65%, providing comparable or even superior benefits to antibiotic supplementation, while simultaneously reducing reliance on chemical additives. Despite the promising effects of biochar on performance, immunity, and environmental parameters, further research is needed to fully elucidate its long-term safety, optimal inclusion levels, and comparative efficacy against other non-antibiotic feed additives under commercial production conditions.

Ethics approval

This study protocol was approved by the Research Animal Ethics Committee at the Islamic Azad University (IR.IAU.AEC.) with the reference number of 162773541 (1402/02/25).

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Conflict of interest

No conflicts of interest are declared by the authors.

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اثر تجویز خوراکی *لاکتی پلانتی باسیلوس پلانتاروم* ریزپوشانی شده بر کارایی و ایمنی‌زایی واکسن *آئروموناس هیدروفیلا* در کپور معمولی

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چکیده

در مطالعه حاضر، تأثیر تجویز خوراکی پروبیوتیک *لاکتی پلانتی باسیلوس پلانتاروم* به صورت آزاد و کپسوله شده با آلژینات/کیتوسان بر ایمنی‌زایی و کارایی واکسن *آئروموناس هیدروفیلا* در ماهی کپور معمول ارزیابی شد. تعداد ۳۶۰ قطعه ماهی کپور معمولی (با وزن ۵/۱±۴۸ گرم) به طور تصادفی به چهار گروه در سه تکرار تقسیم شدند. گروه اول، دوم و سوم در برابر باکتری *آئروموناس هیدروفیلا* واکسینه شده و گروه اول با خوراک پایه، گروه دوم با پروبیوتیک ساده و گروه سوم با پروبیوتیک ریزپوشانی شده تغذیه شدند. گروه چهارم یا گروه شاهد با جیره پایه بدون مکمل تغذیه شد. زیست سنجی و نمونه‌گیری خون و روده در روزهای صفر، ۳۰ و ۶۰ آزمایش انجام شد. شاخص‌های عملکرد رشد (ضریب تبدیل غذایی، نرخ رشد ویژه، نسبت کارایی پروتئین و نسبت کارایی غذا) و همچنین شاخص‌های ایمنی (تیتر آنتی‌بادی، فعالیت لیزوزیم، کمپلمان و باکتری‌کشی، احیای NBT، میزان گلوبولین و فعالیت میلوپراکسیداز) اندازه‌گیری و بین گروه‌ها مقایسه شد. همچنین پارامترهای خونی (RBC، WBC، Hb و Hct)، فعالیت آنزیم‌های روده‌ای (لیپاز، پروتئاز، آمیلاز، ALP) و وضعیت آنتی‌اکسیدانی (سطح MDA، SOD، GSH و فعالیت کاتالاز) و برخی شاخص‌های بیوشیمیایی سرم (گلوکز، اوره، کلسیم، تری‌گلیسرید، ALP، CPK و بیلی‌روبین) اندازه‌گیری و بین گروه‌ها مقایسه شدند. در روز ۶۰ آزمایش، ماهیان باقی‌مانده در هر گروه با سویه بیماری‌زای *آئروموناس هیدروفیلا* مورد چالش قرار گرفتند و مرگ و میر تجمعی به مدت ۱۴ روز ثبت شد. نتایج نشان داد که بالاترین شاخص‌های رشد و فعالیت آنزیم‌های روده‌ای در گروه ۳ که واکسینه شده و با پروبیوتیک میکروکپسوله تغذیه شده بودند مشاهده شد. شاخص‌های ایمنی در تیمارهای ۲ و ۳ به طور معنی‌داری نسبت به گروه کنترل افزایش داشتند. پارامترهای خونی و شاخص‌های بیوشیمیایی سرم بین تیمارها تفاوت معنی‌داری نشان ندادند. تلفات پس از چالش در تیمارهای ۳ (۲۰ درصد) و ۲ (۲۰ درصد) به طور معنی‌داری کم‌تر از گروه کنترل (۶۰ درصد) بود. به طور کلی، می‌توان نتیجه‌گیری کرد که نه تنها تجویز این پروبیوتیک نقش مهمی در بهبود کارایی و ایمنی‌زایی واکسن تزریقی آئروموناس در ماهی کپور معمولی دارد، بلکه میکروکپسوله کردن این پروبیوتیک با آلژینات/کیتوزان اثر آن را بر کارایی و ایمنی‌زایی واکسن افزایش می‌دهد. بنابراین، استفاده از این روش میکروکپسوله‌سازی برای بهبود کارایی پروبیوتیک و واکسن توصیه می‌شود.

کلمات کلیدی: واکسن *ائروموناس هیدروفیلا*، *لاکتی پلانتی باسیلوس پلانتاروم*، ریزپوشانی، ماهی کپور، ایمنی‌زایی

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اثر پروبیوتیک‌های ضد درک حد نصاب بر تعدیل فعالیت آنزیم‌های گوارشی، فلور میکروبی، عملکرد رشد و پارامترهای بیوشیمیایی در ماهی کپور معمولی (*Cyprinus carpio*)

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چکیده

در مطالعه حاضر، دو پروبیوتیک اصلی (*Citrobacter freundii* و *Bacillus foraminis*) با ویژگی ضد درک حد نصاب از روده ماهی کپور معمولی جداسازی شدند و اثرات آن‌ها بر عملکرد رشد، فلور میکروبی روده، شاخص‌های بیوشیمیایی و فعالیت آنزیم‌های گوارشی، مانند الفا آمیلاز، لیپاز، تریپسین، کیموتریپسین، و آلکالین فسفاتاز در ماهی کپور معمولی مورد بررسی قرار گرفت. بچه ماهیان (تعداد ۴۵۰ قطعه با وزن ۱۰±۵۰ گرم) به طور تصادفی به ۶ گروه مساوی (با ۳ تکرار) تقسیم شدند و با جیره‌های حاوی QQ1 (*C. freundii* به میزان ۱×۱۰۹ CFU/g)، QQ2 (*B. foraminis* به میزان ۱×۱۰۹ CFU/g)، WQQ (*L. plantarum* باکتری فاقد ویژگی ضد درک حد نصاب) به میزان ۱×۱۰۹ CFU/g)، QQ1 +QQ2 (*B. foraminis* و *C. freundii* به میزان مساوی ۱×۱۰۹ CFU/g)، گروه ترکیبی (*B. foraminis* و *C. freundii* و *L. plantarum* به میزان مساوی ۱×۱۰۹ CFU/g) و یک رژیم غذایی کنترل (بدون پروبیوتیک) برای ۶۰ روز به طور مداوم تغذیه شدند. نتایج نشان داد که مکمل‌های پروبیوتیک به طور کلی اثرات معنی‌داری بر عملکرد رشد داشتند. گروه‌های ترکیبی و پلانتاروم به ترتیب در روزهای ۳۰ و ۶۰ بهترین اثر را بر نرخ رشد ویژه (SGR) و راندمان مصرف خوراک در کپور معمولی داشتند. فعالیت‌های تریپسین، پروتئاز و کیموتریپسین در روز ۳۰ پس از تغذیه، در تیمار ترکیبی در مقایسه با گروه شاهد و سایر گروه‌ها به طور معنی‌داری افزایش یافت. تغییرات معنی‌داری در فلور باکتریایی روده در تمامی گروه‌های پروبیوتیک نسبت به گروه شاهد مشاهده شد. این نتایج استفاده بالقوه از QQ2 (*B. foraminis*) را به تنهایی یا در ترکیب سایر پروبیوتیک‌ها (تیمار ترکیبی) به عنوان افزودنی در رژیم غذایی کپور معمولی نشان داد اما در طولانی مدت توصیه نمی‌شود. نتایج نشان داد که مکمل‌سازی باکتری‌های جدا شده از روده کپور معمولی (مانند *لاکتوپلانتی باسیلوس پلانتاروم*) می‌تواند عملکرد رشد، میکروبیوتای روده و برخی فعالیت‌های آنزیم گوارشی را در بچه ماهیان کپور معمولی در دوره‌های پرورش طولانی‌مدت بهبود بخشد. بنابراین، می‌توان آن را مانند پروبیوتیک‌های تجاری به عنوان تقویت کننده رشد استفاده نمود.

کلمات کلیدی: کپور معمولی، باکتری‌های روده، عملکرد رشد، فعالیت آنزیم‌های گوارشی، فلور میکروبی

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اثرات مکمل روغن ماهی بر ناهنجاری‌های اسکلتی مادرزادی ناشی از فرمالدئید در موش‌های صحرایی ویستار

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چکیده

نشان داده شده است که فرمالدئید به عنوان یک ترکیب آلی رایج باعث ناهنجاری‌های رشدی و نقص جنین می‌شود. روغن ماهی (FO) به دلیل دارا بودن اسید چرب دوکوزاهگزانوئیک برای رشد و نمو طبیعی جنین توصیه می‌شود. هدف از این مطالعه بررسی پتانسیل روغن ماهی در محافظت از رشد جنین و جلوگیری از ناهنجاری‌های اسکلتی ناشی از تیمار موش‌های صحرایی باردار بود. ۳۰ موش صحرایی باردار ویستار به طور تصادفی در ۵ گروه کنترل، شم (سرم فیزیولوژی؛ گاواژ و تزریق داخل صفاقی)، روغن ماهی (۰/۵ میلی‌گرم/کیلوگرم وزن بدن؛ گاواژ)، فرمالدئید (۱۰ میلی‌گرم/کیلوگرم وزن بدن؛ تزریق داخل صفاقی)، فرمالدئید + روغن ماهی دسته‌بندی شدند. دوره تیمار از روز صفر تا ۲۰ بارداری بود. در روز بیستم بارداری، حیوانات تحت بیهوشی قرار گرفتند و پس از آن لاپاراتومی برای تعیین وزن و طول جنین (CRL) انجام شد. ارزیابی‌های استریومیکروسکوپی اسکلتی جنین‌ها با استفاده از روش رنگ‌آمیزی آلیزارین قرمز/آلیسین آبی انجام شد. علاوه بر این، بیان Runx2 و BMP4 از طریق qPCR ارزیابی شد. یافته‌ها نشان داد که قرار گرفتن در معرض فرمالدئی قبل از تولد به طور قابل توجهی وزن جنین و CRL و همچنین بیان ژن‌های Runx2 و BMP4 را کاهش می‌دهد. علاوه بر این، فرمالدیید وقوع ناهنجاری‌های اسکلتی مادرزادی، از جمله شکاف کام، اسپینا بیفیدا و عدم استخوان‌سازی استخوان‌های جنین را افزایش داد. با این وجود، تجویز همزمان روغن ماهی و فرمالدیید در موش‌های صحرایی باردار، رشد استخوان جنین را بهبود بخشید و ناهنجاری‌های اسکلتی را کاهش داد. روغن ماهی پتانسیل کاهش اثرات تراتوژنیک مواجهه با فرمالدئید را با افزایش بیان ژن‌های مرتبط با استخوان‌زایی نشان داد.

کلمات کلیدی: ناهنجاری‌های مادرزادی اسکلتی، فرمالدئید، روغن ماهی، جنین، موش صحرایی

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مطالعه مورفولوژی و مورفومتری مهره‌های کمری خاجی در خوکچه هندی (*Cavia porcellus*) بر اساس تصاویر سی‌تی اسکن

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چکیده

توموگرافی کامپیوتری (CT) از تکنیک‌های تصویربرداری تشخیصی دقیقی می‌باشد که برای ارزیابی ستون مهره‌ها در دام‌های کوچک و حیوانات اگزوتیک استفاده می‌شود. هدف از این مطالعه بررسی مورفولوژیک و مورفومتریک مهره‌های کمری خاجی نرمال در خوکچه‌هندی (*Cavia porcellus*) بر اساس تصاویر سی‌تی اسکن بود. در این مطالعه توصیفی – مقطعی از ۱۰ خوکچه‌هندی بالغ سالم (۵ نر و ۵ ماده) با میانگین سنی ۱۲±۱/۲۰ ماه و میانگین وزنی ۰/۱۵ ± ۱/۰۴ کیلوگرم استفاده شد. متعاقب بی‌هوشی خوکچه‌هندی با کوکتل داروهای زایلازین (۴ mg/kg) و کتامین (۶۰ mg/kg)، سی‌تی اسکن از مهره‌های کمری خاجی آن در پلن‌های ساجیتال، عرضی و دورسال از قسمت قدامی اولین مهره کمری تا انتهای خلفی خاجی انجام گرفت. براساس نتایج این مطالعه تمامی قسمت‌های مهره‌های کمری خاجی و مفاصل بین مهره‌ای خوکچه‌هندی در تصاویر توموگرافی کامپیوتری قابل مشاهده و ارزیابی هستند. زوائد شوکی مهره‌های کمری در پلن ساجیتال و زوائد مفصلی قدامی و خلفی در بازسازی‌های ساجیتال و عرضی بهتر قابل شناسایی هستند. زوائد پستانی و شیارهای مهره‌ای قدامی و خلفی در پلن دورسال بهتر دیده می‌شوند. دو فرورفتگی جانبی در سوراخ مهره‌ای کودال L_6 در محل اتصال پدیکول و بدنه مهره قابل مشاهده بود که این ویژگی آناتومیکی برای اولین بار گزارش می‌شود. فضاهای بین کمانی (interarcuate spaces) در مهره‌های کمری خوکچه‌هندی بسیار باریک بودند، اما این فضا بین مهره‌های L_6 و S_1 پهن و بزرگ بود، لذا جراحان برای بی‌حسی اپیدورال می‌توانند از این محل نسبت به پونکسیون مایع مغزی نخاعی و تزریق داروهای بی‌هوشی اقدام نمایند. در این مطالعه اندازه‌گیری‌های مورفومتریک از قسمت‌های مختلف تشکیل دهنده مهره‌های کمری خاجی انجام یافته و تحت آنالیز آماری قرار گرفتند. نتایج این مطالعه می‌تواند برای آموزش علوم آناتومی توموگرافی کامپیوتری مهره‌های لومبوساکرال، تفسیر تصاویر CT اسکن و نیز در معاینات بالینی و امور درمانی خوکچه‌هندی (*Cavia porcellus*) مورد استفاده قرار گیرند.

کلمات کلیدی: موفولوژی، مورفومتری، خوکچه‌هندی (*Cavia porcellus*)، توموگرافی کامپیوتری، مهره‌های کمری خاجی

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ارزیابی اثر اکسی‌توسین یا کاربتوسین همراه با تجویز فلونیکسین مگلومین و شستشوی رحم بر درمان اندومتریت پایدار پس از جفت گیری در مادیان‌های دره شوری

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چکیده

اندومتریت پایدار پس از جفت گیری (PBIE) سومین بیماری شایع و یکی از علل اصلی ناباروری در مادیان‌ها است. برخی از مادیان‌ها به درمان‌های رایج شامل شستشوی رحم، آنتی‌بیوتیک‌ها، ضدالتهاب‌ها و عوامل انقباض دهنده‌ی رحم به طور موثر پاسخ نمی‌دهند. اما این مطالعه کاربرد ترکیبی اکسی‌توسین و کربیتوسین به همراه فلونیکسین مگلومین برای درمان PBIE در مادیان دره شوری را مورد بررسی قرار داد. این مطالعه شامل ۴۵ مادیان دره شوری مبتلا به PBIE بود. گروه درمانی ۱: درمان با اکسی‌توسین، فلونیکسین مگلومین و شستشوی رحم با نرمال سالین انجام شد (۱۵ رأس مادیان). گروه درمانی ۲: درمان با کاربتوسین، فلونیکسین مگلومین و شستشوی رحم با نرمال سالین انجام شد (۱۵ رأس مادیان). کنترل: فقط شستشوی رحم با با نرمال سالین انجام شد (۱۵ رأس مادیان). نمونه‌های سیتولوژی پس از تخمک گذاری و قبل از درمان برای تأیید آندومتریت جمع‌آوری شد. میزان بارداری ۱۴ روز پس از تخمک گذاری از طریق سونوگرافی ارزیابی شد. نتایج نشان داد که میزان بارداری به طور معنی‌داری در گروه تحت درمان با کاربتوسین (۸۶ درصد) و گروه تحت درمان با اکسی‌توسین (۶۶ درصد) بالا بود. ارتباط معنی‌داری بین آبستنی و عواملی مانند ادم رحم، اندازه فولیکول و فاصله بین جفت گیری تا تخمک گذاری مشاهده شد. این مطالعه اثربخشی بالقوه استفاده از اکسی‌توسین و کربیتوسین با فلونیکسین مگلومین را برای درمان PBIE در مادیان دره شوری نشان می‌دهد، اگرچه تحقیقات بیش‌تری برای نتیجه‌گیری قطعی ضروری است.

کلمات کلیدی: اندومتریت القا شده پس از جفت گیری، کربیتوسین، اکسی‌توسین، فلونکسین مگلوماین، مادیان‌های دره شوری

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پروفایل بیان ژن‌های سیتوکین گاوی در سلول‌های سوماتیک شیر در مراحل مختلف اولین دوره شیردهی گاوهای شیری هلشتاین

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چکیده

سلول‌های سوماتیک شیر پروتئین‌های محلول متعددی مانند سیتوکین‌ها را تولید می‌کنند که نقش مهمی در ایمنی غدد پستانی ایفا می‌کنند. این مطالعه با هدف بررسی پروفایل‌های بیان ژن‌های سیتوکین پیش التهابی گاو شامل IL-2، IL-6، IL-8، TNF-α، IFN-γ و GM-CSF در سلول‌های سوماتیک شیر در مراحل مختلف شیردهی در گاوهای هلشتاین سالم در اولین دوره شیردهی خود انجام شد. برای این منظور، RNA کل از سلول‌های سوماتیک شیر تعداد ۱۸ رأس گاو شیری سالم در مراحل اولیه، میانی و اواخر دوره شیردهی استخراج شد و واکنش real-time PCR جهت بررسی بیان ژن‌های سیتوکین برای تمام نمونه‌ها انجام شد. نتایج نشان داد که بیان تقریباً تمام ژن‌های سیتوکین به جز ژن TNF-α در حیوانات در میانه نسبت به مرحله اولیه شیردهی به طور معنی‌داری بیش‌تر بود. با این حال، بیان ژن‌های سیتوکین نیز در مرحله اواخر شیردهی در مقایسه با اوایل شیردهی روند بالاتری را نشان داد، اما این تفاوت‌ها تنها برای سطوح mRNA ژن‌های TNF-α و GM-CFSمعنی‌دار بود. علاوه بر این، تفاوت بیان ژن‌های سیتوکین در گاوها در مرحله انتهای شیردهی نسبت به حیوانات در مرحله میانی شیردهی معنی‌دار نبود. در کل دوره شیردهی، سطوح رونویسی mRNA برای IL-6 و IL-2 به ترتیب در غلظت‌های بالا و پایین نسبت به سایر ژن‌های سیتوکین مشاهده شد. همبستگی بین سطوح بیان ژن برای اکثر ژن‌های مورد مطالعه در مراحل مختلف شیردهی تقریباً معنی‌دار نبود. با این حال، ارتباط معنی‌داری بین IL-8 و GM-CSF در کل، مراحل اولیه و اواخر دوره شیردهی یافت شد.

کلمات کلیدی: بیان ژن، سیتوکین، دوره شیردهی، گاو

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مقایسه تجویز داخل صفاقی مدتومیدین و پیرامون برش بوپیواکائین بر کنترل درد بعد از جراحی اواریوهیسترکتومی در سگ

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چکیده

ایجاد شرایط بی‌دردی مؤثر بعد از اعمال جراحی در دامپزشکی به خصوص متعاقب اعمالی چون عقیم‌سازی حیوانات امری بسیار مهم از نظر اخلاقی و مراقبت بالینی به شمار می‌آید. به منظور دستیابی به این هدف از داروهای ضد درد گوناگونی تا به امروز استفاده شده است، اما در حیوانات مختلف مدیریت درد حین و بعد از جراحی نیازمند استراتژی‌های متفاوتی است. در این مطالعه، آثار دو داروی مدتومیدین و بوپیواکائین در اشکال متفاوت تجویز بر روی کنترل درد بعد از عمل جراحی اواریوهیسترکتومی بر روی سگ‌های ماده مورد بررسی قرار گرفت. ۲۵ سگ ماده نژاد بومی (۱-۴ سال سن و ۱۵-۲۵ کیلوگرم وزن) بر اساس نوع دارو و روش تجویز به ۵ گروه ۵ تایی تقسیم شدند: گروه کنترل، گروه مدتومیدین عضلانی (Med-IM)، گروه مدتومیدین صفاقی (Med-IP)، گروه مدتومیدین صفاقی همراه با بوپیواکائین زیر جلدی اطراف برش (Med/Bup) و گروه بوپیواکائین زیرجلدی اطراف برش (Bup-Sc). در تمامی گروه‌ها داروها در سه نقطه زمانی تجویز شد؛ قبل از برش پوست، همزمان با لیگاتور اولین پایه تخمدان و پیش از بستن برش خط وسط شکم. در زمان‌های قبل از جراحی، حین و بعد از اتمام عمل جراحی کیفیت آرامبخشی، کیفیت درد، عمق بیهوشی، کیفیت ریکاوری، علائم حیاتی (دمای بدن، تعداد تنفس، شاخص‌های قلبی عروقی) و شاخص‌های هماتولوژیک، بیوشیمیایی اندازه‌گیری شد. امتیاز درد بعد از جراحی با کمک روش‌های امتیاز توصیفی ساده (SDS)، مقیاس بصری آنالوگ (VAS)، مقیاس درد ملبورن (UMPS) و فرم کوتاه مقیاس درد گلاسگو (CMPS-SF) مورد ارزیابی قرار گرفت. داده‌های به دست آمده با استفاده از نرم‌افزار SPSS و به کارگیری آزمون‌های مناسب مورد ارزیابی و تحلیل قرارگرفت. نتایج این مطالعه نشان داد که تجویز داخل صفاقی داروی مدتومیدین با دوز مشابه تزریق عضلانی این دارو منجر به اختلافات معنی‌داری در امتیاز درد (بر اساس روش‌های CMPS-SF و VAS)، ضربان قلب و سطوح سرمی کورتیزول در زمان‌های به خصوص بعد از عمل جراحی شد. تجویز زیرجلدی اطراف برش بوپیواکائین به تنهایی در مقایسه با تجویز مدتومیدین به تنهایی و یا مدتومیدین و بوپیواکائین همراه با یکدیگر، به طرز معنی‌داری باعث کاهش امتیاز درد بعد از عمل جراحی و کاهش مدت زمان ریکاوری شد. در گروه مدتومیدین صفاقی، حیوانات نیازمند دریافت دوز ضددرد نجات دهنده شدند در حالی که در سایر گروه‌های تیمار این نیاز ایجاد نگردید. مقدار داروهای تجویز شده در این مطالعه اختلالی در عملکردهای فیزیولوژیک هیچ یک از سگ‌ها ایجاد نکرد و شاخص‌های قلبی عروقی، دستگاه تنفس و دمای مقعدی در محدوده طبیعی خود باقی بود. فعالیت سرمی آنزیم‌های کبدی و عملکرد قلبی نیز در محدوده طبیعی خود بود. ارزیابی امتیاز درد با استفاده از روش‌های VAS، UMPS و CMPS-SF هیچ گونه ارجحیت یا برتری در تجویز داخل صفاقی داروی مدتومیدین نسبت به تجویز عضلانی آن (با دوز مشابه) یا تجویز عضلانی همین دارو همراه با بوپیواکائین در کنترل درد بعد از جراحی عقیم سازی سگ ماده و ریکاوری بعد از جراحی، پارامترهای فیزیولوژیک، قلبی عروقی، تنفسی و دمای مقعدی نشان نداد.

کلمات کلیدی: مدیریت درد، اواریوهیسترکتومی، سگ ماده، مدتومیدین، بوپیواکائین

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بهبود سیستم ایمنی و وضعیت آنتی اکسیدانی در بلدرچین ژاپنی با استفاده از بیوچار

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چکیده

این مطالعه به ارزیابی تأثیرات بیوچار حاصل از محصولات جانبی پسته بر عملکرد، متابولیت‌های خونی، پاسخ ایمنی، وضعیت آنتی‌اکسیدانی و انتشار گاز آمونیاک در بلدرچین‌های ژاپنی پرداخت. در مجموع، ۵۰۰ قطعه بلدرچین یک روزه ژاپنی به طراحی کاملاً تصادفی با پنج تیمار غذایی و پنج تکرار (۲۰ قطعه بلدرچین در هر تکرار) به مدت ۳۵ روز تخصیص داده شدند. جیره‌های غذایی آزمایشی شامل موارد زیر بودند: (۱) جیره پایه بدون افزودنی (شاهد)، (۲) جیره پایه با ۰/۰۵ درصد فلوکوئین ۱۰ درصد (شاهد مثبت)، (۳) جیره پایه با ۰/۳۵ درصد بیوچار، (۴) جیره پایه با ۰/۶۵ درصد بیوچار و (۵) جیره پایه با ۱ درصد بیوچار. نتایج نشان داد که وزن در بلدرچین‌های تغذیه شده با ۰/۶۵ درصد بیوچار نسبت به گروه شاهد و مکمل شده با فلومکوئین به طور معنی‌داری بیشتر بود، بدون آن که مصرف خوراک تحت تأثیر قرار بگیرد. ضریب تبدیل نیز در پرندگان تغذیه شده با ۰/۶۵ درصد بیوچار نسبت به شاهد تمایل به معنی‌داری داشت. بلدرچین‌های تغذیه شده با ۱ درصد بیوچار به طور معنی‌داری سطح کلسترول و LDL کمتری داشتند، در حالی که گروه شاهد بالاترین مقدار را نشان دادند. بالاترین درصد لنفوسیت‌ها در بلدرچین‌های تغذیه‌شده با ۱ درصد بیوچار مشاهده شد و افزایش سطح بیوچار در جیره غذایی نسبت لنفوسیت/هتروفیل را به طور معنی‌داری کاهش داد. با این حال، مکمل کردن بیوچار تأثیر معنی‌داری بر سطوح ایمنوگلوبولین‌ها (IgG، IgM، IgY، و IgT) نداشت. نشان‌گرهای آنتی‌اکسیدانی، از جمله ظرفیت آنتی‌اکسیدانی کل، گلوتاتیون پراکسیداز و سوپراکسید دیسموتاز، در پرندگان تغذیه‌شده با ۱ درصد بیوچار پوست پسته بالاترین مقادیر را داشتند ولی تفاوت معنی‌داری بین سطوح ۰/۶۵ درصد و ۱ درصد مشاهده نشد. علاوه بر این، مکمل کردن بیوچار انتشار گاز آمونیاک را به طور معنی‌داری کاهش داد. به طور کلی، افزودن حداقل ۰/۶۵ درصد بیوچار پوست پسته به جیره غذایی بلدرچین‌های گوشتی، عملکرد رشد، پارامترهای خونی، فعالیت آنزیم‌های آنتی‌اکسیدانی و پاسخ ایمنی را بهبود بخشید و جایگزینی دوستدار محیط زیست برای آنتی‌بیوتیک‌ها ارائه داد.

کلمات کلیدی: بیوچار، پوست پسته، متابولیت‌های خونی، پاسخ ایمنی، انتشار گاز آمونیاک

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