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Prophybiotics for *in-ovo* stimulation; validation of effects on gut health and production of broiler chickens

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ABSTRACT Probiotics and phytobiotics have demonstrated effective improvement of gut health in broiler chickens when individually administered *in-ovo.* However, their combined use *in-ovo*, has not been studied to date. We coined the term "prophybiotic" (probiotic + phytobiotic) for such a combination. The current study therefore, aimed to elucidate the effects of combined use of a selected probiotic and a phytobiotic *in-ovo,* on broiler gut health and production parameters, as opposed to use of probiotics alone. ROSS 308 hatching eggs were injected with either *Leuconostoc mesenteroides* (probiotic: **PB**) or *L. mesenteroides* with garlic aqueous extract (prophyiotic: **PPB**) on the 12th day of incubation. Relative abundances of bacteria in feces and cecal content (qPCR), immune related gene expression in cecal mucosa (qPCR) and histomorphology of cecal tissue (PAS staining) were analyzed along with production parameters (hatch quality, body weight, feed efficiency and slaughter and meat quality). PPB treatment increased the abundance of faecalibacteria and bifidobacteria in feces (d 7) and *Akkermansia* sp. in cecal content. Moreover, it decreased *Escherichia coli* abundance in both feces (d 34) and cecal content. PB treatment only increased the faecalibacteria in feces (d 7) and *Akkermansia* sp. in the cecal content. Moreover, PPB treatment resulted in up-regulation of immune related genes (Avian beta defensing 1, Free fatty acid receptor 2 and Mucin 6) and increased the crypt depth in ceca whereas PB treatment demonstrated a higher crypt depth and a tendency to increase Mucin 6 gene expression. Both treatments did not impair the production parameters studied. In conclusion, our results suggest that *in-ovo* PPB treatment may have enhanced potential in boosting the immune system without compromising broiler production and efficiency, as compared to the use of probiotic alone. Our study, highlights the potential of carefully selected PPB combinations for better results in improving gut health of broiler chickens.

Key words: broiler, gut health, *in-ovo* stimulation, production, prophybiotic

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INTRODUCTION

Ensuring optimal gut health in broilers is imperative in broiler production as it impacts many aspects of the industry including the production and welfare of birds and food safety of the broiler meat products (Oviedo-Rondón, 2019). As broilers have been selected intensively for fast growing and efficiency parameters, there is a tradeoff in the energy utilization between the production and immunity (van der Most et al., 2011; Dadfar et al., 2023). In this respect, an impairment or stimulation in gut health may cause a higher energy burden towards maintaining the immunity instead of rapid production. Therefore, it is important to investigate the gut health parameters alongside the production parameters of fast growing broiler chickens in order to maintain the sustainability of broiler production.

The gut microbiome has been identified as a key player in gut health, immunity and metabolism of broiler chickens via training and stimulating immune response, recruitment of immune cells, production of immunostimulant chemicals/signals and direct and indirect pathogen exclusion (Fathima et al., 2022). Unlike

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mammals, broiler chicks mostly hatch in a relatively sterile environment (due to sterilization of eggs and commercial hatchers) without a maternal contact (Kogut, 2019; Dunislawska et al., 2021) and face delays in access to feed (due to longer hatching windows and transportation) (Proszkowiec-Weglarz et al., 2022). Broilers therefore, have a less opportunity to colonize their gut with beneficial commensal bacteria. For this reason, there is a high likelihood that they may be exposed to environmental pathogens given the lack of a strong microbiome to out-compete. To address this, many scientists suggest an early intervention strategy such as *in-ovo* administration of bioactive substances such as probiotics, prebiotics, synbiotics (probiotics + prebiotics) and phytobiotics to manipulate the gut microbiome of broiler chickens (Rubio, 2019). Moreover, the *in-ovo* technology may be efficient when compared to other *in-vivo* methods (in feed/water, microbiome transplants *etc.*), as there are less influence from confounding environmental factors which may reduce the efficiency of delivering the bioactive substances (Kogut, 2019).

Recent evidence indicates that chicken eggs, particularly the yolk sac and amniotic fluid undergo microbiota changes over the course of embryonic development. These changes displayed functional associations that could be linked to early, mid and late stages of embryonic development indicating the role of native *in-ovo* bacteria in the embryonic development of the broiler chickens (Akinyemi et al., 2020). The *in-ovo* administration of prebiotics on 12th embryonic day (which marks the mid phase of the embryonic development), demonstrated a stimulating effect on beneficial groups of bacteria present in the chicken eggs (Siwek et al., 2018). On the contrary, probiotics administered *in-ovo* act as pioneer colonizers laying a foundation for a healthy microbiome. Interestingly, injection with synbiotics is known to shape the gut microbiome by exerting both mechanisms described above in shaping the gut microbiome (Dunislawska et al., 2021). Furthermore, phytobiotics is another category of biotics that has been tested *in-ovo*. *In-ovo* delivery of phytobiotics also demonstrated beneficial effects on hatchability, chick quality, antioxidant activity and gut development via mechanisms such as modulating gut microbiome and gene expression of the host (Akosile et al., 2023).

Similar to the combined administration of probiotics and prebiotics (synbiotics) *in-ovo,* it is of interest to examine the effects of the combined use of probiotics and phytobiotics *in-ovo*, on the gut health and production of broiler chickens, due to their promising benefits imparted individually. We have coined the term **prophybiotics** (**pro**biotics + **phy**tobiotics) to describe this type of combination (Wishna-Kadawarage et al. 2023) that may provide a prophylaxis in the poultry gut. However, to the best of our knowledge, no previous studies examined the potential of prophybiotic (**PPB**) combinations in an *in-ovo* model to validate the possible beneficial effects on the gut health of chickens.

The probiotic *Leuconostoc mesenteroides* (B/00288) which was selected for the current study is currently used in multistrain probiotic supplement for poultry produced by JHJ, Nowa Wieś, Poland, which has resulted in reduction of *Salmonella enteritidis* (Smialek et al., 2019) and *Campylobacter spp.* (Smialek et al., 2018) in the broiler gastrointestinal tract. Likewise, *Leuconostoc mesenteroides* has displayed promising antimicrobial (Zhang et al., 2021, 2023) and probiotic (de Paula et al., 2015) properties in previous studies. Furthermore, *L. mesenteroides* is known to produce prebiotic oligosaccharides which do not stimulate the growth of harmful pathogens such as *Salmonella* and *E. coli* but beneficial bacteria in the gut (Chung and Day, 2004; Miyamoto et al., 2023).

The garlic aqueous extract (0.5% w/v) with *L. mesenteroides* was identified as a compatible PPB pair as this concentration of garlic aqueous extract neither inhibited nor stimulated the growth of *L. mesenteroides, in-vitro* (Wishna-Kadawarage et al., 2023). This indicated that the antimicrobial compounds in garlic such as allicin was non inhibitory to *L. mesenteroides* whereas garlic fructans were not readily utilized by *L. mesenteroides* as an energy source. Therefore, we hypothesized that when combined, the garlic portion of the PPB will not be consumed by *L. mesenteroides,* allowing it to purely act on the host, causing additive or synergistic effects of the combination.

Accordingly, the current study was conducted to validate the effects of *in-ovo* application of the selected PPB (*L. mesenteroides* + garlic aqueous extract) as opposed to the use of probiotics alone on the gut health and production parameters of ROSS 308 broiler chickens. To our knowledge, our study is the first to use a PPB combination as well as a *L. mesenteroides* strain in an *in-ovo* application in poultry.

As the ceca is the major organ which harbors the majority of the gut microbiome of chickens, our investigation was mainly focused on the microbiome, gene expression and histomorphology of the ceca. Additionally, fecal microbiome was analyzed together with the production and meat quality parameters to exemplify how the administration of PPB *in-ovo,* may affect the gut health and production parameters of fast growing broiler chickens.

MATERIALS AND METHODS

Egg Incubation and Experimental Design

A total of 400 ROSS 308 broiler hatching eggs were incubated at the standard conditions (Temperature: 37.5°C and Relative Humidity: 55%) (Midi series I, Fest Incubators, Gostyń, Poland). On the 12th day of incubation, after performing candling and removal of infertile eggs and dead embryos, equal number of eggs were randomly allocated into 4 *in-ovo* treatment groups namely; negative control (**NC**), positive control (**PC**), probiotic (**PB**), and PPB. The eggs of the NC group did not receive any *in-ovo* injection and PC group eggs were injected with 0.2 mL of sterile 0.9% NaCl physiological saline solution (Natrium Chloratum 0.9% Fresenius

KabiPac, Fresenius Kabi, Warsaw, Poland). The PB group eggs were injected with 10^6 CFU of *L. mesenteroides* B/00288 probiotic bacteria suspended in 0.2mL of 0.9% NaCl physiological saline solution per egg. The eggs of PPB group received a total volume of 0.2 mL injection with *L. mesenteroides* probiotic suspension in 0.9% NaCl physiological saline and 0.5% (w/v) garlic aqueous extract in 2:1 ratio by volume. Before the injections were performed, the blunt end (where the air cell is located) of all the eggs was disinfected with 70% ethanol to avoid unnecessary contamination. Next, each egg was candled to locate the air cell and a hole was carefully made into the egg shell (at the site of air cell) using 20 G needles manually. The respective injection solutions were then manually injected into the air cell space of each egg with a 26 G needle insuring no damage to the inner membranes of the egg. The injection holes were then sealed with a drop of non-toxic glue (Elmer's school glue, Elmer's Products Inc., Ohio). The injection was carried out as quickly as possible and the eggs were then transferred back to the incubator to continue the incubation under standard conditions.

Preparation of Injection for PB Group

Leuconostoc mesenteroides (LM) was grown in MRS broth media (BD Difco 288130, Fisher Scientific, Dublin, Ireland) for 15 h (based on our preliminary experiments, at 15 h of incubation LM had attained its peak growth and had started the stationary phase of the growth curve) to obtain the maximum number of cells in a metabolically active phase. The culture was then centrifuged at 4,200 rpm for 20 min in a refrigerated (4°C) centrifuge. The cell pellet was washed twice with sterile 0.9% NaCl physiological saline solution and resuspended in 0.9% NaCl physiological saline. The optical density at 600 nm (OD600) of the solution was adjusted to 0.0311 (using Thermo Scientific Multiskan FC plate reader: Thermo Fisher Scientific, Warsaw, Poland) to obtain a cell density similar to 5×10^6 CFU/mL (based on the regression equation obtained between the CFU/mL and OD600 by the preliminary experiments). From this bacterial suspension, 0.2mL was injected into each egg of the PB group.

Preparation of Injection for PPB Group

Approximately, the same amount of bacteria (10^6 CFU/egg) was delivered of the PPB injection as the PB injection to compare the results of PPB vs. PB alone. However, as the volume of injection material was a constant (0.2 mL/egg) across all treatments, the volume of bacterial suspension here was 2/3 the amount (as the PPB injection consisted of 2 components, the bacterial suspension and garlic aqueous extract, in 2:1 ratio by volume). Therefore, a bacterial suspension with a higher concentration was necessary for the PPB injection mixture. A separate bacterial suspension was prepared by adjusting to a higher OD600 (corresponds to 7.5×10^6 CFU/mL cell density) as described in the preparation of injection for PB group. Similarly, in order to obtain 0.5% (w/v) garlic concentration in the final injection mixture (in which only 1/3 garlic extract is included), 0.15g of finely milled air dried garlic powder was added to 10mL of sterile distilled water, and the protocol to activate the allinase enzyme thereby producing allicin was carried out as described in Wishna-Kadawarage et. al (2023). Both components (the bacterial suspension and garlic aqueous extract) were combined at 2:1 ratio and the mixture was gently mixed. A volume of 0.2mL of this mixture was used to inject each egg in the PPB group.

Hatching and Data Collection

Upon completion of the incubation period, the hatchability of each group was recorded. The chicks hatched from each group were wing tagged for identification. The weight (when the chicks are dried well) and length of 25 randomly selected birds/group were recorded. Chick length was measured from the tip of the beak to the tip of the middle toe by placing the chick face down on a flat surface and straightening the right leg (Sozcu and Ipek, 2015). The chick quality of ten birds (out of the 25 randomly selected birds per a group) was assessed by performing the Pasgar scoring as described in the Lohmann breeder guide ("Lohmann Hatchery Guide,").

Animal Rearing and Sample Collection

The rearing and slaughter of the birds were carried out in accordance with the guidelines of the Ethics Committee for Experiments with Animals and regulations of the Polish Act on the Protection of Animals Used for Scientific or Educational Purposes of 15 January 2015 (which implements Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes).

The chickens belonging to 4 treatment groups were contained in separate pens having uniform optimal, electronically controlled environmental conditions (temperature, lighting regime, air humidity). Broilers were fed *ad libitum* with starter (1-21 d) grower (22−28 d) and finisher (29−35 d) dry mixes containing 22.3, 20.2, and 20.2% crude protein and 12.45, 13.01 and 13.01 MJ/Kg metabolizable energy, respectively, and had unlimited access to drinking water. All the mixtures were prepared according to the dietary requirements of broiler chickens (Smulikowska and Rutkowski, 2018). All the birds were raised until 35 d of age (market age) on deep litter providing the standard care. Eight feces samples from each group were collected on the 7th day (1 wk posthatching) and the 34th day (1 d before sacrifice) to quantify the relative abundance of selected bacterial communities (beneficial and potentially harmful) as a reflection of the gut microbiome in early post hatch and final stages of life of a broiler. The body weight and feed intake per

group were recorded weekly to calculate the feed conversion ratio (FCR = Total feed consumed/Total weight gained) of each group.

On the 35th day after 10 h of fasting, 8 birds per group were sacrificed (by decapitation and suspended to bleed for approximately 90 s) to obtain biological samples (cecal tissue, cecal mucosa, and cecal content). The birds were sacrificed, the luminal content of ceca was carefully transferred to sterile 5 mL micro-centrifuge tubes and placed immediately in dry ice. The samples were transported in dry ice and stored at -80°C until use. Cecal mucosa samples for gene expression analysis were placed in tubes containing stabilization buffer (fix RNA: E0280, EURx, Gdańsk, Poland) for transport at room temperature. Upon transportation, fix RNA was removed and samples were frozen at −80° C until use. The middle part of the cecum was sampled for histology analysis and was directly preserved in Bouin's solution (HT101128, Sigma-Aldrich, Poznan, Poland) until processing.

Slaughter Analysis

After 24 h of cooling, carcasses were subjected to slaughter analysis. Carcass dressing percentage with giblets was estimated as the ratio of chilled carcass with neck, abdominal fat, and edible giblets (gizzard, liver, and heart) to live body weight. Carcass dressing percentage without giblets was estimated as the ratio of a chilled carcass with neck and abdominal fat to live body weight. The percentage of breast muscle, leg muscle (thigh and drumstick), leg bones, giblets, and abdominal fat were calculated as a percentage of the cold carcass weight with giblets.

Meat Quality Analysis

The breast and thigh muscles were dissected from the chilled carcasses and evaluated for physicochemical properties (pH, color, drip loss, thawing loss, cooking loss, shear force, and texture). All meat characteristics were determined following the method described by Połtowicz et al. (2015).

Extraction of DNA

Extraction of DNA from feces samples and luminal content of the ceca was performed using the GeneMATRIX Stool DNA Purification Kit (E3575, EURx, Gdańsk, Poland) optimizing the manufacturer's protocol. The quality and quantity of the extracted DNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Warsaw, Poland). The integrity of the DNA was confirmed by performing electrophoresis on a 2% agarose gel. The DNA samples were stored at −80°C until use.

Extraction of RNA

Isolation of RNA was performed by homogenizing the mucosal tissues in 1mL of RNA extracol solution (E3700, EURx, Gdańsk, Poland) using a TissueRuptor II homogenizer (990890, Qiagen, Wrocław, Poland) followed by centrifugation with 0.2 mL of chloroform (112344305, Chempur, Piekary Śląskie, Poland). RNA isolated in the supernatant was further purified using a Universal RNA purification kit (E3598, EURx, Gdańsk, Poland) following the manufacturer's protocol. The quality, quantity, and integrity of RNA were validated as described in the DNA extraction section. RNA was stored at −80°C until further use.

Analysis of Relative Abundance of Bacteria

The relative abundance of the selected bacterial communities was determined using a quantitative PCR (qPCR) method. In the fecal samples, quantification of the relative abundance of *Lactobacillus* sp., *Bifidobacterium* sp. *Faecalibacterium* sp. (beneficial) and *Escherichia coli* (potentially harmful) was performed. In the luminal content of ceca, *Lactobacillus* sp., *Bifidobacterium* sp., *Prevotella* sp., *Akkermansia* sp. and *Fecalibacterium* sp. (beneficial) and *E. coli* (potentially harmful)

Table 1. Primer sequences for determining the relative abundance of bacterial communities in the feces and luminal content of ceca via qPCR.

Bacterial community	Primer sequence[1] (5′→ 3′)	Reference
Universal bacteria	F: ACTCCTACGGGAGGCAGCAGT R: GTATTACCGCGGCTGCTGGCAC	(Tannock et al., 1999)
Akkermansia sp.	F: CAGCACGTGAAGGTGGGGAC R: CCTTGCGGTTGGCTTCAGAT	(Earley et al., 2019)
Bifidobacterium sp.	F: GCGTGCTTAACACATGCAAGTC R: CACCCGTTTCCAGGAGCTATT	(Penders et al., 2005)
Escherichia coli	F: CATGCCGCGTGTATGAAGAA R: CGGGTAACGTCAATGAGCAAA	(Penders et al., 2005)
Faecalibacterium sp.	F: ACCATGAGAGCCGGGGGG R: GGTTACCTTGTTACGACTT	(Lund et al., 2010)
Lactobacillus sp.	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	(Slawinska et al., 2019)
Prevotella sp.	F: CCAGCCAAGTAGCGTGCA R: TGGACCTTCCGTATTACCGC	(Martin et al., 2002)

[1]F: Forward primer/ R: Reverse primer.

were quantified. All bacterial communities were quantified relative to the universal bacterial quantity in each sample. The primer sequences are indicated in the Table 1.

The qPCR was performed in a total reaction mixture volume of 12.5 μL containing 1 μM of each (forward and reverse) primer (Sigma-Aldrich, Darmstadt, Germany), 20 ng of DNA, and 6.25μl of SG qPCR Master Mix (2x) (0401, EURx, Gdańsk, Poland) in 96 well plates (4TI-0955, AZENTA, Genomed, Warsawa, Poland). The qPCR reaction for each sample was performed using LightCycler 480 II (Roche-Diagnostics, Rotkreuz, Switzerland) and 2 technical replicates. The qPCR protocol included an initial denaturation at 95°C for 5 min, followed by 40 cycles of amplification. Each amplification cycle consisted of a denaturation step at 95°C for 10 s, an annealing step at 58°C for 15 s, and an elongation step at 72°C for 30 s. The average Ct values of the 2 technical replicates obtained were used for data analysis. A standard curve for each primer pair was performed using five 2× dilutions (1x, 0.5x, 0.25x, 0.125x, and 0.0625x) of pooled bacterial DNA of relevant samples of all treatment groups. Then the PCR efficiency for each primer pair was determined using the LightCycler 480 II software (Roche-Diagnostics). The relative abundances of the bacteria in the luminal content of ceca were calculated using the following formula as described in Slawinska et al. (2019):

$$\text{Relative Abundance [\%]} = (\text{E universal})^{\text{Ct universal}}/(\text{E target})^{\text{Ct target}}$$

E universal: qPCR Efficiency of universal bacteria primers

Ct universal: Ct value of qPCR reaction for universal bacteria

E target: qPCR Efficiency of target bacteria primers

Ct target: Ct value of qPCR reaction for target bacteria

Analysis of Immune Related Gene Expression

The genes coding for immune related components (pro- and anti-inflammatory cytokines: *IL1-β, IL2, IL4, IL6* and *IL10*, pro-inflammatory chemokine: *IL8*, free fatty acid receptor 2 (***FFAR2***), host defense peptides: *AVBD* and *CATHL2* and barrier function related components: *MUC6* and *CLDN1*) were quantified by a quantitative reverse transcription PCR (**RT-qPCR**) method. The relative gene expression was calculated against the expression of *ACTB* and *G6PDH* genes as the reference genes. The primer details are listed in the Table 2.

Reverse transcription of the RNA samples was performed using the smART First Strand cDNA Synthesis Kit (0804, EURx, Poland) according to the manufacturer's protocol. The qPCR was then performed 20 ng of complementary DNA in the reaction mixture as described in the analysis of relative abundance of bacteria. The qPCR protocol for gene expression analysis included an initial denaturation for 15 min (95°C), followed by 40 cycles of amplification (Denaturation: 95°C for 15 s, annealing: 58°C for 30 s and elongation: 72°C for 30 s). The average Ct values of the 2 technical replicates obtained were used for data analysis wherein relative gene expression was calculated using ΔΔCt method (Livak and Schmittgen, 2001).

Table 2. Primer sequences for determining the relative gene expression in cecal mucosa via qPCR.

Gene name	Gene symbol	Primer sequence[1] (5′→ 3′)	Reference
Actin, beta	*ACTB*	F: CACAGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	(Sevane et al., 2014)
Glucose-6-Phosphate Dehyfrogenase	*G6PDH*	F: CGGGAACCAAATGCACTTCGT R: GGCTGCCGTAGAGGTATGGGA	(Sevane et al., 2014)
Avian beta-defensin 1	*AVBD1*	F: AAACCATTGTCAGCCCTGTG R: TTCCTAGAGCCTGGGAGGAT	(Slawinska et al., 2019)
Cathelicidin 2	*CATHL2*	F: AGGAGAATGGGGTCATCAGG R: GGATCTTTCTCAGGAAGCGG	(Slawinska et al., 2019)
Claudin 1	*CLDN1*	F: TCTTCATCATTGCAGGTCTGTC R: AACGGGTGTGAAAGGGTCAT	(Slawinska et al., 2019)
Free fatty acid receptor 2	*FFAR2*	F: GCTCGACCCCTTCATCTTCT R: ACACATTGTGCCCCGAATTG	(Slawinska et al., 2019)
Interleukin 1 beta	*IL1-β*	F: GGAGGTTTTTGAGCCCGTC R: TCGAAGATGTCGAAGGACTG	(Dunislawska et al., 2017)
Interleukin 2	*IL2*	F: GCTTATGGAGCATCTCTATCATCA R: GGTGCACTCCTGGGTCTC	(Pietrzak et al., 2020)
Interleukin 6	*IL6*	F: AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT	(Chiang et al., 2009)
Interleukin 8	*IL8*	F: AAGGATGGAAGAGAGGTGTGCTT R: GCTGAGCCTTGGCCATAAGT	(Sławinska et al., 2014)
Interleukin 10	*IL10*	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG	(Rothwell et al., 2004)
Mucin 6	*MUC6*	F: TTCAACATTCAGTTCCGCCG R: TTGATGACACCGACACTCCT	(Slawinska et al., 2019)

[1]F: Forward primer/ R: Reverse primer.

Analysis of Cecal Histology

Histomorphology of the cecal samples was performed in a histological laboratory according to the methodology of Bogucka et al. (2016) using the paraffin technique. Briefly, the samples which were preserved in Bouin's solution were taken out and sliced into approximately 1 cm lengths. The tissue pieces were put into a tissue processor (Microm STP 120, Thermo Shandon, Runcorn, United Kingdom) for overnight incubation in which the tissues were subsequently dehydrated, cleared, and infiltrated with paraffin. Next, the processed tissues were embedded into paraffin blocks manually in a transfer station (TES 99, Medite, Burgdorf, Germany). Using a rotational microtome (Finesse ME+, Thermo Shandon, Runcorn, United Kingdom), 7 μm thick sections of each tissue sample were cut and adhered to glass slides covered with egg white and glycerin. Next, the slides were de-waxed and hydrated before the staining.

PAS reaction (Dubowitz et al., 1973) was performed on microscopic preparations. An Evolution 300 microscope (Delta Opitcal, Warsaw, Poland) equipped with a digital camera ToupCam (TP605100A, ToupTek, Hangzhou, Zhejiang, China) was used to capture microscopic images of caeca on a computer disk. The height and width of villi and crypt depth were measured (10 measurements for each parameter per a chicken) using the Multiscan 18.03 microscopic images software (Computer Scanning Systems II, Warsaw, Poland). The villus height to crypt depth ratio (VH/CD) was also calculated for each bird. The surface area of the villi was calculated according to the formula of Sakamoto et al. (2000).

$$\text{Surface area of villi} = (2\pi) \times (\text{VW}/2) \times (\text{VH})$$

VW= villus width,
VH = villus height.

Statistical Analysis of Data

The production, meat quality, bacterial abundance and histology data were analyzed using a linear mixed model in R (version 4.3.1) using "lmer" function in "lme4" package after removing the outliers (values which are greater than Quartile 3 + 1.5 × interquartile range and below Quartile 1 + 1.5 × interquartile range). The treatment effect was used as the fixed effect and the sex of the bird was considered as a random effect to account for the possible confounding variation due to sex. Wald chi square test (for the significance of the fixed effect) and Tukey's HSD test (for mean comparison) were performed to identify the significantly different means (P- value < 0.05). In case the assumptions of normality of residuals (tested by Shapiro-Wilk test) and equal variances (tested by Levene's test) were not met, the non-parametric analysis, Kruskal-Wallis test followed by Dunn's test was performed to identify the significantly different means. Regarding the fecal bacteria, where significant differences among the *in-ovo* treatment groups were observed, we conducted separate Wilcoxon Rank-Sum tests for each treatment group to assess the variations in relative abundance between the early (d 7) and late (d 34) life stages. All microbiological data from the fecal samples were utilized to conduct a Between-Class Analysis (**BCA**) employing the "bca" function within the "ade4" package. This analysis aimed to visualize the separation between groups at both early (d 7) and late (d 34) life stages. For the gene expression analysis, ΔCt values of each treatment group was compared against that of the positive control group using 2 sample 2 test to identify significant differences in the treatments (P- value < 0.05).

RESULTS AND DISCUSSION

In-ovo stimulation has shown promising potential in improving the gut health of broiler chickens. We hypothesized *in-ovo* stimulation using a novel approach, PPB (probiotic + phytobiotics) may provide broiler chickens with a lifelong competitive advantage against environmental pathogens. The selected PPB, *L. mesenteroides* (probiotic) in combination with garlic aqueous extract (phytobiotic), displayed promising potential in improving the gut health of broiler chickens without compromising the production and meat quality parameters, when compared to using the probiotic alone. Thus, it can be suggested that garlic aqueous extract imparts an additive or synergistic effect when combined with a compatible probiotic for *in-ovo* stimulation.

Hatch Properties

The highest hatchability was obtained from the negative control group (91.7%) and the PPB group (89.5%) displayed the highest hatchability among the *in-ovo* injected groups (positive control: 86.9% and probiotic: 85.5%). Differences in chick length and chick quality

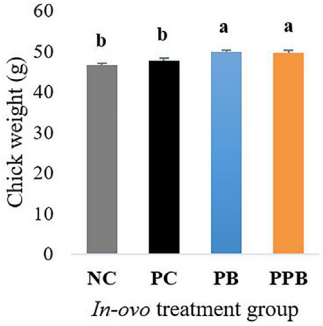

Figure 1. The weight of chicks at hatch across *in-ovo* treatment groups. Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending order). Abbreviations: NC: negative control group, PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group.

Table 3. Body weight of chickens of *in-ovo* treatment groups.

Day	Body weights[2] (g)				Treatment effect[3,4]
	NC[1]	PC[1]	PB[1]	PPB[1]	
1	**48.9 ± 2.9[b]**	**48.0 ± 3[b]**	**53.0 ± 2.9[a]**	**52.4 ± 3.9[a]**	***
7	**180.5 ± 25.8[b]**	**177.3 ± 23[c]**	**206.1 ± 25.6[a]**	**190.2 ± 30.7[ab]**	***
14	**480.2 ± 71.5[b]**	**500.0 ± 47.2[ab]**	**536.9 ± 79.9[a]**	**521.2 ± 62.1[ab]**	**
21	1014.4 ± 143.1	1011.3 ± 113.5	1042.8 ± 141.6	1052.7 ± 129.4	NS
28	1681.5 ± 197.9	1663.8 ± 191.5	1718.3 ± 230.7	1711.9 ± 200.6	NS
35	2437.5 ± 254.9	2433.6 ± 301.7	2502.3 ± 255.7	2455.6 ± 266.3	NS

[a,b,ab]Homogenous means have been indicated by similar letters (in descending order).
[1]NC: negative control, PC: positive control, PB: probiotic group, PPB: prophybiotic group.
[2]Data are represented as mean ± SD.
[3]Significant codes: *P*- values < 0.0001: ***, < 0.001: **, < 0.05: *, <0.1: T, >0.1: NS
[4]Significantly different data is in bold.

(Pasgar score) were not statistically significant between the groups (*P*- value > 0.05) whereas the chick weight was significantly higher (*P*- value <0.05) in the PB and PPB groups when compared to control groups (Figure 1).

These results suggest that the *in-ovo* administration of the selected PPB and probiotic was safe and also beneficial for the embryonic development, enabling a successful hatching of quality chicks. Previous literature also indicated that injection of embryonic d 12 is safe and less likely to decrease the hatchability (Siwek et al., 2018) whereas other studies which injected synbiotics displayed a higher (Dunislawska et al., 2017) and lower (Asaadi et al., 2021) hatchability based on the bioactive substances used.

Body Weight and Feed Efficiency

Previous studies have shown inconsistent effects as a result of *in-ovo* administration of bioactive substances on growth parameters, some of which showed no significant benefits whereas others displayed significant benefits (Siwek et al., 2018). This inconsistency may be based on the differences between trials such as the bioactive used and the date of injection. However, here, our intention was not to improve the production of the broiler chickens as broiler chickens are already intensively selected for production and efficiency parameters. Our aim was to improve gut health and immunity without causing an energy burden thus compromising the production parameters. Interestingly, our results indicated a higher body weight in the chickens from both the probiotic and PPB groups compared to control groups from hatch to 2 wk of age (Table 3), demonstrating the beneficial effects of these treatments in the early life of the broilers. However, no difference was observed in the body weight among the groups from the 21st day onwards. Additionally, the weekly FCR (Figure 2A) and the overall FCR (for the entire production lifetime) (Figure 2B) did not reveal clear evidence that any group had a higher FCR when compared to the others. Therefore, we suggest that our treatments do not compromise the production or feed efficiency of fast growing broiler chickens.

Slaughter and Meat Quality Analysis

The results of the slaughter analysis is summarized on Table 4. There was a statistically significant reduction in the cooling losses of the chickens treated

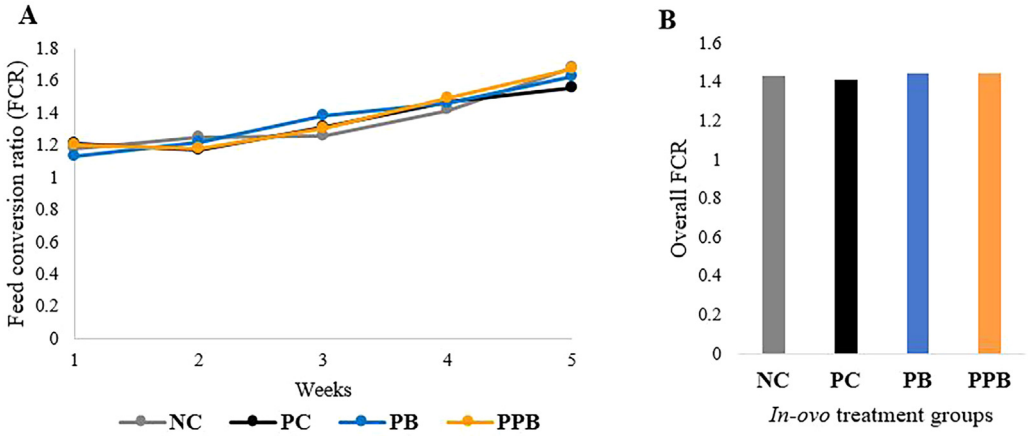

Figure 2. Feed conversion ratio (FCR) of the chickens of *in-ovo* treatment groups. (A) Weekly FCR. (B) Overall FCR. Abbreviations: NC: Negative control group, PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group.

Table 4. Slaughter analysis of the chickens of *in-ovo* treatment groups.

Parameter	Slaughter analysis[2]			Treatment effect[3,4]
	PC[1]	PB[1]	PPB[1]	
Cooling losses (%)	**1.79 ± 0.21**[a]	**1.35 ± 0.29**[b]	**1.547 ± 0.09**[b]	***
Dressing percentage with giblets (%)	79.81 ± 1.14	79.51083 ± 1.25	79.81917 ± 1.24	NS
Dressing percentage without giblets (%)	76.83 ± 1.19	76.49 ± 1.25	76.70 ± 1.3	NS
Breast muscle (%)	**31.35 ± 2.05**[a]	**29.39 ± 1.53**[b]	**30.77 ± 2.37**[ab]	*
Leg muscles (%)	19.19 ± 1.47	19.39 ± 1.27	18.89 ± 2.07	NS
Giblets (%)	3.75 ± 0.42	3.93 ± 0.24	3.91 ± 0.3	NS
Liver (%)	2.23 ± 0.3	2.42 ± 0.3	2.34 ± 0.19	NS
Gizzard (%)	0.96 ± 0.2	0.92 ± 0.19	0.97 ± 0.12	NS
Heart (%)	0.53 ± 0.06	0.55 ± 0.07	0.53 ± 0.05	NS
Leg bones (%)	**3.98 ± 0.48**[b]	**4.44 ± 0.49**[a]	**4.17 ± 0.4**[ab]	**T (*P*- value: 0.06)**
Abdominal fat (%)	1.83 ± 0.3	1.94 ± 0.46	1.7 ± 0.34	NS

[a,b,ab]Homogenous means have been indicated by similar letters (in descending order).
[1]PC: positive control, PB: probiotic group, PPB: prophybiotic group.
[2]Data are represented as mean ± SD.
[3]Significant codes: *P*- values < 0.0001: ***, < 0.001: **, < 0.05: *, <0.1: T, >0.1: NS
[4]Significantly different data is in bold.

with both the probiotic and PPB. The breast muscle percentage of the probiotic group was statistically lower as compared to the positive control although the PPB group displayed a similar percentage to the positive control. Additionally, there was a statistical tendency for higher leg bone percentage in the probiotic group as compared to the positive control. The remaining components studied were not statistically different between the groups.

Meat quality analysis indicated that most of the parameters studied were not affected by the 2 *in-ovo* treatments (PB and PPB) (*P*- value > 0.05 when compared to the positive control). The parameters that displayed a statistically significant difference are summarized in Table 5. Briefly, probiotic treatment resulted in the breast meat being more chewy and gummy whereas the PPB treatment resulted in more springiness in the breast muscle. Both treatments resulted a lower pH in the breast muscle after 15 min postmortem, when compared to that of the positive control. However, the pH at 24 h remained similar (*P*- value > 0.05) in all groups. Interestingly, the losses after thawing of both breast and leg muscles, were lowest in the PPB group. Nevertheless, a higher drip loss after 24 h cooling was observed in leg muscles of the PPB group as compared to others.

Both slaughter and meat quality analysis further indicated that our *in-ovo* treatments did not influence most of the quality parameters apart from some beneficial changes observed. Overall, it can be suggested that both probiotic and PPB *in-ovo* treatments did not adversely affect the production, efficiency, or meat quality parameters of the broilers in this study.

Relative Abundance of Bacteria in Feces

The relative abundance of *Lactobacillus* sp. did not differ in the fecal samples among the groups at either time point. However, a significant increase in *Bifidobacterium* sp. (*P*- value < 0.05) in the PPB group and *Faecalibacterium* sp. (*P*- value = 0.06791) in both the probiotic and PPB groups was observed on d 7, when compared to the positive control (Figure 3). *Faecalibacterium* sp. is known to modulate gut health by producing anti-inflammatory metabolites (Lenoir et al., 2020) and imparting anaerobisation in the gut environment (by consuming the trace amounts of oxygen) creating an unfavorable environment for pathogens such as *E. coli*

Table 5. Significant changes in the meat quality of the chickens of *in-ovo* treatment groups.

Parameter	Meat quality analysis[2]			Treatment effect[3]
	PC[1]	PB[1]	PPB[1]	
Breast muscle quality				
Chewiness	10.255 ± 2.83[b]	12.642 ± 3.24[a]	11.191 ± 1.35[ab]	T (*P*- value: 0.07676)
Gumminess	29.316 ± 6.96[b]	35.018 ± 8.79[a]	29.925 ± 3.11[ab]	T (*P*- value: 0.07074)
Springiness	0.348 ± 0.03[b]	0.361± 0.02[ab]	0.372 ± 0.03[a]	T (*P*- value: 0.0923)
Thawing loss (%)	5.373 ± 1.32[a]	4.271 ± 1.98[ab]	3.150 ± 1.1[b]	**
pH15 min	6.597 ± 0.14[a]	6.338 ± 0.13[b]	6.361 ± 0.18[b]	***
Leg muscle quality				
Drip loss 24h (%)	0.57 ± 0.12[b]	0.60 ± 0.08[b]	0.65 ± 0.05[a]	T (*P*- value: 0.06)
Thawing loss (%)	3.05 ± 1[a]	3.60 ± 1.39[a]	2.29 ± 0.64[b]	*

[a,b,ab]Homogenous means have been indicated by similar letters (in descending order).
[1]PC: positive control, PB: probiotic group, PPB: prophybiotic group.
[2]Data are represented as mean ± SD.
[3]Significant codes: *P*- values < 0.0001: ***, < 0.001: **, < 0.05: *, <0.1: T

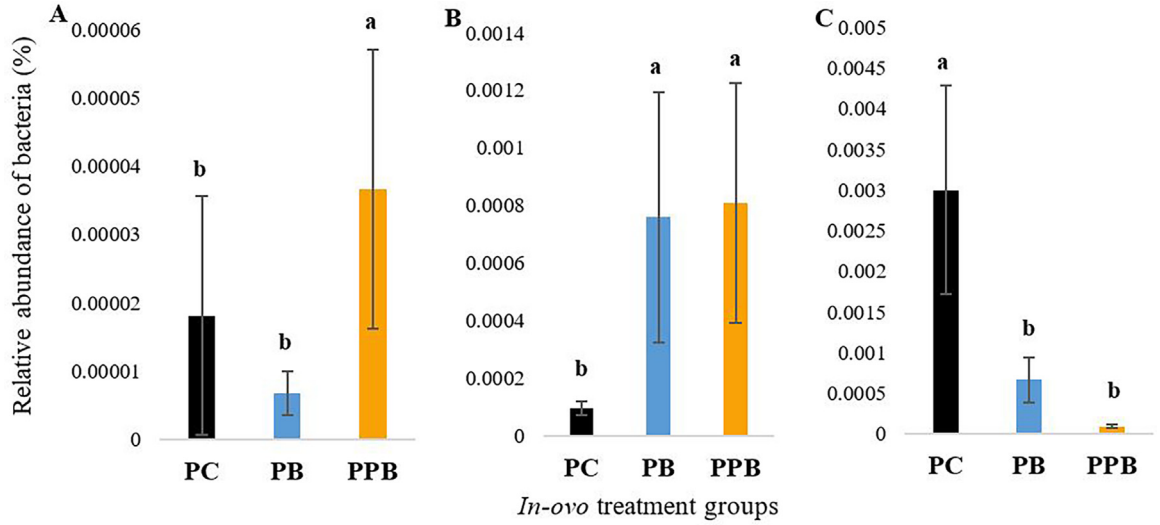

Figure 3. The relative abundance of bacterial communities in the feces of chickens of different *in-ovo* treated groups. (A) D 7 − *Bifidobacterium* sp. (B) D 7 − *Faecalibacterium* sp. (C) D 34 − *E. coli.* Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending order). Abbreviations: PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group.

and *Salmonella* (Rychlik, 2020). In particular, *F. prausnitzii* produces butyrate, the main energy source for colonocytes, by fermenting prebiotic fibers (Ferreira-Halder et al., 2017). Similarly, bifidobacteria are associated with many beneficial effects in the gut such as production of metabolites which are harmful to gram negative pathogenic bacteria, fermentation of prebiotic fibers and production of Vitamin B (Abd El-Hack et al., 2020). Therefore, by increasing the abundance of both bifidobacteria and faecalibacteria in the chicken gut (as reflected by the fecal samples), the use of PPB combination displays promise for use in modulating the gut microbiome of broiler chickens.

The probiotic species used, *L. mesenteroides* is known to produce exopolysaccarides which display prebiotic properties (Pan et al., 2020; Miyamoto et al., 2023) whereas garlic was previously reported as a rich source of fructans (70%−80% of dry weight) which also have proven prebiotic potential (Lu et al., 2021). In the human gut, studies have shown an increased abundance of bacteria belonging to the *Faecalibacterium* (Panyod et al., 2022) and *Bifidobacterium* (Ettehad-Marvasti et al., 2022) genera in the presence of fructans. Therefore, it is possible that garlic aqueous extract has an additive or synergistic role in influencing changes in the microbiome when used in combination with *L. mesenteroides.*

However, towards the end of the production (d 34) the relative abundance of these beneficial bacteria in the feces was similar among all groups (P- value > 0.05). Interestingly, the relative abundance of the bifidobacteria was statistically similar between the d 7 and d 34 in the positive control and probiotic groups (P- value > 0.05) whereas PPB group displayed a reduced number of bifidobacteria from d 7 to d 34 (P- value < 0.05). Therefore, the reason for observing a significantly higher relative abundance of bifidobacteria in the PPB group at the beginning of life but not towards the end, is likely due to the reduction of bifidobacteria abundance in feces from d 7 to d 34. Faecalibacteria, however, displayed a higher relative abundance at d 7 but no significant difference at d 34 in both probiotic and PPB groups when compared to the positive control. In spite of this, the relative abundance of faecalibacteria at d 7 and d 34 remained statistically similar in all *in-ovo* treatments. This contrast in the results may be due statistics (a large variation observed within the treatment groups) or a change in the total gut microbiome, striving for homeostasis despite the effects of the *in-ovo* treatments.

Conversely, the relative abundance of *E. coli* did not differ significantly among the groups in the feces at d 7, although it was significantly reduced in feces at d 34 in the PPB group when compared to the positive control and probiotic groups. Moreover, the Wilcoxon Rank-Sum test revealed that the relative abundance of *E. coli* in the PPB group was significantly reduced from d 7 to d 34, providing a possible explanation for the observed between-group significance at d 34. This suggests that although the changes in the beneficial bacteria in feces did not last until the end of the production lifespan, the positive effects created by *in-ovo* treatment of PPB provided a lifelong competitive advantage against potentially harmful *E. coli.*

The BCA of overall bacterial abundance further displayed a distinct separation in the birds belonging to the treatment groups (PB and PPB) from the positive control group at the beginning of the life (d 7) whereas towards the end of the production life span (d 34), the treatment groups displayed more overlap with the positive control (Figure 4). Previously, Li et al. (2022) reported that the gut microbiome of broiler chickens changes with age respective to different developmental changes. Therefore, it can be suggested that the maturation of the gut microbiome with age and other developmental factors created a more uniform gut microbiome structure in these chickens later in their lives irrespective of the *in-ovo* treatment.

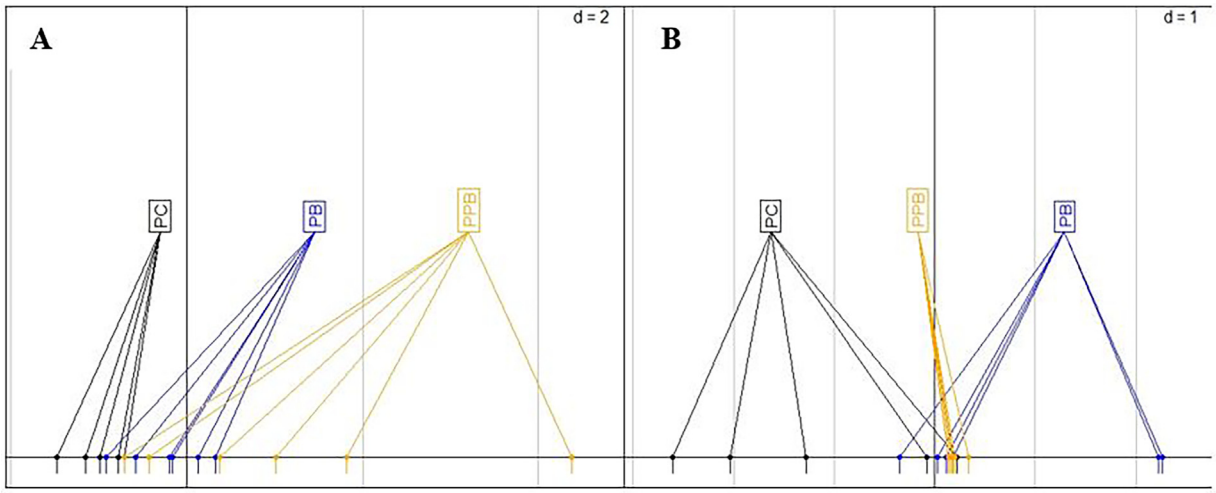

Figure 4. The between-class analysis plot for microbiological data in feces (A) D 7 (B) D 34. Abbreviations: PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group.

Relative Abundance of Bacteria in the Ceca

There was no significant difference in the relative abundance of *Lactobacillus* sp., *Bifidobacterium* sp. and *Prevotella* sp. in the cecal content of the birds across the groups. However, a significant reduction (*P*- value < 0.05) of *Escherichia coli* in the luminal content of ceca of PPB group when compared to the positive control and probiotic groups (which displayed statistically similar means) was observed (Figure 5). This result further supports our theory that *in-ovo* treatment with PPB provides the broilers with competitive advantage against potentially harmful *E. coli* in the gut. In addition, the relative abundance of *Akkermansia* sp. was increased (*P*- value < 0.05) in both probiotic and PPB groups when compared to the PC group (Figure 5). *Akkermansia* sp. particularly, *A. muciniphila* is known to impart beneficial effects in maintaining the gut health by degrading mucin to produce short chain fatty acids (**SCFA**) providing nutrients to epithelial cells and other gut microbiota, increasing goblet cell counts, up-regulating mucus layer turnover, promoting gut barrier function via tight junction protein expression (Yang et al., 2022) and production of antimicrobial peptides in the gut (Paone and Cani, 2020). Therefore, an increased abundance of *Akkermansia* sp. highlights that our PPB and probiotic *in-ovo* treatments support barrier function in the ceca of broiler chickens. Conversely, the abundance of faecalibacteria reduced in the luminal content of the ceca of the PPB group when compared to the probiotic and control groups (*P*- value < 0.05) (Figure 5). As *F. prausnitzii* and *A. muciniphila* possess similar functions in modulating gut health (anti-inflammation, SCFA production, enhance gut barrier function *etc.*), it is unclear if this reduction was a consequence of increased *Akkermansia* sp., balancing the microbiome in the ceca or a functionally important change.

Expression of Immune Related Genes in Cecal Mucosa

Interestingly, there was no significant difference between the *in-ovo* treatments in terms of expression of

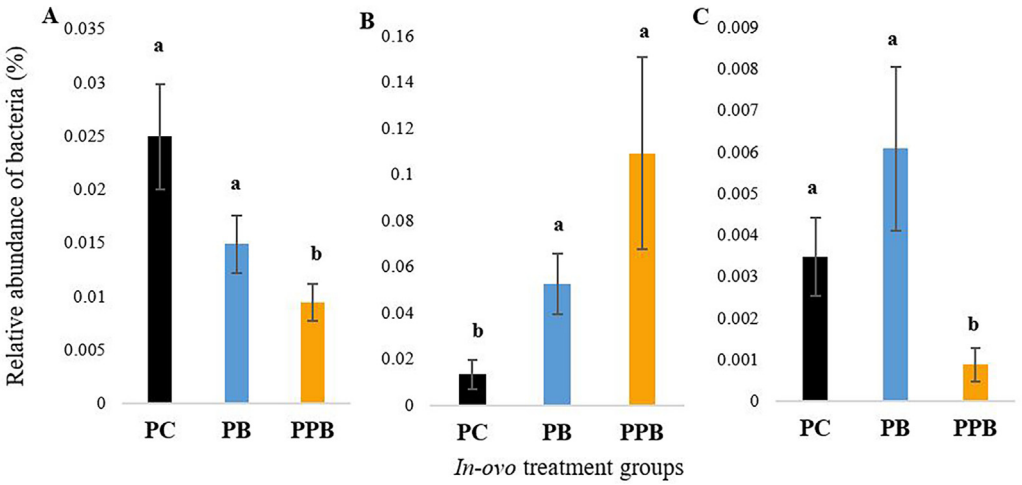

Figure 5. The relative abundance of bacterial communities in the luminal content of ceca of chickens of different *in-ovo* treated groups. (A) *Faecalibacterium sp.* (B) *Akkermansia sp.* (C) *Escherichia coli.* Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending order). Abbreviations: PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group.

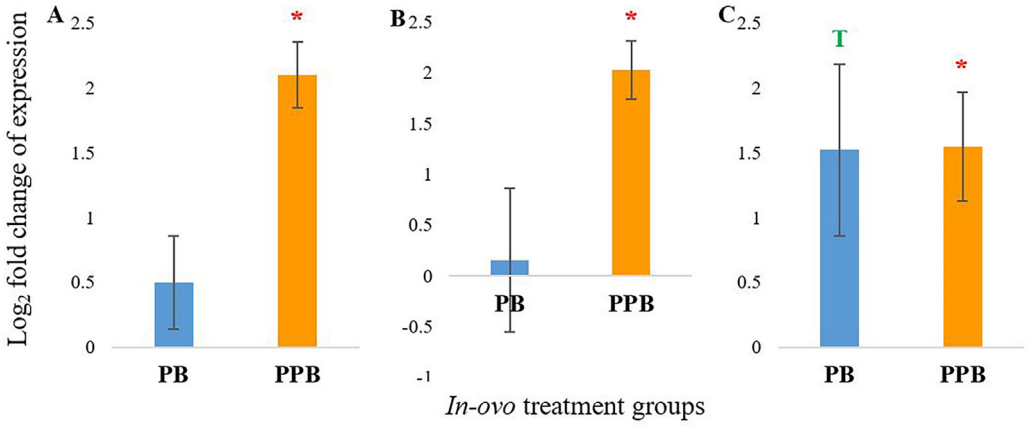

Figure 6. Immune-related gene expression in the cecal mucosa of chickens of different *in-ovo* treated groups. (A) *AVBD1* (B) *FFAR2* (C) *MUC6*. Error bars: ± SE. Red color asterick (*) indicates significant changes (*P*- value < 0.05) The letter T in green indicates there is a tendency (*P*-value = 0.0637). Abbreviations: PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group.

the genes coding for the anti- and pro-inflammatory cyto/chemokines (*IL1-β, IL2, IL4, IL6. IL8* and *IL10*), the tight junction protein; *CLDN* or the host defense protein; *CATHL2* studied in the mucosa of the ceca. This suggests that there was no inflammation as a result of the treatment, thus no dysbiosis or any other stress (Fathima et al., 2022) in cecal mucosa.

However, the PPB group resulted in an up-regulation in the expression of *AVBD1* and *FFAR2* in the cecal mucosa when compared to the positive control group (*P*- value < 0.05) (Figure 6). The *AVBD1* gene is responsible for production of avian *β* defensing 1 which is a host defense peptide belonging to the innate immune response (Lyu et al., 2020). Defensins display a broad

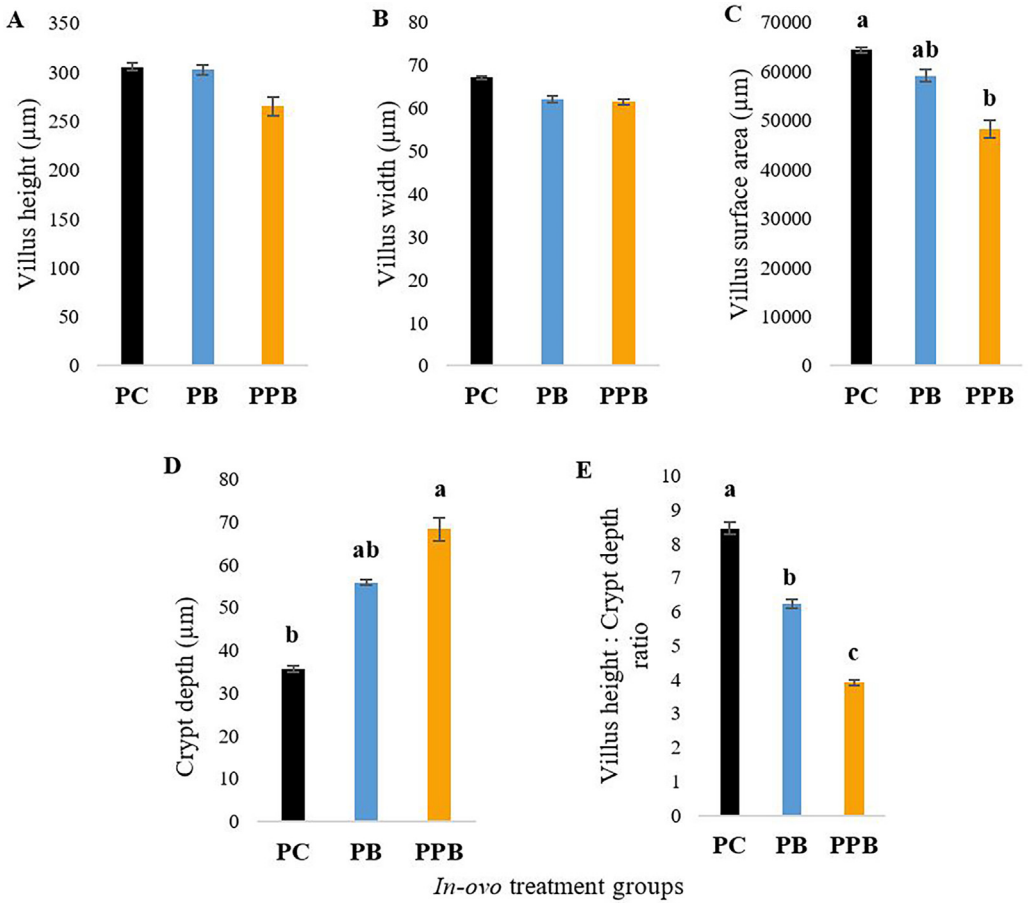

Figure 7. The analysis of histomorphological parameters of the cecal tissue of the *in-ovo* treated chickens. (A) Villus Height. (B) Villus Width. (C) Villus Surface Area. (D) Crypt depth. (E) Villus Height to Crypt Depth Ratio. Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending order). Abbreviations: PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group.

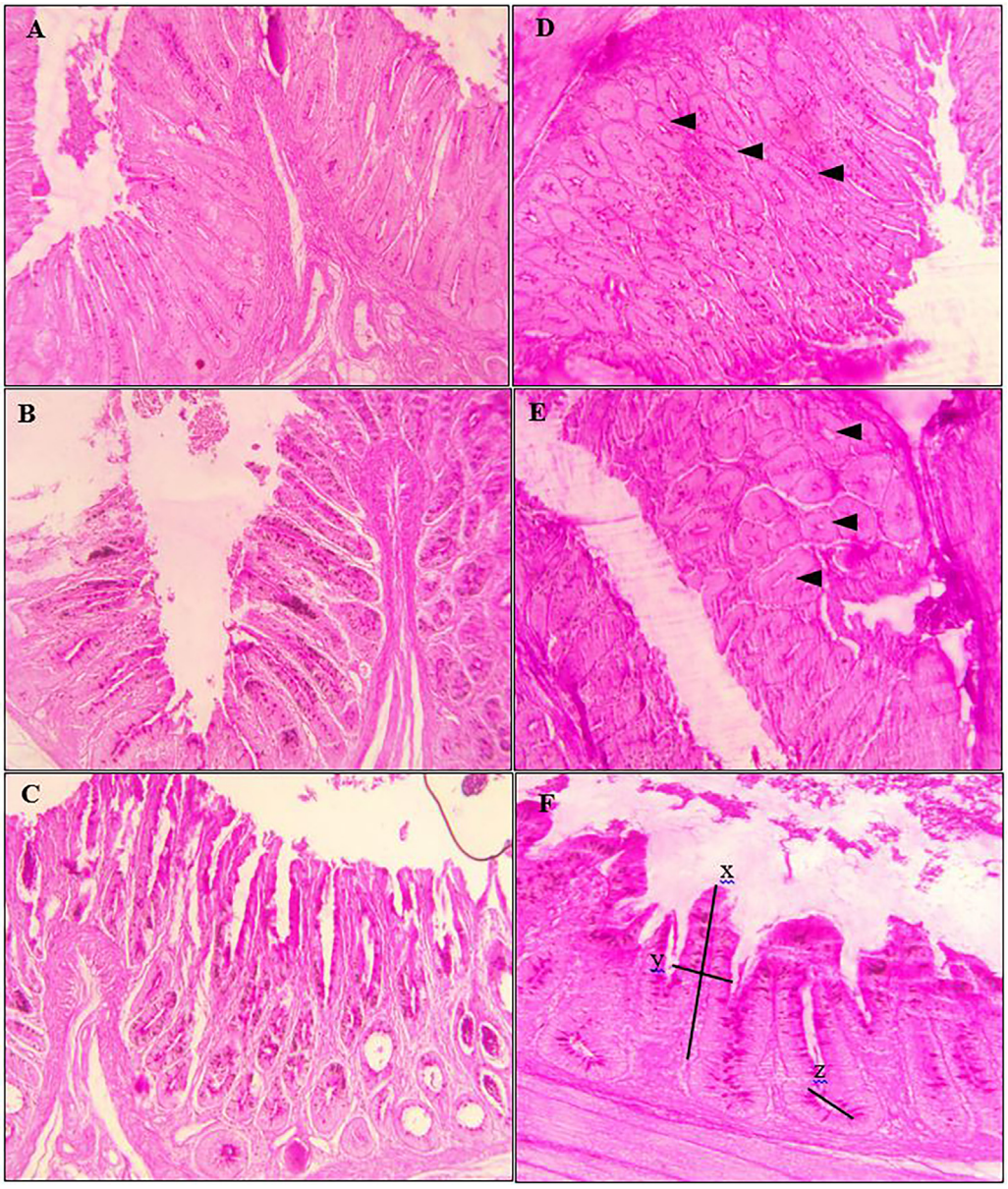

Figure 8. Histomorphological analysis of cecal tissue of *in-ovo* treated birds with Periodic acid−Schiff (PAS) staining (Magnification 100×). (A) Positive control (PC). (B) Probiotic (*Leuconostoc mesenteroides*) group (PB). (C) Prophybiotic (*Leuconostoc mesenteroides* + 0.5% (w/v) garlic aqueous extract) group (PPB). (D) Arrangement of crypts in multiple layers in the PB group. (E) Arrangement of crypts in multiple layers in PPB group. Arrowheads ◄ pointing the crypts. (F) Measurements of villus height (x), villus width (y), and crypt depth (z).

spectrum of antipathogenic properties and fight infection (Zhang and Sunkara, 2014). Although the production of defensins is mostly up-regulated during infection, it has also known that SCFA such as acetate and butyrate stimulates the production of defensins in epithelial cells without inducing inflammation (Zhang and Sunkara, 2014; Chen et al., 2020). As we observed no sign of inflammation (differential expression of interleukins) and higher *AVBD1* expression along with the higher abundance of *Akkermansia* sp. (which produce SCFAs) in the PPB group, it is possible that this higher expression of *AVBD1* is induced by higher production of SCFAs via modulating the gut microbiome rather than an indication of infection.

Moreover, Schlatterer et al. (2021) reported that SCFAs recruit immune cells particularly leucocytes to regulate immune responses in the gut epithelium and the key receptor which is found in these immune cells (through which immune response is mediated) is free fatty acid receptor 2 (*FFAR2*). As our PPB treatment induced the expression of *FFAR2*, there may be a greater recruitment of immune cells, particularly leucocytes in the cecal mucosa possibly via higher SCFA production as a result of microbiome modulation (increased *Akkermansia* sp. abundance). This is further indicated by the observed higher *AVBD* expression as leucocytes are one of the major producers of β defensins (Flaherty, 2012). Schlatterer et al. (2021) claimed that targeted administration of SCFAs thereby activating free fatty acids receptors could be a novel approach in combatting infection. Therefore, we suggest that our PPB *in-ovo* treatment could be a novel and promising

approach to mitigate pathogenic stress in broiler chickens.

Interestingly, the expression of *MUC6* was also higher in both PPB (P- value < 0.05) and probiotic (P- value: 0.0637) treatments. This gene encodes one of the secretory mucins, mucin 6, a component of the mucus layer which influences gut barrier function (Forder et al., 2012) indicating that our treatments influenced mucin production in the cecal mucosa of broiler chickens thereby providing a protective barrier against pathogen colonization.

Histology of Cecal Tissue

The effects of the *in-ovo* treatments on the histolomorphological parameters are shown on Figure 7. There was no statistical difference in villi height among the groups (P- value > 0.05) whereas crypt depth was significantly increased (P- value < 0.05) thus, decreasing the villi height to crypt depth (VH:CD) ratio (P- value < 0.05), in treatment groups when compared to the positive control. Moreover, the crypts of the probiotic and PPB treatment groups were arranged in multiple layers (Figures 8D and 8E, respectively) providing more crypts in the cecal tissue of these birds.

Crypts are generally, considered as villus factories and their depth/size reflect the rate of cell renewal in the mucosa (Sobolewska et al., 2017). Therefore, a higher crypt depth (PPB > PB) and a large number of crypts arranged in multilayers may indicate a higher tissue renewal or stem cell proliferation and differentiation in the treatment groups. In agreement, we observed a greater abundance of *Akkermansia* sp. (PPB > PB) in the cecal content which is known to activate the Wnt/β-catenin signaling pathway stimulating the proliferation of intestinal stem cells (Zhu et al., 2020). This indicates that the PPB treatment followed by probiotic treatment stimulated the efficient development of the mucosal tissue in the ceca to possibly maintain a higher mucin production rate thereby providing protection against pathogen invasion and substrates for SCFA production. Supporting this theory further, more intense PAS staining reaction was apparent in the ceca of chickens belong to treatment groups when compared to the positive control (Figures 8A−8C) suggesting a possible higher glycoprotein production in the cecal mucosa of these chickens.

However, the villus width tended to be decreased in the 2 treatment groups when compared to the positive control (P- value: 0.0501). Consequently, the surface area of the villi was the highest in the positive control group whereas the PPB group displayed the least surface area (P- value: 0.0106). Although, a reduction in the surface area of the gut is generally a sign of reduction in absorption and thus metabolic efficiency, we did not observe any compromising of the production parameters such as body weight, feed efficiency and meat quality. Therefore, we suggest, that it may be an adaptation of the ceca to reduce the surface area to maintain a higher renewal rate and mucin production without causing an energy burden to the birds.

CONCLUSION

The current study highlights the positive effects of administering a PPB combination *in-ovo*, on gut health and production parameters in broiler chickens. More beneficial effects were observed in the PPB treated birds when compared with the probiotic alone group. The PPB treatment beneficially modulated the gut microbiome, upregulated the expression of the genes related certain innate immune parameters and modified the histology of the ceca. Together with production data, our results suggest that the PPB treatment maintains the immune system on standby providing prophylaxis to the host without causing inflammation or an energy burden for production and efficiency. Combining probiotics along with phytobiotics (PPB) is a promising *in-ovo* application which may confer lifelong benefits to the gut health of broiler chickens. Our results encourage further research to elucidate the synergistic potential of different PPB combinations in order to overcome challenges in the gut health of broiler chickens with the aim of reducing the use of antibiotics in poultry production going forward.

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Author Contributions: All authors contributed to the study conception and design. Conducting *in-ovo* experiment, data collection and analysis were performed by Ramesha N. Wishna Kadawarage. The first draft of the manuscript was prepared by Ramesha N. Wishna Kadawarage. Funding acquisition, supervision, review and editing was by Rita Hickey and Maria Siwek. Supervising the *in-vivo* experiment and the meat quality analysis was done by Katarzyna Połtowicz. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

Abd El-Hack, M. E., M. T. El-Saadony, M. E. Shafi, S. Y. A. Qattan, G. E. Batiha, A. F. Khafaga, A.-M. E. Abdel-Moneim, and M. Alagawany. 2020. Probiotics in poultry feed: a comprehensive review. J. Anim. Physiol. Anim. Nutr. 104:1835–1850.

Akinyemi, F. T., J. Ding, H. Zhou, K. Xu, C. He, C. Han, Y. Zheng, H. Luo, K. Yang, C. Gu, Q. Huang, and H. Meng. 2020. Dynamic distribution of gut microbiota during embryonic development in chicken. Poult. Sci. 99:5079–5090.

Akosile, O. A., F. O. Kehinde, A. I. Oni, and O. E. Oke. 2023. Potential implication of in ovo feeding of phytogenics in poultry production. Transl. Anim. Sci. 7:txad094.

Asaadi, S., M. Daneshyar, and Y. A. Alijoo. 2021. The effects of in ovo injection of synbiotic on hatchability, chick quality, blood indices and performance of newly hatched chicks of Japanese Quail. Iran. J. Anim. Sci. Res. 13:389–404.

Bogucka, J., A. Dankowiakowska, G. Elminowska-Wenda, A. Sobolewska, A. Szczerba, and M. Bednarczyk. 2016. Effects of prebiotics and synbiotics delivered in ovo on broiler small intestine histomorphology during the first days after hatching. Folia Biol. (Praha) 64:131–143.

Chen, J., Z. Zhai, H. Long, G. Yang, B. Deng, and J. Deng. 2020. Inducible expression of defensins and cathelicidins by nutrients and associated regulatory mechanisms. Peptides 123:170177.

Chiang, H.-I., L. R. Berghman, and H. Zhou. 2009. Inhibition of NF-kB 1 (NF-kBp50) by RNA interference in chicken macrophage HD11 cell line challenged with Salmonellaenteritidis. Genet. Mol. Biol. 32:507–515.

Chung, C. H., and D. F. Day. 2004. Efficacy of Leuconostoc mesenteroides (ATCC 13146) isomaltooligosaccharides as a poultry prebiotic. Poult. Sci. 83:1302–1306.

Dadfar, M.-J., R. V. Torshizi, A. Maghsoudi, A. Ehsani, and A. A. Masoudi. 2023. Trade-off between feed efficiency and immunity in specialized high-performing chickens. Poult. Sci. 102:102703.

de Paula, A. T., A. B. Jeronymo-Ceneviva, L. F. Silva, S. D. Todorov, B. D. G. M. Franco, and A. L. B. Penna. 2015. Leuconostoc mesenteroides SJRP55: a potential probiotic strain isolated from Brazilian water buffalo mozzarella cheese. Ann. Microbiol. 65:899–910.

Dubowitz, V., M. H. Brooke, and H. E. Neville. 1973. Muscle Biopsy: A Modern Approach. W. B. Saunders, Philadelphia, USA.

Dunislawska, A., A. Slawinska, M. Siwek, and M. Bednarczyk. 2021. Epigenetic changes in poultry due to reprogramming of the gut microbiota. Anim. Front. 11:74–82.

Dunislawska, A., A. Slawinska, K. Stadnicka, M. Bednarczyk, P. Gulewicz, D. Jozefiak, and M. Siwek. 2017. Synbiotics for broiler chickens—in vitro design and evaluation of the influence on host and selected microbiota populations following in ovo delivery. PLOS ONE 12:e0168587.

Earley, H., G. Lennon, Á. Balfe, J. C. Coffey, and D. C. Winter. 2019. The abundance of Akkermansia muciniphila and its relationship with sulphated colonic mucins in health and ulcerative colitis. Sci. Rep. 9:15683.

Ettehad-Marvasti, F., H.-S. Ejtahed, S.-D. Siadat, A.-R. Soroush, Z. Hoseini-Tavassol, S. Hasani-Ranjbar, and B. Larijani. 2022. Effect of garlic extract on weight loss and gut microbiota composition in obese women: A double-blind randomized controlled trial. Front. Nutr 9. Available at https://www.frontiersin.org/articles/10.3389/fnut.2022.1007506 (verified 25 October 2023).

Fathima, S., R. Shanmugasundaram, D. Adams, and R. K. Selvaraj. 2022. Gastrointestinal microbiota and their manipulation for improved growth and performance in chickens. Foods 11:1401.

Ferreira-Halder, C. V., A. V. de S. Faria, and S. S. Andrade. 2017. Action and function of *Faecalibacterium prausnitzii* in health and disease. Best Pract. Res. Clin. Gastroenterol. 31:643–648.

Flaherty, D. K. (Ed.). (2012). Mosby, Saint Louis.

Forder, R. E. A., G. S. Nattrass, M. S. Geier, R. J. Hughes, and P. I. Hynd. 2012. Quantitative analyses of genes associated with mucin synthesis of broiler chickens with induced necrotic enteritis. Poult. Sci. 91:1335–1341.

Kogut, M. H. 2019. The effect of microbiome modulation on the intestinal health of poultry. Anim. Feed Sci. Technol. 250:32–40.

Lenoir, M., R. Martín, E. Torres-Maravilla, S. Chadi, P. González-Dávila, H. Sokol, P. Langella, F. Chain, and L. G. Bermúdez-Humarán. 2020. Butyrate mediates anti-inflammatory effects of Faecalibacterium prausnitzii in intestinal epithelial cells through Dact3. Gut Microbes 12. Available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7567499/ (verified 26 October 2023).

Li, M.-H., J.-X. Meng, W. Wang, M. He, Z.-Y. Zhao, N. Ma, Q.-B. Lv, Y.-F. Qin, H.-L. Geng, Q. Zhao, H.-B. Ni, and X.-X. Zhang. 2022. Dynamic description of temporal changes of gut microbiota in broilers. Poult. Sci. 101:102037.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. San Diego Calif. 25:402–408.

Lohmann Hatchery Guide. Available at https://lohmann-breeders.com/e-guide/hatchery-guide/29/(verified 11 October 2023).

Lu, X., N. Li, R. Zhao, M. Zhao, X. Cui, Y. Xu, and X. Qiao. 2021. *In vitro* prebiotic properties of garlic polysaccharides and its oligosaccharide mixtures obtained by acid hydrolysis. Front Nutr. 8:798450, doi:10.3389/fnut.2021.798450.

Lund, M., and L. Bjerrum. 2010. Quantification of Faecalibacterium prausnitzii- and Subdoligranulum variabile-like bacteria in the cecum of chickens by real-time PCR. Poult. Sci. 89:1217–1224.

Lyu, W., L. Zhang, Y. Gong, X. Wen, Y. Xiao, and H. Yang. 2020. Developmental and tissue patterns of the basal expression of chicken avian β-Defensins. BioMed Res. Int. 2020:e2567861.

Martin, F. E., M. A. Nadkarni, and N. A. Jacques. 2002. Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathological changes in chronic pulpitis. J. Clin. Microbiol. 40:1698–1704.

Miyamoto, J., H. Shimizu, K. Hisa, C. Matsuzaki, S. Inuki, Y. Ando, A. Nishida, A. Izumi, M. Yamano, C. Ushiroda, J. Irie, T. Katayama, H. Ohno, H. Itoh, K. Yamamoto, and I. Kimura. 2023. Host metabolic benefits of prebiotic exopolysaccharides produced by Leuconostoc mesenteroides. Gut Microbes. 15:2161271.

Oviedo-Rondón, E. O. 2019. Holistic view of intestinal health in poultry. Anim. Feed Sci. Technol. 250:1–8.

Pan, L., Y. Han, and Z. Zhou. 2020. In vitro prebiotic activities of exopolysaccharide from *Leuconostoc pseudomesenteroides* XG5 and its effect on the gut microbiota of mice. J. Funct. Foods 67:103853.

Panyod, S., W.-K. Wu, P.-C. Chen, K.-V. Chong, Y.-T. Yang, H.-L. Chuang, C.-C. Chen, R.-A. Chen, P.-Y. Liu, C.-H. Chung, H.-S. Huang, A. Y.-C. Lin, T.-C. D. Shen, K.-C. Yang, T.-F. Huang, C.-C. Hsu, C.-T. Ho, H.-L. Kao, A. N. Orekhov, M.-S. Wu, and L.-Y. Sheen. 2022. Atherosclerosis amelioration by allicin in raw garlic through gut microbiota and trimethylamine-N-oxide modulation. NPJ Biofilms Microb. 8:4.

Paone, P., and P. D. Cani. 2020. Mucus barrier, mucins and gut microbiota: the expected slimy partners? Gut 69:2232–2243.

Penders, J., C. Vink, C. Driessen, N. London, C. Thijs, and E. E. Stobberingh. 2005. Quantification of Bifidobacterium spp., Escherichia coli and Clostridium difficile in faecal samples of breast-fed and formula-fed infants by real-time PCR. FEMS Microbiol. Lett. 243:141–147.

Pietrzak, E., A. Dunislawska, M. Siwek, M. Zampiga, F. Sirri, A. Meluzzi, S. Tavaniello, G. Maiorano, and A. Slawinska. 2020. Splenic gene expression signatures in slow-growing chickens stimulated in ovo with Galactooligosaccharides and challenged with heat. Animals 10:474.

Połtowicz, K., J. Nowak, and D. Wojtysiak. 2015. Effect of Feed restriction on performance, carcass composition and physicochemical properties of the *M. Pectoralis* superficialis of broiler chickens. Ann. Anim. Sci. 15:1019–1029.

Proszkowiec-Weglarz, M., K. B. Miska, L. E. Ellestad, L. L. Schreier, S. Kahl, N. Darwish, P. Campos, and J. Shao. 2022. Delayed access to feed early post-hatch affects the development and maturation of gastrointestinal tract microbiota in broiler chickens. BMC Microbiol 22:206.

Rothwell, L., J. R. Young, R. Zoorob, C. A. Whittaker, P. Hesketh, A. Archer, A. L. Smith, and P. Kaiser. 2004. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. J. Immunol. 173:2675–2682.

Rubio, L. A. 2019. Possibilities of early life programming in broiler chickens via intestinal microbiota modulation. Poult. Sci. 98:695–706.

Rychlik, I. 2020. Composition and function of chicken gut microbiota. Anim. Open Access J. MDPI 10:103.

Sakamoto, K., H. Hirose, A. Onizuka, M. Hayashi, N. Futamura, Y. Kawamura, and T. Ezaki. 2000. Quantitative study of changes in intestinal morphology and mucus gel on total parenteral nutrition in rats. J. Surg. Res. 94:99–106.

Schlatterer, K., A. Peschel, and D. Kretschmer. 2021. Short-Chain Fatty Acid and FFAR2 Activation - A New Option for Treating Infections? Front Cell Infect Microbiol. 11:785833, doi:10.3389/fcimb.2021.785833.

Sevane, N., F. Bialade, S. Velasco, A. Rebolé, M. L. Rodríguez, L. T. Ortiz, J. Cañón, and S. Dunner. 2014. Dietary inulin supplementation modifies significantly the liver transcriptomic profile of broiler chickens. PloS One. 9:e98942.

Siwek, M., A. Slawinska, K. Stadnicka, J. Bogucka, A. Dunislawska, and M. Bednarczyk. 2018. Prebiotics and synbiotics – in ovo delivery for improved lifespan condition in chicken. BMC Vet. Res. 14:402.

Slawinska, A., A. Dunislawska, A. Plowiec, M. Radomska, J. Lachmanska, M. Siwek, S. Tavaniello, and G. Maiorano. 2019. Modulation of microbial communities and mucosal gene expression in chicken intestines after galactooligosaccharides delivery in ovo. PLOS ONE 14:e0212318.

Sławinska, A., M. Z. Siwek, and M. F. Bednarczyk. 2014. Effects of synbiotics injected in ovo on regulation of immune-related gene expression in adult chickens. Am. J. Vet. Res. 75:997–1003.

Smulikowska, S., and A. Rutkowski. 2018. Recommended Allowances and Nutritive Value of Feedstuffs for Poultry. 5th ed. Kielanowski Institute of Animal Physiology and Nutrition, PAS, Jabłonna and PB WPSA, Poznań, Poland (in Polish).

Smialek, M., S. Burchardt, and A. Koncicki. 2018. The influence of probiotic supplementation in broiler chickens on population and carcass contamination with *Campylobacter spp.* - Field study. Res. Vet. Sci. 118:312–316.

Smialek, M., E. Kaczorek, E. Szczucińska, S. Burchardt, J. Kowalczyk, B. Tykałowski, and A. Koncicki. 2019. Evaluation of *Lactobacillus spp.* and yeast based probiotic (Lavipan) supplementation for the reduction of Salmonella Enteritidis after infection of broiler chickens. Pol. J. Vet. Sci. 22:5–10.

Sobolewska, A., J. Bogucka, A. Dankowiakowska, G. Elminowska-Wenda, K. Stadnicka, and M. Bednarczyk. 2017. The impact of synbiotic administration through in ovo technology on the microstructure of a broiler chicken small intestine tissue on the 1st and 42nd day of rearing. J. Anim. Sci. Biotechnol. 8:61.

Sozcu, A., and A. Ipek. 2015. Quality assessment chicks from different hatcher temperatures with different scoring methods and prediction of broiler growth performance. J. Appl. Anim. Res. 43:409–416.

Tannock, G. W., A. Tilsala-Timisjarvi, S. Rodtong, J. Ng, and K. Munro. 1999. Identification of Lactobacillus Isolates from the Gastrointestinal Tract, Silage, and Yoghurt by 16S-23S rRNA Gene Intergenic Spacer Region Sequence Comparisons. Appl. Environ. Microbiol. 65:4264.

van der Most, P. J., B. de Jong, H. K. Parmentier, and S. Verhulst. 2011. Trade-off between growth and immune function: a meta-analysis of selection experiments. Funct. Ecol. 25:74–80.

Wishna-Kadawarage, R. N., M. Jensen, S. Powałowski, R. M. Hickey, and M. Siwek. 2023. In-vitro screening of compatible synbiotics and (introducing) "prophybiotics" as a tool to improve gut health. Int. Microbiol. Online ahead of print.

Yang, W.-Y., C.-H. Chou, and C. Wang. 2022. The effects of feed supplementing *Akkemansia muciniphila* on incidence, severity, and gut microbiota of necrotic enteritis in chickens. Poult. Sci. 101:101751.

Zhang, H., H. HuangFu, G. Qin, G. Wu, L. Wang, and Z. Tan. 2023. Transcriptomic and metabolomic insights into the antimicrobial effect of Leuconostoc mesenteroides or lactic acid on pathogenic Gallibacterium anatis. Chem. Biol. Technol. Agric. 10:118.

Zhang, H., H. HuangFu, X. Wang, S. Zhao, Y. Liu, H. Lv, G. Qin, and Z. Tan. 2021. Antibacterial activity of lactic acid producing *leuconostoc mesenteroides* QZ1178 against pathogenic *Gallibacterium anatis*. Front Vet. Sci. 8:630294, doi:10.3389/fvets.2021.630294.

Zhang, G., and L. T. Sunkara. 2014. Avian antimicrobial host defense peptides: from biology to therapeutic applications. Pharmaceuticals 7:220–247.

Zhu, L., X. Lu, L. Liu, J. Voglmeir, X. Zhong, and Q. Yu. 2020. *Akkermansia muciniphila* protects intestinal mucosa from damage caused by *S. pullorum* by initiating proliferation of intestinal epithelium. Vet. Res. 51:34.