

Modulation of intestinal histology and immune functions using prebiotic and organic acid supplementation with stocking density in local quails

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The research examined the impact of prebiotic and organic acid additives, along with high stocking density, on the growth performance, biochemical parameters, histological characteristics, and immune function of local quail. A total of 276 one-day-old local quails were randomly assigned to five treatment groups, with three replicates per group and housed in boxes for 42 days. The dietary treatments were designed as follows: A negative control (12 birds/ replicate), positive control (20 birds/ replicate), group with 1% prebiotic (Jerusalem artichoke) additive at a high stocking density of 20 birds/ replicate, group with 0.1% organic acid additive with high stocking density and a group with a combination of 0.5% prebiotic and 0.05% organic acid additives with high stocking density. The results showed that quails receiving prebiotic alone or a combination of prebiotic and organic acid supplements with high stocking density performed best compared to both control groups. The cholesterol and blood sugar levels were reduced in both the prebiotic alone and when combined with organic acid. The lactic acid bacteria count in the caecum digesta were significantly higher in the

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prebiotic alone and in combination with organic acid compared to both controls. The jejunum villus height was significantly increased with prebiotic alone and with prebiotic mixed with organic acid compared with both controls. All additives significantly enhanced the size of the Fabricius follicles. In summary, supplementing quail diets with prebiotics, alone or in combination with organic acids, under high stocking density conditions improved production performance, biochemical parameters, jejunum histology, and immune function in local quails.

KEY WORDS: antibiotic alternatives / intestinal health / stress / bacterial count / quail nutrition

Japanese quails (*Coturnix coturnix japonica*) have become increasingly significant in the avian world due to their small size, ease of management, ability to house many birds in compact spaces, and high egg production. As quail populations grew, there was a corresponding increase in the intensification of production systems. It was initially domesticated by the Chinese as an amateur bird, several hundred years ago, for ‘singing’ males. Later, the Japanese began keeping quail in enclosed small cages. In 1910, Kotaro Oda discovered their extremely high laying rate. In the 1950s, scientists became interested in using quail in laboratory studies; In the 1970s, quail were kept on many farms in Japan, Italy, France, and Germany (stocking rates of 2-3 million). The first quails were imported to Poland in the 1960s.

Stocking density is a key environmental stressor that can impact the health and performance of birds. These stressors can lead to physiological changes, behavioral shifts, reduced egg and meat quality, tissue and intestinal damage, and increased mortality. Nasr *et al.* [2021] note that high stocking density has harmful effects, including decreased feed intake, lower feed conversion efficiency, and reduced final weight. It also leads to increased footpad dermatitis, scratches, poor feather condition, bruising, and higher condemnation rates. Various feeding strategies have been explored to mitigate the negative effects of stress on birds. One such approach is the use of prebiotics, which help reduce stress and promote better well-being in birds [Mirza 2015, Mohammed and Kareem 2022].

Prebiotics are specifically defined as non-digestible food components that positively affect the host by promoting the growth and/or activity of beneficial bacteria in the intestines, thereby improving the host’s microbial balance [Sobolewska *et al.*, 2017]. Generally, prebiotics can be fermented by beneficial bacteria in the intestines to produce lactic acid, short-chain fatty acids, or antimicrobial substances such as bacteriocins, which help combat harmful bacteria [Bogusławska-Tryk *et al.* 2012, Mirza 2015]. Oligosaccharides and monosaccharides can help reduce pathogen colonization by blocking the receptor sites on epithelial cell surfaces, preventing pathogens from attaching [Pourabedin and Zhao 2015, Kareem *et al.* 2016].

Organic acids (OAs) are carboxylic acids with an R-COOH structure commonly used in poultry feed additives due to their effectiveness in enhancing growth performance and promoting intestinal health [Dai *et al.* 2021]. OAs can lower the pH in the intestinal lumen and boost digestive enzyme activity, thereby enhancing the apparent digestibility of nutrients [Ma *et al.* 2021]. In particular, OAs can influence the intestinal microbiota of broilers through their antimicrobial effects. They achieve

this by disrupting the semipermeable peptidoglycan or phospholipid membranes of bacteria, releasing hydrogen ions. This decrease in pH causes the bacterial cells to collapse [Hu et al. 2020, Dai *et al.* 2021]. Meanwhile, growing evidence suggests that OAs enhance intestinal barrier function and immune response in broilers by altering their intestinal microbiota [Sabour *et al.* 2019, Dai *et al.* 2021]. However, it is still unclear whether dietary OAs can improve the intestinal barrier and growth performance of broilers that are affected by high stocking density stress.

High stocking density is recognized as a major stress factor that can impair growth performance, intestinal health, and immune function in poultry. Prebiotics and organic acids have been reported to improve gut microbial balance, enhance nutrient utilization, and support immune responses under various stress conditions. Therefore, we hypothesized that high stocking density would adversely affect the productive performance, intestinal histology, and immune status of local quails, whereas dietary supplementation with prebiotics and/or organic acids would mitigate these negative effects and improve bird health and performance. Accordingly, this study was conducted to evaluate the effectiveness of prebiotics and organic acids as feed additives in alleviating the detrimental impacts of high stocking density on growth performance, intestinal histology, and immune function of local quails.

Material and methods

Design of experiment

This experiment is designed in the Grdarasha Field Research, College of Agricultural Engineering Sciences, Salahaddin University, Erbil. The study examined the effects of prebiotics, organic acids, and their combination on performance, biochemical parameters, histology, and immune function under high stocking density conditions in local quail. A total of 276 one-day-old local quails were randomly assigned to five treatments, with three replicates per treatment, and housed in boxes (60×30×25 cm) for a 42-day trial period. The ambient temperature was maintained at 35-37°C during the first week and gradually reduced by 2-3°C weekly until reaching 24-26°C. Relative humidity was maintained between 55 and 65% throughout the trial. A lighting schedule of 23 h light and 1 h darkness was provided during the first week, followed by 20 h light and 4 h darkness until the end of the experiment. Adequate ventilation was provided to ensure continuous air exchange and maintain acceptable air quality within the experimental house. The dietary treatments were designed as follows: Negative control (12 birds per replicate, 36 birds per treatment), Positive control (20 birds per replicate, 60 birds per treatment), 1% prebiotic (Jerusalem artichoke containing inulin as the prebiotic source) with stocking density (20 birds per replicate, 60 birds per treatment), 0.1% organic acid additive with stocking density (20 birds per replicate, 60 birds per treatment), and 0.5% prebiotic + 0.05% organic acid additives with stocking density (20 birds per replicate, 60 birds per treatment). The chicks had free access to obtain diet and water *ad libitum*. The composition of the control diet is presented in Table 1.

Table 1. The composition of the standard diet for the experiment (%)

Ingredients	Composition (%)
Maize	19.5
Soybean meal	34.1
Wheat	40.0
Limestone	1.5
Premix ¹	2.5
Di calcium phosphate	0.6
Enzyme	0.1
Antioxidant	0.1
Oil	1.6
Total	100
Calculated values ²	
ME (kcal/kg)	3000
Crude protein (%)	23
Lysine (%)	1.19
Methionine (%)	0.47
Calcium (%)	0.81
Available phosphate (%)	0.45

¹Premix: each 1 kg contains: Crude protein 15%, Crude fat 2%, Crude fiber 4%, Calcium 4%, Phosphorus (Av.) 8.6%, Methionine 5.2%, Methionine + cystine 5.4%, Lysine 2.5%, Sodium 5%, Vit. A 480.000IU, Vit. D 3120.000IU, Vit. E 1000mg, Vit. K 80mg, Vit. B₁ 80mg, Vit. B₂ 40mg, Vit. B₃ 600 mg, Vit. B₆ 120 mg, Vit. B₁₂ 1mg, Niacin 1.200 mg, Folic acid, 40mg, Biotin 6mg, CholinChlorid 12.000 mg, Iron 2.800mg, Zinc 2.400 mg, Copper 320mg, Manganese 3.000mg, Iodine 1100 mg, Selenium 8 mg.

²Food requirements were estimated according to [NRC, 1994].

Growth Performance

The initial and final weights of the quails were recorded using a digital scale, and feed intake was monitored throughout the experimental period. The feed conversion ratio (FCR) was calculated as feed intake divided by weight gain. Carcass weight was also measured. The growth performance was evaluated using the following formulas:

Weight gain (g) = BW at the end of the experiment - BW at the beginning of the experiment
 BW - body weight; FCR = final feed intake / final weight gain.

Biochemical Parameters

On day 42, six birds were randomly selected from each treatment, and blood samples were collected from the wing vein and stored in sterilized tubes for the analysis of serum biochemical parameters and lipid profiles (including cholesterol, LDL, HDL, and triglycerides) using the Roche Cobas® 6000 analyzer series (Switzerland).

Analysis of the caecum microbiota

At the end of the experiment, six birds were randomly selected from each treatment, and their cecal digesta were carefully collected under sterile conditions for the analysis

of intestinal microbes. (*Lactobacillus* spp. and total coliform bacteria). As a result, serial dilutions were prepared over a range of 10^{-1} to 10^{-7} . Target bacterial groups were counted by plating 0.1 ml from each dilution onto sterile selective media, including MacConkey agar for total coliforms and MRS (De Man-Rogosa-Sharpe) agar for lactic acid bacteria (Sigma-Aldrich, UK). The colony-forming units (CFU) were then calculated based on the number of microbial colonies. For fresh cecal digesta, the CFU/ml values were calculated using logarithmic transformations [Doski and Kareem 2024].

Histology examination of Jejunum and bursa of Fabricius

At the end of the experiment, six birds were randomly selected from each group and euthanized by cervical dislocation. The jejunum of the small intestine and the bursa of Fabricius were dissected and fixed in 10% buffered formalin for histological analysis. The tissue processing involved a series of dehydration steps, wax impregnation, and clearing. Tissue sections were prepared by cutting with a microtome to a thickness of 5 μm and then mounted on slides. A standard routine protocol was followed using hematoxylin and eosin staining. The stained slides were examined under a light microscope, and images were captured using a digital camera at 10 $\times$ magnification. The crypt depth (μm), villus height of the jejunum, and follicles' area in the Fabricius bursa were measured using Image J software [Mirza 2015].

Statistical analysis

Data were analyzed using one-way ANOVA in SPSS, version 27. The summary statistics included means and standard errors. Duncan's test was used at the 0.05 significance level to identify significant differences between the various parameters.

Results and discussion

The results of this study showed that supplementing with the plant additive Jerusalem artichoke as a prebiotic had no positive impact on the growth performance of local quails. The highest final live body weight and weight gain were observed in the group receiving the combination of prebiotic and organic acid supplements under high stocking density, compared to the other treatments. Additionally, no significant differences ($P>0.05$) were observed in the carcass weight of local quails at the end of the experimental period (Tab. 2).

The cholesterol content and HDL levels in male quails were significantly ($P<0.05$) reduced in the prebiotic and prebiotic + OA combination groups, compared to the positive control, negative control, and OA groups (Tab. 3). In contrast, triglyceride and LDL levels were significantly higher ($P<0.05$) in the positive control group than in all other treatments. Furthermore, blood sugar levels in male quails were significantly ($P<0.05$) lower when prebiotic or the prebiotic + OA combination was added under high stocking density, compared to all other treatments. Additionally, the prebiotic additive significantly reduced blood sugar levels compared to the other additives.

Table 2. Influence of prebiotic and organic acid on growth performance and carcass weight of local quails with stocking density at six weeks of age (mean $\pm$ standard error)

Parameters	Treatments ¹				P value
	Negative CON	Positive CON	prebiotic	organic acid	mixed (Pre+OA)
Initial weight (g)	35.7 $\pm$ 1.10 ^a	37.16 $\pm$ 0.33 ^a	35.83 $\pm$ 0.60 ^a	35.16 $\pm$ 0.83 ^a	34.90 $\pm$ 0.90 ^a
Final weight (g)	211.03 $\pm$ 1.27 ^a	216.16 $\pm$ 0.44 ^a	209.83 $\pm$ 2.60 ^a	208.33 $\pm$ 3.67 ^a	227.23 $\pm$ 11.04 ^a
Weight gain (g/bird)	175.33 $\pm$ 1.93 ^a	179.00 $\pm$ 0.5 ^a	174.00 $\pm$ 2.84 ^a	173.16 $\pm$ 4.20 ^a	192.65 $\pm$ 11.33 ^a
Feed intake (g/bird)	563.07 $\pm$ 11.03 ^a	547.85 $\pm$ 4.16 ^a	547.25 $\pm$ 1.36 ^a	561.41 $\pm$ 7.70 ^a	564.39 $\pm$ 11.64 ^a
FCR*	3.21 $\pm$ 0.04 ^a	3.06 $\pm$ 0.03 ^a	3.14 $\pm$ 0.05 ^a	3.24 $\pm$ 0.12 ^a	2.95 $\pm$ 0.13 ^a
Dressing percentage (%)	61.88 $\pm$ 2.03 ^a	60.57 $\pm$ 2.16 ^a	63.12 $\pm$ 1.39 ^a	60.60 $\pm$ 1.54 ^a	61.59 $\pm$ 1.55 ^a

^{abc}Data in the same row direction with different letters differ significantly ($P < 0.05$), *FCR – feed conversion ratio.

¹Negative control (12 birds per replicate), Positive control (20 birds per replicate), 1% prebiotic (Jerusalem artichoke containing inulin) with HSD, 0.1% organic acid additive with HSD, and 0.5% prebiotic + 0.05% organic acid additives with HSD.

Table 3. Influence of prebiotic and organic acid on biochemical parameters of local quails with stocking density at six weeks of age (mean $\pm$ standard error)

Parameters	Treatments ¹				P value
	Negative CON	Positive CON	prebiotic	organic acid	mixed (Pre+OA)
Cholesterol (mg/dL)	254.80 $\pm$ 6.35 ^a	245.36 $\pm$ 6.81 ^{ab}	189.53 $\pm$ 7.56 ^d	224.50 $\pm$ 12.89 ^{bc}	211.79 $\pm$ 10.33 ^{cd}
Triglyceride (mg/dL)	237.90 $\pm$ 14.55 ^a	285.50 $\pm$ 14.74 ^b	218.73 $\pm$ 9.13 ^a	225.16 $\pm$ 13.66 ^a	219.86 $\pm$ 6.78 ^a
HDL (mg/dL)	75.80 $\pm$ 2.82 ^a	71.06 $\pm$ 4.42 ^a	34.43 $\pm$ 2.75 ^b	64.96 $\pm$ 2.91 ^a	42.83 $\pm$ 2.58 ^b
LDL (mg/dL)	116.66 $\pm$ 4.84 ^b	151.00 $\pm$ 3.78 ^a	101.33 $\pm$ 9.15 ^b	115.00 $\pm$ 5.35 ^b	113.33 $\pm$ 4.15 ^b
Blood sugar (mg/dL)	283.16 $\pm$ 5.69 ^{ab}	278.23 $\pm$ 6.96 ^b	217.20 $\pm$ 3.87 ^d	299.56 $\pm$ 6.49 ^a	241.60 $\pm$ 8.15 ^c

^{abc}Data in the same row direction with different letters differ significantly ($P < 0.05$).

¹Negative control (12 birds per replicate), Positive control (20 birds per replicate), 1% prebiotic (Jerusalem artichoke containing inulin) with HSD, 0.1% organic acid additive with HSD, and 0.5% prebiotic + 0.05% organic acid additives with HSD.

Yalçın *et al.* [2014] reported that the yeast cell wall derived from baker's yeast is an effective prebiotic feed additive for broilers, as it enhances growth performance, boosts humoral immune response, and reduces abdominal fat. In another study conducted with laying hens, Yalçın *et al.* [2014] concluded that the yeast cell wall had beneficial effects on the production of low-cholesterol eggs and on enhancing the humoral immune response.

Serum glucose levels were lower in prebiotic treatment than in other treatments. The elevated glucose levels associated with increased feed intake may suggest that the birds were experiencing stress. Sabater and Forbes [2015] noted that elevated serum glucose concentrations could be attributed to factors such as stress, with increased glucose levels potentially resulting from stress or other contributing factors.

The study demonstrated the effect of prebiotic, organic acid, and their combination under high stocking density on bacterial counts in the cecal digesta at 42 days of age. The results showed that the lactic acid bacteria (LAB) counts in treatments 3 and 4 were significantly ($P<0.05$) higher in the cecal digesta than in the negative and positive control groups. Meanwhile, no significant differences were observed between treatments in the coliform bacteria counts (Tab. 4).

Table 4. Effect of prebiotic and organic acid with stocking density on Bacteria counting (Log CFU/mL) in caecum digesta of local quails at 42 days of age

Treatments ¹	Caecum microbiota	
	lactic acid bacteria	total coliform bacteria
Negative CON	8.58±0.04 ^c	5.74±0.06 ^a
Positive CON	8.50±0.02 ^c	5.72±0.04 ^a
Prebiotic (Pre)	8.86±0.04 ^a	5.59±0.07 ^a
Organic acid (OA)	8.73±0.02 ^b	5.64±0.02 ^a
Mixed (Pre+OA)	8.82±0.03 ^{ab}	5.62±0.04 ^a
<i>P</i> -value	<0.001	0.288

^{abc}Differences in letters among the groups within the same column are statistically different, $P<0.05$.

¹Negative control (12 birds per replicate), Positive control (20 birds per replicate), 1% prebiotic (Jerusalem artichoke containing inulin) with HSD, 0.1% organic acid additive with HSD, and 0.5% prebiotic + 0.05% organic acid additives with HSD.

Abdeldayem *et al.* [2024] indicated that butyric acid, as an organic acid, significantly ($P<0.05$) reduced the counts of cecal *E. coli*, *Salmonella*, *Enterococcus*, and coliform bacteria. At the same time, it significantly increased the levels of lactic acid bacteria compared to the control group. Also, according to Martin-Gallausiaux *et al.* [2021]. The gut microbiota plays a key role in nutrient digestion and absorption, maintaining energy balance, and supporting immune system development. In quails, supplementation with butyric acid as an organic acid, starting at 50 mg/kg of the diet, was found to modulate the caecal microbiota significantly. In this study, the numbers of *E. coli*, *Salmonella*, *coliforms*, and *Enterococcus* were reduced, while the count of lactic acid bacteria increased.

The study highlights the effects of prebiotics, organic acids, and their combination on alleviating stress caused by high stocking density, particularly on jejunum histology in quails at 42 days of age. The jejunum villus height was significantly ($P<0.05$) greater in the groups treated with prebiotic, organic acid (OA), and the combination of prebiotic + OA, compared to both the positive and negative control groups. The

Table 5. Effect of prebiotic and organic acid with stocking density on histology of jejunum of local quails at 42 days of age (mean $\pm$ standard error)

Treatments ¹	Histology of Jejunum	
	villi height (μm)	crypt depth (μm)
Negative CON	671.43 $\pm$ 8.88 ^c	95.66 $\pm$ 0.87 ^a
Positive CON	672.90 $\pm$ 8.04 ^c	93.26 $\pm$ 0.52 ^a
Prebiotic (Pre)	792.76 $\pm$ 10.82 ^a	93.00 $\pm$ 1.42 ^a
Organic acid (OA)	742.30 $\pm$ 8.78 ^b	95.13 $\pm$ 0.93 ^a
Mixed (Pre+OA)	782.76 $\pm$ 9.98 ^a	94.33 $\pm$ 1.47 ^a
<i>P</i> -value	<0.001	0.628

^{abcd}Differences in letters among the groups within the same column are statistically different, $P < 0.05$.

¹Negative control (12 birds per replicate), Positive control (20 birds per replicate), 1% prebiotic (Jerusalem artichoke containing inulin) with HSD, 0.1% organic acid additive with HSD, and 0.5% prebiotic + 0.05% organic acid additives with HSD.

highest villus height was observed in the group with the addition of 1% prebiotic. Meanwhile, no significant ($P > 0.05$) differences were observed in the crypt depth of local quails at the end of the experimental period (Tab. 5).

The results clearly demonstrated the differences between the treatment groups and the control group at the final stage of the study (Fig. 1).

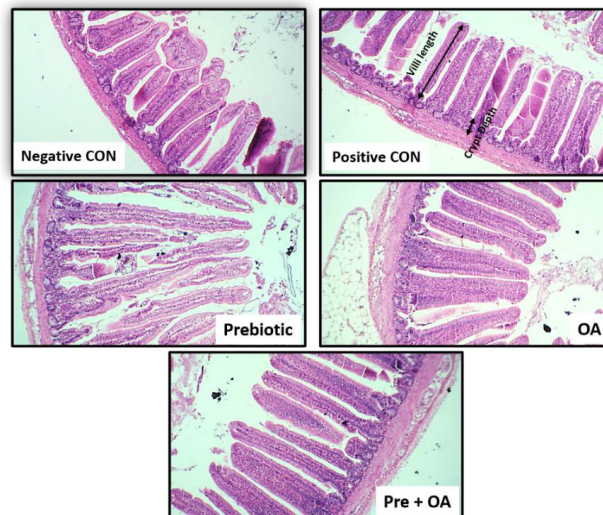


Fig. 1. A haematoxylin and eosin-stained section of the jejunum from birds fed diets at 42 days of age. The groups included: Negative control (12 birds per replicate), Positive control (20 birds per replicate), 1% prebiotic (Jerusalem artichoke containing inulin) with HSD, 0.1% organic acid additive with HSD, and 0.5% prebiotic + 0.05% organic acid additives with HSD. VL stands for Villus height, CD stands for Crypt depth (Magnification 10 $\times$).

Mirza [2015] revealed that treatments incorporating two types of inulin as prebiotics had highly significant ($P<0.01$) effects on the villus height of the jejunum in specific pathogen-free chicks at 14 and 21 days of age. The small intestine is lined by a single layer of epithelial cells, including absorptive, goblet, and enteroendocrine cells, which cover the villi and crypts. This layer is coated with mucus produced by goblet cells, serving as the interface between the gut and the microbiota [Casteleyn *et al.* 2010]. The villi of the small intestine, which are the functional units of the organ, can be described as projections of the lamina propria into the gut lumen that increase the surface area. Their size and length vary along the small intestine, becoming progressively different from the proximal to the distal regions [Rubin and Langer 2022]. Since chickens have a relatively small large intestine, the ileum serves as the final section of the digestive tract responsible for nutrient absorption [Smith *et al.* 2022]. According to the researchers, the increase in villi surface area is another factor that influences the absorptive capacity of the intestines [Saki *et al.* 2017]. The results of this study suggest that the addition of prebiotics and organic acids increases villus height and reduces stress associated with stocking density in birds. This has important implications for villus height, as longer gut villi are considered a sign of better intestinal health.

According to intestinal morphology, prebiotics increased intestinal villus height in broiler diets. A healthy population of beneficial bacteria in the digestive tract enhances detoxification and elimination processes [Teitelbaum and Walker 2002].

Figure 2 presents the histological results of the bursa in local quails at 42 days of age. Furthermore, the effects of the three additive supplements were illustrated by microscopic images of tissue sections from the Bursa of Fabricius (Fig. 3). Compared to both the negative and positive control diets, all additive supplements significantly ($P<0.05$) increased the size of the follicles in the Bursa of Fabricius. However, no significant difference was observed between the prebiotic and organic acid additives under high stocking density.

Mirza [2015] showed that the weight of the Bursa of Fabricius and its histology was higher in birds at 14 and 21 days of age when supplemented with both commercial inulin and inulin from Jerusalem artichoke. The Bursa of Fabricius is an immune system organ responsible for the maturation of B lymphocytes [Schat 2022]. Birds with small follicles did not produce any detectable antibodies against IBDV or the subsequently injected antigen. It was demonstrated that the ability to produce Ig responses was linked to the presence of larger follicles. However, the small follicles could not support the entire developmental pathway of bursal B cells [Mirza 2015]. Villi are the primary structures responsible for nutrient absorption in the small intestine, and enhancing gut morphology positively improves growth performance. The findings of Rodjan *et al.* [Rodjan *et al.* 2018] showed that organic acids in broiler diets affect gut morphology, with significant differences in villus height and crypt depth observed compared with both the positive and negative control groups.

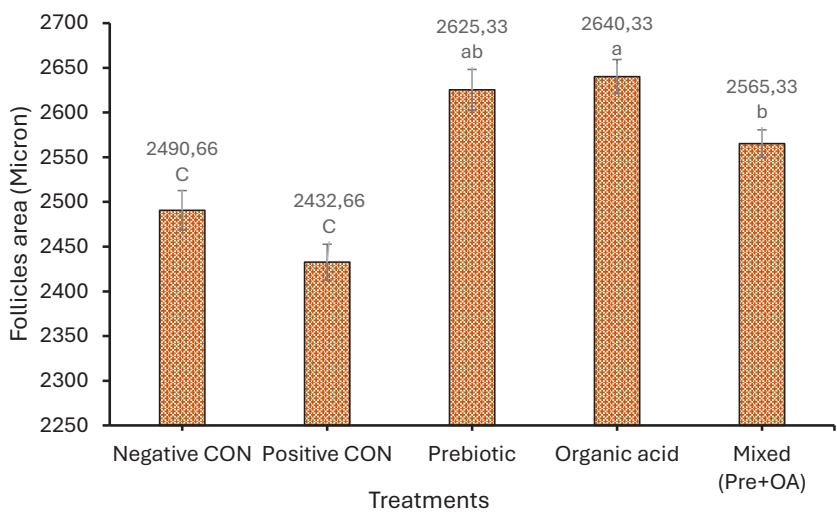


Fig. 2. The effect of prebiotic and organic acid with stocking density on the follicles area (Micron) of the Bursa of Fabricius after local quails at 42 days after the end of the study. Negative control (12 birds per replicate), Positive control (20 birds per replicate), 1% prebiotic (Jerusalem artichoke containing inulin) with HSD, 0.1% organic acid additive with HSD, and 0.5% prebiotic + 0.05% organic acid additives with HSD.

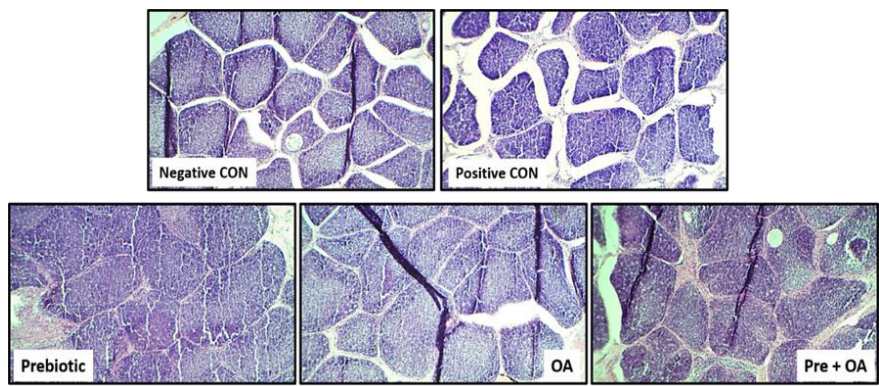


Fig. 3. Follicles Bursa of Fabricius section stained with haematoxylin and eosin at the age of 42 days. The groups included: Negative control (12 birds per replicate), Positive control (20 birds per replicate), 1% prebiotic (Jerusalem artichoke containing inulin) with HSD, 0.1% organic acid additive with HSD, and 0.5% prebiotic + 0.05% organic acid additives with HSD. (Magnification 10 $\times$).

Conclusions

In conclusion, supplementing local quail feed with prebiotics and organic acids under high stocking density showed a numerical increase was observed by increasing the number of birds per unit area without negatively affecting the performance. LAB

was increased in the cecal digesta with prebiotic and organic acid supplementation. Additionally, villus height in the jejunum was enhanced, along with an increase in the size of the follicles in the Bursa of Fabricius.

Ethics approval

The Scientific Ethical Committee of Salahaddin University's Animal Resources Department College of Agricultural Engineering Sciences approved this work on May 30, 2024 (Ref. No.:1432).

Data availability statement

The datasets used and/or analysed in the current study are available from the corresponding authors on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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