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Effects of *Lactiplantibacillus plantarum* and Galactooligosaccharide Administered In Ovo on Hatchability, Chick Quality, Performance, Caecal Histomorphology and Meat Quality Traits of Broiler Chickens

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ABSTRACT

The presented study explored the promising alternatives of in ovo injection with *Lactiplantibacillus plantarum* (LP) and galactooligosaccharide (GOS) in the poultry industry. The study aimed to assess the effects of probiotic and prebiotic on various aspects of poultry production. The study involved 300 Ross broiler eggs, individually candled on Day 7 of embryonic development. The eggs were sorted into four groups: negative control (no injection), positive control (0.9% physiological saline injection), GOS 3.5 mg/egg and LP 1×10^6 CFU/egg. The groups used during the incubation period were the same for the animal trial; each pen/group had 25 chickens. At the end of the experiment, 8 chickens from each group were slaughtered for tissue sample collection and 12 chickens were slaughtered to determine slaughter yield, carcass and meat quality. All data were analysed by one-way ANOVA or repeated measured ANOVA except for the parameters that did not meet the assumption of normality, the Kruskal–Wallis test (Dunn's test) was used. Key findings revealed that hatchability remained unaffected across groups, indicating the safety of the in ovo injections. Both LP and GOS enhanced chick quality, as evidenced by improved body weight, Pasgar score and chick length. The in ovo administration of LP increased the body weight of the chickens during the first-week post-hatch (7 days of age) without impacting feed intake and feed conversion ratio in the later stages. The study demonstrated no adverse effects on meat quality due to the in ovo injection of LP and GOS. Additionally, a positive impact on caecal histomorphology was observed and early gut colonization of beneficial bacteria (*Lactobacillus* spp. and *Bifidobacteria* spp.) indicated potential benefits for intestinal health in broilers. In conclusion, the in ovo inoculation of 1×10^6 LP and 3.5 mg of GOS per egg increased the relative abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. and showcased promising enhancements in chick quality without compromising growth performance, meat quality and caecal histomorphology. These findings suggest a positive outlook for these substances as a viable alternative for improving poultry health and productivity.

1 | Introduction

In the past few years, great attention has been focused on commercial broiler production due to its short production cycle compared to other livestock species, excellent carcass traits and efficient feed conversion rate. However, modern commercial broiler chickens are susceptible to adverse stimuli from the external environment, causing poor intestinal health and growth performance and reducing meat quality (Ahmed et al. 2023). In recent years, bioactive substances such as probiotics, prebiotics and synbiotics have shown beneficial effects in reducing the incidence of disease infection and mortality while improving feed efficiency, carcass and histological traits, and growth performance in the livestock industry, especially the poultry sector (Dankowiakowska et al. 2019; Oladokun et al. 2021; Wishna-Kadawarage et al. 2024).

During the perinatal period, the supplementation of appropriate nutrition promotes immune system development, stabilizes the gut microbiota and thus reduces the occurrence of pathogen infection (Hou and Tako 2018). The supplementation of these bioactive substances is added to the diet, water or by spray (Bednarczyk et al. 2016). However, this strategy does not aid in early gut colonization by beneficial bacteria during embryonic development as it is done post-hatch. In addition, the quality of the water and the amount of feed mixed with the supplemented bioactive substances may reduce the beneficial effects of the bioactive substances (Bednarczyk et al. 2016). In light of this, another strategy (in ovo technology) has been reported to avert the above-mentioned issues. The in ovo strategy involves the in ovo administration of bioactive substances on Day 17, 18, and so forth, and is often referred to as in ovo feeding while the in ovo delivery of prebiotics, synbiotics and or probiotics on Day 12 is regarded as in ovo stimulation (Siwek et al. 2018). The in ovo stimulation is an effective and efficient intervention strategy as it ensures early gut colonization as early as the 12th day of embryonic development thus influencing a balanced and healthy gut during embryonic development and subsequently in the life of birds (Dunislawska et al. 2017) and therefore improving production performance (Duan et al. 2021). In addition, the in ovo stimulation of bioactive substances has an advantage when compared to in-feed or in-water supplementation as it ensures a precise dosage for each embryo (Siwek et al. 2018).

Probiotics are living microorganisms that confer benefits to the host's health by improving its nutritional and intestinal microbial balance (Majidi-Mosleh et al. 2017). Prebiotics are selectively fermented ingredients that exert positive changes in the gastrointestinal microbiota, thus conferring benefits upon host health (Oladokun and Adewole 2020) while synbiotic is the synergistic combinations of probiotics with prebiotics that subsequently improve host health and performance (Mookiah et al. 2014).

In previous studies, it has been demonstrated that the supplementation of probiotics and prebiotics in poultry diets enhances barrier functions and improves the growth performance and health status of chickens (Deng et al. 2012; Dankowiakowska et al. 2019). It has been also shown that Lactic acid bacteria (LAB) can improve growth performance (Khochamit et al. 2020), modulate the gut microbiota and reduce pathogens and disease infection in poultry (Adhikari and Kim 2017; Kim et al. 2020). The use of *Lactiplantibacillus plantarum* (LP) as a probiotic supplement in broilers' diets has been reported to improve growth performance stimulate immunity and enhance balance gut microflora (Chen et al. 2023; Liu et al. 2023). Additionally, the in ovo administration of LP has several beneficial effects on chick's gut microbiota such as pathogen exclusion, promoting intestinal health and immune functions, antimicrobial and antibacterial effects, lactic acid, and acetic acid to inhibit bacteria, and other harmful microbes and the production of volatile fatty acids, while providing metabolic energy to the host (Alizadeh et al. 2021; Shehata et al. 2021; Guo et al. 2023). On the other hand, the galactooligosaccharides (GOS) (trade name: Bi^{2}tos, Clasado Biosciences Ltd., Reading, UK) have the potential to improve gut health, immunity, antioxidant and production performance of broilers (Slawinska et al. 2020). Furthermore, the same author demonstrated that the in ovo delivery of GOS during embryonic development selectively stimulated the gut microbiota by increasing the presence of beneficial bacteria (*Lactobacilli* and *Bifidobacteria*) and improved gut barrier and epithelial integrity, growth performance, feed and growth efficiency and also mitigated the adverse effects of heat stress (Slawinska et al. 2020).

Despite the numerous studies reporting the potential effects of probiotics and prebiotics to promote embryonic development, growth and poultry, there have been so many inconsistent results. Studies on the in ovo delivery of GOS and LP on Day 12 of incubation are scarce. Thus, there is a dire need to validate the impact of in ovo administration of this prebiotic and probiotic on the growth and intestinal health, carcass and meat quality of broiler chickens. Therefore, this study was designed to determine the effects of in ovo administration of LP and GOS on embryonic development, chick quality, production performance, carcass traits, meat quality, intestinal health in reference to caecal histomorphometry parameters and the presence of beneficial bacteria in the gut microflora.

2 | Materials and Methods

2.1 | In Ovo Injection and Experimental Settings

In this study, we evaluated the effects of in ovo delivery of LP and GOS on the hatchability, chick quality parameters, growth performance, gut histomorphology, bacterial composition, carcass trait and meat quality of Ross 308 broiler chickens. We used two control groups: positive control (PC) injected with 0.2 mL of 0.9% saline solution and negative control (NC) which was left un-injected (Table 1).

2.2 | Preparation of Bioactive Substances (GOS and LP)

For the preparation of GOS, an amount of 3.5 mg GOS/egg was dissolved in 0.2 mL physiological saline solution and delivered in ovo into the air chamber on Day 12 of egg incubation (Slawinska et al. 2020).

The probiotic (LP) was grown in MRS broth media for 15 h (based on our preliminary experiments, at 15 h of incubation, this probiotic reached its peak growth at 37°C in which the number of

TABLE 1 | Experimental design for the in ovo experiment.

Groups	In ovo injection treatments	Dose of bioactive/egg
NC	No injection	—
PC	0.9% Physiological saline	0.2 mL
Prebiotic (GOS)	Galactooligosaccharides dissolved in 0.9% saline solution	3.5 mg GOS (in 0.2 mL)
Probiotic (LP)	*Lactiplantibacillus plantarum* bacterial suspension in 0.9% saline solution	10^6 CFU (in 0.2 mL)

Abbreviations: CFU, colony-forming unit; GOS, galactooligosaccharide; LP, *Lactiplantibacillus plantarum*; NC, negative control; PC, positive control.

active and viable cells can be obtained (Wishna-Kadawarage et al. 2024). Using a refrigerated centrifuge, the probiotic (LP) cells were centrifuged at 4200 rpm for 20 min at 4°C. Next, the cell pellets obtained from each culture were then washed twice with sterile 0.9% saline solution and resuspended in 0.9% saline solution. This was followed using a microplate reader (Thermo Scientific Multiskan FC plate reader: Thermo Scientific, Poland) by adjusting the optical density at 600 nm (OD600) of the solution to obtain the cell density similar to 5×10^6 CFU/mL (based on the regression equation obtained from our preliminary study between the CFU/mL and OD600). Finally, 200 μL of this cell suspension was used for in ovo injection for each egg.

2.3 | Egg Incubation and In Ovo Injection

In this experiment, a total of 300 ROSS 308 broiler eggs were incubated. The incubation parameters were maintained in optimum conditions (temperature: 37.5°C, relative humidity: 65% and egg turning every 1 h) (Midi series I, Fest Incubators, Poland) throughout the incubation process. All eggs were candled on the seventh day of egg incubation and nonviable and dead embryos were discarded. The remaining eggs were then randomly allotted to the four treatment groups (Table 1) and placed back into the incubator. Next, on the 12th day of egg incubation, all eggs were disinfected with 70% ethanol to avoid any possible contamination before injection and the blunt end of each egg (air chamber) was identified. Subsequently, a 20G needle was used to carefully make a hole in the egg air chamber. The respective doses (as described in Table 1) were manually injected into the air chamber of each egg using a 26G needle assuring no damage to the inner membranes of the egg. A drop of organic glue (Elmer's school glue, Elmer's Products Inc., USA) was used to seal the holds of each egg. The negative group (NC) was left not injected.

2.4 | Hatchability and Chick Quality Analysis

The hatchability was calculated based on the fertile eggs after candling. At the end of the incubation and hatching, the hatchability rate of each group was recorded and calculated by using the equation below:

$$\text{Hatchability} = (\text{No. of chicks hatched}/\text{No. of hatching eggs}) \times 100.$$

Upon recording the hatchability, all chicks were wing-tagged. Next chick quality assessment was performed using the Pasgar score, chick-hatchling weight and chick length. In each treatment group, 25 well-dried chicks were randomly selected, and their weight was recorded using an electronic balance. For the length measurement, the same 25 chicks were measured by placing the chick face down on a flat surface and straightening the right leg. The length (cm) was measured from the tip of the beak to the tip of the middle toe using a ruler (Sozcu and Ipek 2015). Using the Pasgar scoring method (Mukhtar, Khan, and Anjum 2013), the quality of 10 birds (out of the 25 randomly chosen birds/group) were selected to determine the quality of 1-day-old chicks for each of the treatment groups.

2.5 | Birds and Management

The rearing and management of birds were conducted in accordance with the guidelines of the Ethics Committee for Experiments with Animals and the 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. All birds of each experimental group were housed in separate pens with similar environmental conditions in all the pens to ensure optimal conditions throughout the trial period. In the experiment, there were 25 birds/pen. All of them were used for production performance evaluation (BW, ADG, ADFI and FCR), 8 birds from each group were used for sample collection for transcriptomic and histological analysis while 12 birds (with a body weight closest to the average per group) from each group were used for meat quality analysis). The size of each pen was $1.5\,m \times 2\,m = 3\,m^2$. Feed and water were provided ad libitum at all times during the rearing period. The birds were fed with the following three types of diets throughout the experimental period: starter (1–21 days), grower (22–28 days) and finisher (29–35 days) containing 22.3%, 20.2% and 20.2% crude protein and 12.45, 13.01 and 13.01 MJ/kg metabolizable energy, respectively. The dietary mixtures were in accordance with broiler chicken dietary requirements (Smuliikowska and Rutkowski 2018) listed in Table 2. The initial temperature for the chicks was 32°C–33°C in the first day of age and was gradually decreased until reaching about 21°C at the end of the trial period (35 days).

2.6 | Growth Performance

The weekly feed intake and body weight of each bird from the respective groups were recorded to determine the feed conversion ratio (FCR).

TABLE 2 | Dietary composition fed to Ross 308 broiler chicken during three growing phases.

Dietary composition	Starter (1–14 days)	Grower (15–22 days)	Finisher (16–35 days
Dry matter (%)	91.19	91.19	91.61
Crude protein (%)	22.3	20.2	20.2
Metabolize energy (mJ/kg)	12.45	13.01	13.01
Crude fat (%)	5.02	6.88	6.36
Crude fibre (%)	2.64	2.16	2.23
Crude ash (%)	5.49	5.19	5.09
Lysine	11.60	11.33	11.86
Methionine	6.06	5.15	4.87
Arginine	14. 131	12.77	12.62
Cystine	3.033	3.03	2.84
Alanine	10.65	9.96	9.97
Glycine	9.14	8. 19	8.29
Valine	9.85	8.93	9.11
Leucine	17.41	16.421	16.62
Tyrosine	7.25	7.19	6.601
Phenylalanine	10.56	9.84	9.74
Histidine	6.74	6.14	6.36
Calcium (g/kg)	9.20	9.14	8.74
Sodium (g/kg)	1.45	1.43	1.52
Phosphorous (g/kg)	6.65	6.25	6.36
Chlorides (g/kg)	2.60	2.65	2.74

2.7 | Sample Collection and Carcass Traits

During the rearing period, eight faeces samples were collected from each group on 7, 14, 21, 28 and 34 days to determine the relative abundance of bacteria to determine the bacterial composition of the gut microbiota in different developmental stages of the birds. Additionally, the caecal content (from the ceca) was also sampled to determine the relative bacterial abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. At the end of the rearing period (35 days), 12 birds of average body weight from each group were selected after a fasting period of 12 h whereas free access to water was ensured. Next, the birds were slaughtered by decapitation and left to bleed for about 90 s. After 5 min of bleeding, each bird was scalded, feathers removed, and eviscerated. The carcasses with and without giblets were weighed and the carcass yield was calculated as a percentage of the live weight. Additionally, organs and tissues such as the liver, gizzard, heart, breast muscles, leg muscles (thigh and drumstick), leg bones and abdominal fat were excised and weighed individually using an electronic scale. The percentage of each organ and tissue was then expressed as a percentage of the chilled carcass weight with giblets.

2.8 | Meat Quality Analysis

The carcasses were air chilled at 4°C and then breast muscle and thigh muscles were used for the meat quality analysis. The pH was recorded at 15 min and 24 h (pH15, pH24) post-mortem using a portable CyberScan10 pH meter (Eutech Instruments Pte Ltd., Singapore). The meat colour was determined and recorded as lightness (L^*), redness (a^*) and yellowness (b^*). Other parameters such as drip losses, cooking losses, losses after thawing, shear force, hardness, springiness, cohesiveness, gumminess, chewiness, resilience and adhesiveness were determined. The meat quality analysis was performed as described by Połtowicz, Nowak, and Wojtysiak (2015).

2.9 | Ceca Histomorphology Analysis

The middle part of the caecum was obtained for histomorphometry analysis and was directly immersed in Bouin's solution (HT101128, Sigma-Aldrich, Poland) until further use. Chicken caeca histomorphology was performed in a histological laboratory according to the methodology described by Bogucka et al. (2016) using the paraffin technique and a microscopic magnification of 100. Samples of the caeca—ca. 2 cm long—were collected from eight chickens from each group. The caecal sections were fixed in Bouin's fluid, dehydrated, cleared and infiltrated with paraffin in a tissue processor Microm STP 120 (Thermo Shandon, Chadwick Road, Astmoor, Runcorn, Cheshire, UK), embedded in paraffin blocks using the dump station (Medite, Burgdorf, Germany) and cut on a rotary microtome (Finesse ME+, Thermo Shandon, Chadwick Road, Astmoor, Runcorn, Cheshire, UK) into 10-μm-thick sections. After placing the sections on a glass slide, which had previously been covered with egg white and glycerin, the slides were de-waxed and hydrated. Next, a PAS reaction (Dubowitz Brooke, and Neville 1973) was performed. Evolution 300 microscope (Delta

Optical, Poland) equipped with a digital camera ToupCam (TP605100A, ToupTek, China) was used to record microscopic images of caeca on a computer disk. Histological measurements (10 villi/chicken): height and width of intestinal villi, intestinal crypt depth and thickness of the muscle membrane were made using Multiscan 18.03 microscopic images software (Computer Scanning Systems II, Warsaw, Poland). Based on the data obtained, the ratio of the height of the villus to the depth of the crypts (VH/CD) was calculated. The surface of the villi was calculated according to the formula given by Sakamoto et al. (2000): $(2\pi) \times (VW/2) \times (VH)$, where VW is the villus width and VH the villus height.

2.10 | Bacterial DNA Extraction

The GeneMATRIX Stool DNA Purification Kit (E3575, EURx, Gdańsk, Poland) was used for the extraction of DNA from faecal samples and the caecal content of birds. Next, a NanoDrop 2000 spectrophotometer (ThermoScientific, Warsaw, Poland) was used to evaluate the quality and quantity of the isolated DNA and gel electrophoresis was performed using 2% agarose gel to determine DNA integrity. All extracted DNA samples were kept at −80°C until further analysis.

2.11 | Relative Abundance of Bacteria Quantification Using Quantitative PCR (qPCR)

The relative abundance of *Lactobacillus* spp., *Bifidobacteria* spp. in faeces samples and caecal content were evaluated using a qPCR method. All the bacteria were determined in relation to the universal bacterial quantity in each sample. The primer sequences used are highlighted in Table 3.

A total reaction mixture volume of 12.5 μL constituting of 1 μM of each (forward and reverse) primer (Sigma-Aldrich, Darmstadt, Germany), 10–20 ng of DNA, and 6.25 μL of SG qPCR Master Mix (2×) (0401, EURx, Gdańsk, Poland) was used for qPCR using a 96-well plates (4TI-0955, AZENTA, Genomed, Warsawa, Poland). In each sample, two technical replicates were prepared, and the qPCR was done using Light-Cycler 480 II (Roche-Diagnostics, Rotkreuz, Switzerland). The steps in the qPCR process involved an initial denaturation at 95°C for 5 min. Next was followed by 40 cycles of amplification and a denaturation step at 95°C for 10 s for each amplification. This was followed by an annealing step at 58°C for 15 s, and finally an elongation step at 72°C for 30 s. The average Ct values of the two replicates from each sample were recorded and used for statistical analysis. Therein, five dilutions (1×, 0.5×, 0.25×, 0.125× and 0.0625×) of bacterial DNA pooled together from each treatment group were used to determine the standard curve relevant samples of all treatment groups. Next, the primer efficiency was evaluated PCR efficiency using the Light-Cycler 480 II software (Roche-Diagnostics) as described by Slawinska, Dunislawska, et al. (2019) and Wishna-Kadawarage et al. (2024):

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

where E universal is the efficiency of qPCR with primers for all bacteria; Ct universal, the Ct values for reaction with primers for all bacteria; E target the the efficiency of qPCR with primers specific for *Bifidobacterium* spp. or *Lactobacillus* spp.; and Ct target is the Ct values for reaction with primers for *Bifidobacterium* spp. or *Lactobacillus* spp.

2.12 | Data Analysis

Before the analysis, a normality test was performed on all data. Thus, the normal distribution of the data and equal variances were tested using the Shapiro–Wilk and Levene's tests, respectively. Afterward, the hatch parameters (chick weight, chick length and chick quality) were analysed using a one-way ANOVA. The body weight of chickens was analysed using a repeated measures ANOVA taking into account repeated measures over time (7, 14, 21, 28 and 35 days) in STATISTICA software 14.0.0.15. The other parameters such as FI, FCR, slaughter parameters, meat quality and relative abundance of bacteria were analysed using one-way ANOVA and for the parameters that did not meet the assumption of normality, the Kruskal–Wallis test was performed and then the Dunn's test was used to check for differences between the treatments. Tukey's HSD test was performed to compare means for identifying the statistically different groups ($p < 0.05$). GraphPad Prism version 10.1.2 (324) was used for graphing and visualization of the results obtained.

3 | Results

3.1 | Hatchability

The results of the hatchability (fertile eggs after candling) were similar across all groups, with NC 92%, PC 86%, GOS 90% and LP 86%.

TABLE 3 | Primer sequences used for evaluating the bacteria relative abundance in faecal and caecal content using qPCR.

Bacteria	Primer sequence (5′ → 3′)	References
Universal bacteria	F: ACTCCTACGGGAGGCAGCAGT R: GTATTACCGCGGCTGCTGGCAC	Tannock et al. (1999)
Lactobacillus spp.	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	Slawinska, Dunislawska, et al. (2019)
Bifidobacterium spp.	F: GCGTGCTTAACACATGCAAGTC R: CACCCGTTTCCAGGAGCTATT	Penders et al. (2005)

Abbreviations: F, forward primers; R, reverse primers.

3.2 | Chick Quality Parameters

The results of the chick quality (hatchling weight, length and Pasgar score are presented in Figures 1A–C). Our current study demonstrated a significant increase ($p < 0.05$) in BW of the newly hatched chicks in the LP and GOS groups (Figure 1A) (50 and 47 g) as compared to our control groups (NC and PC). Regarding chick length (Figure 1B) and Pasgar score (Figure 1C), we found no significant effects; however, the chicks in the LP and GOS were longer (18.47 and 18.20 cm) as compared to the control groups. Furthermore, the Pasgar score showed the GOS experimental group having the highest score (9.3) with intermediate values between the other treatments.

3.3 | Growth Performance

The results of the growth performance are presented in Table 4. In this study, we observed a significant increase in BW on 7 days ($p < 0.05$) in the LP group as compared to the PC group. The GOS group and LP had a BW of 179.60 and 195.2 g, respectively. In Days 14, 21, 28 and 35, no significant effect of on BW was found. However, GOS and LP in ovo-treated chickens had a numerically higher body weight at Day 35 as compared to the NC and PC groups. The in ovo stimulation of either GOS or LP did not cause any significant effects on ADG, ADFI and FCR ($p > 0.05$) throughout the rearing period.

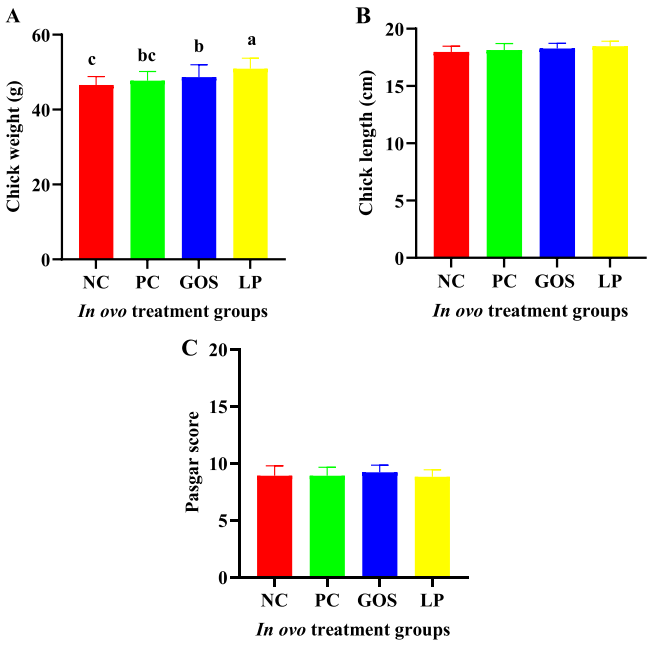

FIGURE 1 | The assessment of chick quality parameters: (A) hatchling weight (g), (B) length (cm) and (C) Pasgar score of the four in ovo treatment groups. Error bars: ±SD. Tukey HSD test ($p < 0.05$) was used to check for significant differences with different letters a, b, c. GOS, galactooligosaccharides; LP, *Lactiplantibacillus plantarum*; NC, negative control; PC, positive control. [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Slaughter Analysis, Carcass Traits and Meat Quality

The results of the carcass traits and the meat quality analysis (in both breast muscles and leg muscles) are presented in Tables 5 and 6, respectively. No significant effects on the dressing percentage and the other carcass traits were observed due to the in ovo treatments. However, significant changes in cooling losses (lower cooling losses) were observed in the carcasses of chickens from the LP group ($p < 0.05$) (Table 5). Regarding the other parameters (Table 5) determined, there were no significant changes between our in ovo injected groups and the control groups (NC and PC). Regarding the meat quality, presented in Table 6, we found a higher pH at 15 min post-mortem ($p < 0.05$) in GOS and LP as compared to the control group (Table 6). However, no significant changes were found upon measurement of the pH at 24 h post-mortem in chickens. In addition, the effects of GOS and LP on meat colour were also evaluated. The results presented in Table 6 revealed no significant changes in meat colour upon in ovo administration of either GOS or LP. Furthermore, as shown in Table 6, there were no significant differences in drip loss, thawing loss, cooking loss, as well as shear force and other parameters evaluated in this study.

3.5 | Relative Bacterial Abundance in Faecal Samples

The results of the relative bacterial abundance (*Bifidobacterium* spp. and *Lactobacillus* spp.) in chicken's faeces from different time points (Days 7, 21 and 34) are reported in Figures 2 and 3 respectively. On 7 and 21 days of the bird's life, we observed no pronounced changes in the relative abundance of *Bifidobacterium* in the chickens in ovo treated with either GOS or LP (Figure 2). However, nearing the end of the rearing period (Day 35), a significant increase ($p < 0.001$) of *Bifidobacterium* spp. was observed in both GOS and LP as compared to the control group. The result of the *Lactobacillus* spp. (Figure 3) showed a substantial increase ($p < 0.05$) in the relative abundance of *Lactobacillus* in both of our treatment groups on days 7, 21 and 35 as compared to the control group. From the results, the GOS had a higher influence on the presence of *Lactobacillus* spp. and *Bifidobacteria* spp. (Figures 2 and 3).

3.6 | Relative Bacterial Abundance in Caecal Content

The changes in the relative bacterial abundance in chicken caecal content upon in ovo delivery are reported in Figure 4A,B. Our results showed a significant increase ($p < 0.05$) in the relative abundance of *Lactobacillus* spp. compared to the control group (Figure 4A). In addition, a pronounced increase ($p < 0.05$) in the relative abundance of *Bifidobacterium* spp. was found in both GOS and LP as compared to the control group (Figure 4B). Comparing the results, we demonstrated that LP had more influence on the relative abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. in the caecal content of chicken (Figure 4A,B) whereas in faeces GOS had more influence on the prevalence of these beneficial bacteria (Figures 2 and 3).

TABLE 4 | Effects of in ovo injection of GOS and LP on chicken growth performance from Day 1 to Day 35.

Items	Treatment groups				SD	*p* value
	NC	PC	GOS	LP		
BW (g)						
Day 1	48.32[c]	47.99[bc]	49.47[b]	53.45[a]	2.509	0.001
Day 7	180.50[b]	177.34[b]	179.60[b]	195.23[a]	24.140	0.021
Day 14	480.20	490.81	485.93	518.80	66.130	0.914
Day 21	1014.40	1011.25	1017.70	1044.30	113.941	0.999
Day 28	1681.50	1663.40	1655.40	1716	175.018	0.885
Day 35	2437.50	2433.60	2526.90	2499.70	302.093	0.790
ADG (g)						
Days 1–7	18.88	18.91	19.51	21.89	1.423	0.619
Days 8–14	42.81	44.78	43.76	46.22	1.461	0.319
Days 15–21	76.32	74.34	75.96	75.07	0.890	0.662
Days 22–28	97.68	93.22	91.10	95.96	2.913	0.528
Days 29–35	105.60	109.961	124.50	111.95	8.108	0.190
ADFI (g)						
Day 1–7	22	22	22	23	0.554	0.195
Day 8–14	54	52	5414	56	2.002	0.875
Day 15–21	96	98	97	100	2.304	0.804
Day 22–28	138	137	136	137	3.842	0.711
Day 29–35	177	170	172	170	4.616	0.490
FCR (g/g)						
Day 1–7	1.44	1.20	1.32	1.137	0.205	0.662
Day 8–14	1.53	1.17	1.35	1.27	0.194	0.069
Day 15–21	1.52	1.31	1.37	1.37	0.306	0.055
Day 22–28	1.71	1.47	1.57	1.47	0.148	0.374
Day 29–35	2.05	1.55	1.47	1.56	0.216	0.209

Note: Data are presented as mean and pooled standard deviation (SD). Values in a row with different superscript letters (a, b) indicates significant difference ($p < 0.05$). Abbreviations: GOS, galactooligosaccharides; LP, *Lactiplantibacillus plantarum*; NC, negative control; PC, positive control; SD, standard deviation.

3.7 | Caecal Histomorphology Analysis

The results of the caecal histomorphology parameters are shown in Table 7. In the current study, the in ovo administration of either GOS or LP significantly increased ($p < 0.05$) in the villus height villus width of adult chickens as compared to the control (PC group). Surprisingly, we found a significant increase ($p < 0.05$) in villus surface area in the control as compared to GOS and LP groups. On the other hand, a deeper crypt depth ($p < 0.05$) was observed in the LP group as compared to the GOS. Additionally, no significant changes were found in the muscle membrane and villus height/crypt depth ratio between the groups.

4 | Discussion

With the intensification and expansion of the broiler industry, innovative techniques and alternative nutritional strategies are required to maintain chicken health and productivity and food safety. The probiotic LP (B/00081) is a commercialized product that is part of 'LAVIPAN' a probiotic premix produced by JHJ, Nowa Wieś, Poland. The probiotic LP inhibits pathogen infection (Smialek et al. 2018), improves antioxidant capacity (Ciszewski et al. 2023) and modulates the immune system (Alizadeh et al. 2021) without compromising production performance (Gao et al. 2024). On the other hand, the prebiotic Bimuno galactooligosaccharide is produced by Clasado Biosciences Ltd., Reading, UK and was primarily used in humans. The supplementation of GOS in poultry diet demonstrated an increased number of *lactobacilli* and *bifidobacteria* in chickens (Bednarczyk et al. 2016), improved health and immune functions (Slawinska et al. 2016), and growth performance (Slawinska et al. 2020). Therefore, this research was undertaken to explore the impacts of the in ovo administration of either GOS or LP on Day 12 of embryonic development on hatchability, chick quality, and overall performance while promoting intestinal health and meat quality traits. The novelty of this study relies on involving the in ovo administration for a commercial prebiotic GOS or commercial probiotic LP to find a sustainable alternative modulation strategy thereby contributing to improving chicken welfare and food safety standards.

TABLE 5 | Effects of in ovo injection of GOS and LP on chicken carcass traits and slaughter analysis parameters.

Parameters	Treatment groups			SD	*p* value
	PC	GOS	LP		
Cooling losses (%)	1.79[a]	1.58[ab]	1.31[b]	0.326	0.004
Dressing percentage with giblets %	79.81	80.19	80.32	1.103	0.690
Dressing percentage without giblets %	76.83	77.19	77.35	1.166	0.945
Breast muscles %	31.35	30.60	31.34	1.760	0.554
Leg muscles %	19.19	18.47	18.70	1.436	0.459
Giblets %	3.75	3.73	3.70	0.356	0.987
Liver %	2.23	2.25	2.20	0.25	0.897
Gizzard %	0.96	0.94	0.93	0.186	0.937
Heart %	0.55	0.54	0.57	0.073	0.775
Leg bones %	3.98	4.03	4.15	0.836	0.677
Abdominal fat %	1.83	1.90	1.89	0.310	0.865
Breast muscles (g)	615.025	606.18	621.66	57.216	0.790
Leg muscles (g)	377.12	366.22	369.65	39.040	0.775
Giblets (g)	73.59	74.008	73.25	8.320	0.994
Liver (g)	43.91	44.70	43.63	6.110	0.993
Gizzard (g)	18.88	18.48	18.35	3.513	0.958
Heart (g)	10.80	10.84	11.28	1.816	0.802
Leg bones (g)	78.18	80.21	82.73	13.486	0.895
Abdominal fat (g)	35.92	37.61	37.53	6.330	0.793

Note: Data are presented as mean and pooled standard deviation (SD). Values in a row with different superscript letters (a, b) indicates significant difference ($p < 0.05$). Abbreviations: GOS, galactooligosaccharides; LP, *Lactiplantibacillus plantarum*; NC, negative control; PC, positive control; SD, standard deviation.

4.1 | Hatchability

Hatchability remains one of the most important parameters for a successful in ovo injection and the hatchery industry. In this study, we successfully performed in ovo delivery of LP and GOS through the air sac on Day 12 of incubation, with no negative effect on embryo viability. In the present study, hatchability rates were similar across the groups. However, the NC group had the highest hatchability rate (92%). Interestingly, the GOS group had a higher hatchability rate (90%) as compared to LP and PC 86% respectively. Our results revealed that the in ovo injection did not negatively affect hatchability. A similar result was reported by Pruszynska-Oszmalek et al. (2015), Bednarczyk et al. (2016), Slawinska, Mendes, et al. (2019) and Slawinska et al. (2020) confirming that the in ovo administration of probiotics, prebiotics and/or synbiotics did not lower hatchability. Another study reported an increased hatchability rate of 96% and 91% upon in ovo injection of *Bacillus Subtilis* (Oladokun and Adewole 2021, 2022). It is also reported that LP 1×10^6 CFU/egg and LP 1×10^6 CFU/egg + 2 mg/egg Astragalus polysaccharide did not affect hatchability (Duan et al. 2021). The developing embryo is sensitive to homoeostatic disturbances; therefore, during in ovo injection, several critical factors such as embryo age, type and dose of bioactive use, time and site of injection require vital consideration before in ovo injection (Bednarczyk et al. 2016; Siwek et al. 2018). The in ovo injection of LP and Astragalus polysaccharide has several benefits such as early gut colonization, improved embryo viability and pathogens exclusion (Duan et al. 2021). We observed a high hatchability rate without impairing embryonic development following a validated protocol for in ovo injection of bioactive substances on Day 12 of embryonic development as reported by Bednarczyk et al. (2016) and Siwek et al. (2018). Furthermore, our results demonstrated that *the* in ovo delivery of GOS and LP on Day 12 of egg incubation was safe and also provided beneficial effects to developing embryos. In a review by Siwek et al. (2018), it is reported that in ovo injection of bioactives on Day 12 of egg incubation is safe and less likely to reduce or have adverse effects on hatchability.

4.2 | Chick Quality

In our current study, we used three chick quality parameters (chick hatchling weight, length and Pasgar score). Our results (Figures 1A–C) show the effects of the prebiotic (GOS) and probiotic (LP) administered in ovo on the quality of 1-day-old chicks. The current study revealed that the BW of the newly hatched chicks (Figure 1A) was significantly higher ($p < 0.05$) in the LP and GOS groups (50 and 47 g) as compared to our control groups (NC and PC). This may be explained due to the balanced gut provided by the bioactive substance which probably enhanced embryonic development, immune function, and improved gut and nutrient absorption consequently causing a significant increase in the body weight of newly hatched chicks (Gao et al. 2024). In terms of chick length and Pasgar score (Figure 1B,C), we found no significant effects; however, the chicks in the LP and GOS were longer (18.47 and 18.20 cm) as compared to the control groups. Furthermore, the Pasgar score showed the GOS experimental

TABLE 6 | Effects of in ovo injection of GOS and LP on meat quality parameters.

Parameters	Treatment groups			SD	*p* value
	PC	GOS	LP		
Breast muscle					
pH 15 min	6.37[b]	6.45[a]	6.40[a]	0.160	0.002
pH 24 h	5.94	5.98	6.03	0.450	0.804
*L**	52.60	56.66	58.10	6.836	0.570
*a**	9.88	10.68	10.24	1.596	0.844
*b**	14.24	15.05	15.54	2.391	0.771
Drip losses 24 h (%)	0.93	0.84	1.00	0.420	0.896
Drip losses 48 h (%)	1.84	1.75	1.89	1.142	0.769
Thawing losses (%)	4.93	3.55	3.66	2.093	0.321
Cooking losses (%)	24.73	31.13	27.60	6.070	0.431
Shear force (N)	13.06	13.00	12. 58	2.714	0.967
Hardness	64.28	73.20	75.53	16.526	0.426
Springiness	0.32	0.35	0.35	0.053	0.537
Cohesiveness	0.38	0.44	0.44	0.070	0.153
Gumminess	26.87	32.62	33.37	8.810	0.977
Chewiness	9.40	11.38	11.50	3.126	0.307
Resilienceness	0.19	0.23	0.22	0.100	0.066
Adhesiveness	−0.06	−0.05	−0.06	0.100	0.721
Leg muscle					
pH 15 min	6.38[b]	6.43[a]	6.62[a]	**0.153**	**0.012**
pH 24 h	6.24	6.30	6.34	0.126	0.221
*L**	49.83	49.71	49.36	1.883	0.895
*a**	15.23	15.85	15.31	1.203	0.571
*b**	11.14	11.30	11.20	0.913	0.902
Drip losses 24 h (%)	0.57	0.58	0.58	0.090	0.987
Drip losses 48 h (%)	0.75	0.80	0.71	0.126	0.648
Thawing losses (%)	3.05	2.95	2.41	1.030	0.415
Cooking losses (%)	30.45	28.27	27.99	2.920	0.213

Note: Data are presented as mean and pooled standard deviation (SD). Values in a row with different superscript letters (a, b) indicate a significant difference ($p < 0.05$).
Abbreviations: GOS, galactooligosaccharides; LP, *Lactiplantibacillus plantarum*; NC, negative control; PC, positive control; SD, standard deviation.

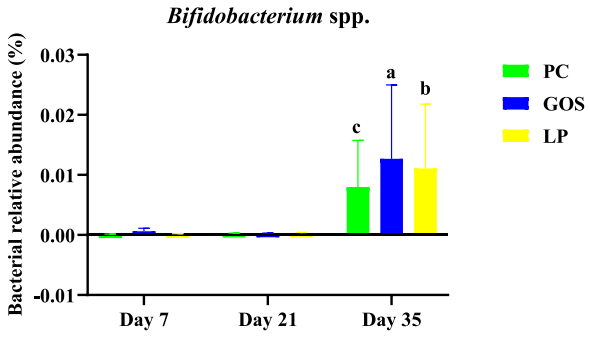

FIGURE 2 | The bacterial relative abundance of *Bifidobacterium* spp. in the faeces of in ovo treated chickens on Days 7, 21 and 35. Error bars: ±SE. a, b letters having different superscripts differ significantly ($p < 0.05$). GOS, galactooligosaccharide; LP, *Lactiplantibacillus plantarum*; PC, positive control. [Color figure can be viewed at wileyonlinelibrary.com]

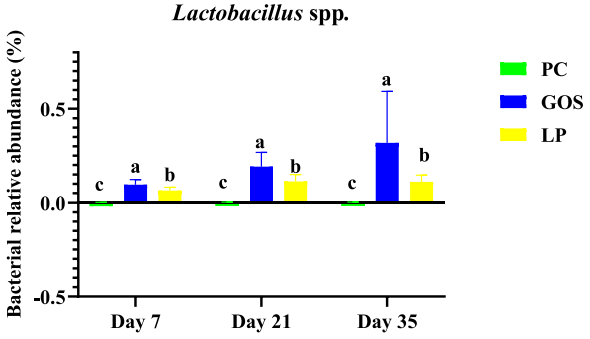

FIGURE 3 | The bacterial relative abundance of *Lactobacillus* spp. in the faeces of in ovo-treated chickens on Days 7, 21 and 35. Error bars: ±SE. a, b letters having different superscripts differ significantly ($p < 0.05$). GOS, galactooligosaccharide; LP, *Lactiplantibacillus plantarum*; PC, positive control. [Color figure can be viewed at wileyonlinelibrary.com]

group having the highest score (9.3) with intermediate values between the other treatments. This is evidence that the bioactive substances used in this study promoted embryo development and viability, and chick quality which may subsequently have a positive impact on the future performance of these chickens Bilalissi et al. (2019) and Akosile et al. (2023). This result is in agreement with other authors (O'Dea et al. 2006). Similar to our findings, Bilalissi et al. (2019) reported no adverse effects on chick quality when 50 μg *Moringa oleifera* was in ovo injected on 17 days of incubation as compared to the control.

4.3 | Growth Performance

From previous research, it is reported that the addition of probiotics in chicken feed could improve the feed intake, weight gain and feed efficiency in broilers (Jha et al. 2020; Ye et al. 2021). On the other hand, 70% of the total production cost in the broiler industry is feed, thus efficient utilization of feed by chickens has been associated with an increase in economic returns (Dankowiakowska et al. 2019). In this study, we observed a significant increase in BW on Day 7 ($p < 0.05$) in the LP group as compared to the PC group (Table 4). However, in other time points (Days 14, 21, 28 and 35), there were no pronounced changes in BW among the group. Interestingly, the GOS and LP groups had a slightly higher BW of 179.60 and 195.2 g, respectively, on Day 35 (at the end of the rearing period) as compared to the control group. No significant effects were observed in the ADG, ADFI and FCR among the groups throughout the trial period (Table 4). Our result is in line with that of Maiorano et al. (2012) and Tavaniello et al. (2023), who reported no significant effect of synbiotics injected in ovo on the growth performance of birds but observed a slightly higher BW in synbiotic-injected groups as compared to the control. Yet still, similar results on increased FI and BW on synbiotics in ovo-injected chickens on Day 7 (Duan et al. 2021). Contrary to our findings, Awad et al. (2009) reported that prebiotics significantly increased the BW of 35-day-old chickens. Our results showed that GOS and LP improved the early growth performance of chicks (Table 4). The varying results revealed that the supplementation of different bioactive substances and doses could lead to varying growth performance of birds.

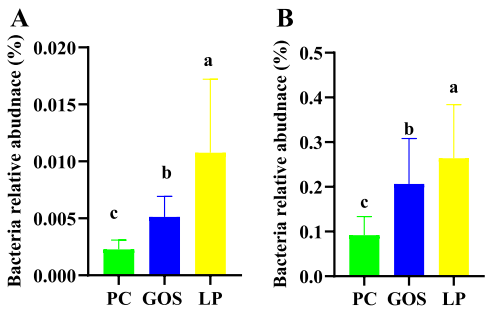

FIGURE 4 | The relative abundance of bacteria in the caecal content of in ovo-treated chickens: (A) *Lactobacillus* spp. and (B) *Bifidobacterium* spp. Error bars: ±SE. a, b letters having different superscripts differ significantly ($p < 0.05$). GOS, galactooligosaccharide; LP, *Lactiplantibacillus plantarum*; PC, positive control. [Color figure can be viewed at wileyonlinelibrary.com]

The results showed similar feed intake among the treatments, displaying no significant differences. However, GOS showed the lowest feed intake as compared to LP and PC treatments. The growth performance was not affected and this could be attributed to GOS's ability to improve nutrient utilization in these chickens (Table 4) (Slawinska et al. 2020). On the other hand, *Lactobacillus* is reported to increase the content of acetic, propionic, butyric, and total short-chain fatty acids and the increased production of these SCFAs such as butyric, therefore promoting the growth performance and nutrient digestibility in broiler chickens (Duan et al. 2021; Guo et al. 2023). This may explain the significant increase in body weight in the early life of birds (Day 7 of rearing) as compared to the other groups. Our results showed that the FCR was lower than 1.6 in all groups except the NC group. This indicates that the in ovo treatment of GOS and LP has beneficial effects on chicken growth and performance.

Furthermore, the absence of major effects of the treatment on body weight, feed intake and FCR (Table 4) can be explained by the fact that the current experiment was conducted with Ross 308 broilers which had been genetically selected for their fast growth performance. From the literature, the variable effects of bioactive substances delivered in ovo on broiler performance can be related to different factors such as type and dose of bioactive substances, environmental factors and endogenous factors related to animals and the complex interactions that occur in the gastrointestinal tract (GIT) (Tavaniello et al. 2023). However, the aim of the in ovo injection of GOS and LP is to

TABLE 7 | Caecal histomorphology of the chickens from the three in ovo treatment groups.

	Treatment groups				
Traits	**PC**	**GOS**	**LP**	**SD**	***p* value**
VH	296.31^{b}	337.93^{a}	326.1215^{a}	61.376	0.025
CD	39.38^{b}	40.20^{ab}	43.91^{a}	5.596	0.033
VW	52.59^{b}	69.48^{a}	69.96^{a}	31.62	0.047
VA	50260.61^{a}	75128.22^{b}	75349.80^{b}	47306.330	0.039
MM	149.51	120.11	148.05	34.410	0.063
VH/CD	7.75	6.80	7.44	0.716	0.084

Note: Villus height is measured in μm while villus surface area is measured in μm^2. Data are presented as mean and pooled standard deviation (SD).(SD). Values in a row with different superscript letters (a, b) indicates significant difference ($p < 0.05$).
Abbreviations: CD, crypt depth; GOS, galactooligosaccharides; LP, *Lactiplantibacillus plantarum*; MM, muscle membrane; VA, villus area; VH, villus height; VW, villus width.

maintain the health of the chickens, rather than cause an increase in performance. In conclusion, the in ovo delivery of either GOS or LP improved intestinal health and feed bioavailability, which are correlated to increased feed consumption and growth performance of broilers (Liu et al. 2023). Our findings demonstrated that GOS and LP administered in ovo increased beneficial bacterial community which could improve intestinal health while not impairing the growth performance of broilers (Figures 2–4).

4.4 | Slaughter Analysis, Carcass Traits and Meat Quality

The carcass traits and the results of the meat quality analysis (in both breast muscles and leg muscles) are presented in Tables 5 and 6. No significant effects on the dressing percentage and the other carcass traits were observed due to the in ovo treatments. However, we found that carcasses of birds from the LP group were characterized by lower ($p < 0.05$) cooling losses (Table 5) of whole carcass weight during storage (by 0.39 percentage points). The characteristics of carcasses from broilers are an important indicator for determining poultry production performance and meat quality. The beneficial effects of supplementation with synbiotics on the increase of breast muscle yield and decrease of abdominal fat with no effect on the carcass yield and leg muscle yield of broilers have been reported (Cheng et al. 2017). According to Dankowiakowska et al. (2019), carcass yield and breast muscle yield were not affected by prebiotics and synbiotics administrated in ovo. Similarly to them, in the present study, the in ovo treatment (groups GOS and LP) did not affect the carcass yield of birds (Table 5). Moreover, the weight and proportion of breast muscles, tight muscles, thigh bones, liver, heart, gizzard and abdominal fat were not affected (Table 5). Our findings are consistent with those reported by Tavaniello et al. (2020) in Ross 308 broilers and in slow-growing Hubbard chickens (Tavaniello et al. 2022). According to them, GOS did not affect carcass and breast muscle yield. In contrary to our findings, Maiorano et al. (2012) reported a reduced carcass yield and an increased pectoral muscle yield in the group in ovo treated with a commercial synbiotic.

The meat colour, pH value, water holding capacity and texture are major indicators of chicken meat quality widely used for its assessment (Połtowicz, Nowak, and Wojtysiak 2015; Tavaniello et al. 2020). The pH value is one of the most vital physical parameters of the meat. It has a central role in determining the activities of protein both in fresh and processed meat products, and thus it is used to assess meat quality (Tavaniello et al. 2019). Postmortem pH reduction results from the conversion of muscle glycogen into lactic acid, and is important because it influences meat colour, texture and water-holding capacity. In our study (Table 6), we observed a higher pH at 15 min postmortem in GOS and LP as compared to the control group. However, the ultimate pH measured at 24 h post-mortem observed in the current study did not differ between the groups and can be considered normal values for breast and leg muscles in broiler chickens. The lack of differences in pH 24 between the groups was linked with no differences in several breast and leg meat quality characteristics such as colour, water holding and texture (Table 6). Our results were consistent with those reported by Tavaniello et al. (2023).

Colour is one of the main sensory features for evaluating meat quality and is one of the main criteria used by consumers to evaluate the quality during purchasing. In our study, we did not find any significant effect of in ovo administration of either GOS or LP on the meat colour of broiler chickens (Table 6). The L^*, a^* and b^* values observed were within the acceptable range, despite the L^* value of breast muscles in the LP group (58.10) being slightly higher than that reported for the acceptable range of chicken meat colour (50–56) (Lee et al. 2022).

The water-holding capacity of meat is a very significant characteristic that can influence the quality of meat products and may cause economic losses (Tavaniello et al. 2019). It is important to note that water loss reduces meat's nutritional value because some nutrients may be lost in the exudate, resulting in less tender meat, which is worse in flavour (Cramer et al. 2018; Angwech et al. 2019). In our study, no significant effect on drip loss, thawing loss, cooking loss, as well as shear force and other texture parameters were observed (Table 5).

With an increased amount of beneficial bacteria in the chicken gut (Figures 2–4) and growth performance (Table 4) not affected, this might indicate a healthy gut, and improved metabolic activities which subsequently did not cause any adverse effects on the carcass and meat quality traits (Dankowiakowska et al. 2019; Duan et al. 2021).

4.5 | Relative Bacterial Abundance in Faecal Samples

In this study, we observed a significant increase in the relative abundance of *Bifidobacterium* in the group in ovo treated with GOS and LP on Day 35 of adult chickens ($p < 0.001$) and not on Days 7 and 21 (Figure 2). In addition, the relative abundance of *Lactobacillus* spp. was significantly increased on Days 7, 21, and 35 in the GOS and LP compared to the control group (Figure 3). In terms of the relative abundance of *Bifidobacterium* spp., a significant effect was found in the GOS group as compared to the PC and LP groups. The in ovo administration of GOS during embryonic development increased the relative abundance of *Bifidobacterium* spp. in the caecum and decreased the relative abundance of *Lactobacillus* spp. in the ileum (Slawinska, Dunislawska, et al. 2019). The competitive exclusion of *Lactobacillus* spp. can be attributed to the *bifidogenic* effect of GOS prebiotic (Slawinska, Dunislawska, et al. 2019). For this reason, GOS promotes the growth of *Bifidobacterium* spp. (Slawinska, Dunislawska, et al. 2019). As a result of the complex carbohydrate structure of GOS, it passes the upper GIT without degradation (Slawinska, Dunislawska, et al. 2019). The genome of *Bifidobacterium* spp. contains carbohydrate-degrading enzymes with high affinity to GOS (Slawinska, Dunislawska, et al. 2019). In the study of Jung et al. (2008), GOS supplementation increased the abundance of *Bifidobacteria* and *Lactobacilli* in animal faeces (Slawinska, Dunislawska, et al. 2019). *Lactobacillus* spp. are usually considered beneficial to the host organism, mainly because they produce lactic and acetic acids, which leads to reduced pH and inhibition of pathogen bacteria (Dunislawska et al. 2017). The prevalence of *Lactobacillus* spp. and *Bifidobacterium* spp. in the GOS and LP may be explained due to the increased *Lactobacillus* spp. and thus lead to butyrate-production and fibrolytic species, which have significant effects on

chicken intestinal health. Yet another study demonstrated that in ovo injection of LP significantly increased the relative abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. as compared to the control group (Duan et al. 2021).

4.6 | Relative Bacterial Abundance in Caecal Content

The caecum is one of the most vital intestinal organs in chickens and hence it is actively involved in regulating immunologic health functions and metabolic activities and therefore increasing nutrient digestion and absorption while maintaining energy balance (Liu et al. 2023). According to our result (Figure 4), we observed a significant difference in the relative abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. (Figure 4A,B). Our results showed that both *Lactobacillus* spp. (Figure 4A) and *Bifidobacterium* spp (Figure 4B) were significantly higher in the LP ($p < 0.05$) as compared to GOS and PC groups (Figure 4A,B). These results validate that the in ovo supplementation of either LP or GOS increases beneficial bacteria (*Lactobacillus* spp. and *Bifidobacterium* spp.) in chicken GIT leading to early gut colonization and subsequently inhibiting pathogens and other harmful bacteria. This finding is consistent with that of Liu et al. (2023). The in ovo delivery of GOS and *Lactobacillus* spp. increased the relative abundance of *Bifidobacterium* and *Lactobacillus* spp. in the caecum of chickens respectively (Dunislawska et al. 2017; Slawinska, Dunislawska, et al. 2019). Similarly, Yang et al. (2022) reported a significant increase in the relative abundance of *Lactobacillus and Bifidobacterium* in the caecum of chicken when in ovo injected with GOSs. Therefore, our study validated that the in ovo delivery of LP and GOS enhances early gut colonization by beneficial bacteria and consequently improves chicken health and performance by excluding the growth of harmful bacteria.

4.7 | Caecal Histomorphology Analysis

The caecum is the primary site of fermentation in chickens, hosting the highest concentration and activity of anaerobic bacteria (Dunisławska et al. 2023). The administration of a synbiotic at an early stage of embryonic development influenced the growth of *Clostridium* bacteria, which in turn significantly affected intestinal health (Dunislawska et al. 2017). In animal nutrition, LAB are considered beneficial to the host as they lower the pH by producing lactic and acetic acids. The in ovo administration of probiotics, prebiotics and synbiotics stabilizes the microbial community in the GIT of chickens. In adult chickens, the caecum is the site of the GIT which is considered to have the highest number of microorganisms, and its effect on health and performance has been demonstrated by Dunislawska et al. (2017, 2023). Intestinal morphological parameters, including the villus height, villus width, crypt depth and villus length-to-crypt depth ratio are good indicators of gut health and the functional capacity of the intestine (Oladokun, Dridi, and Adewole 2023). The increased villus height, villus width villus height to crypt depth ratio, and decreased crypt depth are associated with an increased epithelial turnover and improved digestive and absorptive functions (Munyaka et al. 2012). In our study, the ceca of ROSS 308 broiler chicken (Table 7) were analysed at the end of the rearing period (35 days). As shown in Table 7, our study demonstrated that in ovo administration of GOS and LP exerted positive effects ($p < 0.05$) on the villus height, villus width villus surface area and crypt depth of chicken caecum as compared to the PC group. According to Sobolewska et al. (2017) longer villi and their increased villus surface area indicate increased feed absorption, hence improving chicken health. Crypts are typically viewed as the production sites for the cells that make up the villi. The depth and size of these crypts indicate the rate of cell renewal and proliferation (Sobolewska et al. 2017; Wishna-Kadawarage et al. 2024). Therefore, a higher crypt depth on the in ovo injected groups (GOS and LP) as compared to the PC groups (Table 7) demonstrates an increased renewal of tissues. This reveals that GOS and LP enhanced the development of the mucosal tissue in the ceca to possibly ensure an increase in mucin production and consequently inhibit pathogen invasion and substrates for SCFA production (Wishna-Kadawarage et al. 2024). Our study (Table 7) revealed no significant effect on the muscle membrane and villus height-to-crypt depth ratio across all groups. However, the height-to-crypt depth ratio is within the normal range. Therefore indicating a relatively balanced state of cell proliferation and renewal in the caecal mucosa, which is important for nutrient absorption and gut barrier function. Regarding the muscle membrane thickness, it is considered an indicator of the structural integrity and contractility of the caecal wall (Wiersema et al. 2021).

5 | Conclusions

Our study demonstrated that the in ovo injection of GOS 3.5 mg/egg and LP 1×10^6 CFU improves chick quality, caecal histological parameters (villi height, villi width and crypt depth) without negatively affecting hatchability, body weight gain, FCR, meat quality and carcass traits. In addition, the in ovo injection of GOS and LP significantly increased the relative abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. in the faeces on Days 7 and 21, and more pronounced on Day 35 and caecal content on Day 35 of the in ovo-treated chickens, thus ensuring a healthy gut. Furthermore, GOS 3.5 mg/egg and LP 1×10^6 CFU exerted positive effects on cooling losses with no effect on other carcass traits and meat quality and significantly improved gut health of chickens and body weight gain in the early life of chickens. From our results, we recommend further research to be studied in other to improve the caecal histological parameters and body weight of chickens in their late growth and developmental stages (market age) without negatively affecting their health.

Author Contributions

M. Mangan: conceptualization, methodology, formal analysis, data curation, investigation, visualization, software, writing–original draft. **M. Siwek:** conceptualization, methodology, investigation, funding acquisition, administration, supervision, writing–review and editing. **K. Połtowicz:** conceptualization, methodology, validation, resources, investigation, writing–review and editing. **P. Reszka:** methodology, formal analysis, investigation, validation, data curation, software, writing–review and editing. All authors read and approve the final manuscript for submission and publication.

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Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data collected from this study will become available upon reasonable request.

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