Annex No. 3 to
Instructions for printing, collecting, registering and making available doctoral dissertations by scientific councils of disciplines (artistic disciplines) conducting proceedings for the award of a doctoral degree

5. POLISH ABSTRACT

Wpływ wybranych substancji bioaktywnych dostarczanych *in ovo* na zdrowie jelit i wyniki produkcyjne kurcząt brojlerów.

Mgr. Modou Mangan

Słowa kluczowe: Przeciwutleniacz, Ekspresja genów, Mikrobiota jelitowa *In ovo*, Prebiotyk, Probiotyk

W okresie okołowylęgowym kurczęta brojlery są narażone na działanie różnorodnych drobnoustrojów, które mogą znajdować się na powierzchni skorupy jaja lub w jego bezpośrednim otoczeniu. Obecność tych mikroorganizmów może zakłócać równowagę mikrobiologiczną oraz negatywnie wpływać na zdrowie jelit i ogólną wydajność produkcji drobiu. W odpowiedzi na te wyzwania, zastosowanie związków bioaktywnych metodą in ovo w 12. dniu rozwoju embrionalnego może wspomóc kolonizację przewodu pokarmowego korzystną mikroflorą bakteryjną, łagodząc jednocześnie wpływ niekorzystnych czynników środowiskowych.

Celem niniejszej pracy doktorskiej była ocena wpływu stymulacji *in ovo* galaktooligosacharydem (3,5 mg/jajo) oraz *Lactiplantibacillus plantarum* (1×10^6/jajo) na zdrowie jelit kurcząt brojlerów. W ramach badań analizowano również względną liczebność bakterii, histomorfologię jelita ślepego, ekspresję genów związanych z układem immunologicznym i aktywnością przeciwutleniającą, a także różnorodne metabolity osocza oraz wskaźniki wydajności produkcji.

Na potrzeby badań przeprowadzono test *in vitro*, który umożliwił ocenę kinetyki wzrostu zastosowanych związków bioaktywnych oraz ich potencjału przeciwutleniającego. Test antyoksydacyjny oparty na metodzie 2,2-difenylo-1-pikrylohydrazylu (DPPH) wykazał wysoką aktywność neutralizacji wolnych rodników przez *Lactiplantibacillus plantarum* (68,89%), co wskazuje na ich skuteczność w łagodzeniu stresu oksydacyjnego u kurcząt. Przeprowadzono także badania *in vivo*, aby potwierdzić wpływ galaktooligosacharydu (3,5 mg/jajo) oraz *Lactiplantibacillus plantarum* (1×10^6/jajo) na kluczowe wskaźniki zdrowotne i produkcyjne.

Uzyskane wyniki wykazały, że zastosowany probiotyk i prebiotyk wspomagały wczesną kolonizację jelit przez pożyteczne bakterie, takie jak *Lactobacillus spp*. i *Bifidobacteria spp*., co korzystnie wpłynęło na histomorfologię jelita ślepego, aktywność przeciwutleniaczy oraz ekspresję genów związanych z układem

the highest antioxidant potential (68.89%). Galactooligosaccharide 3.5mg/egg (selected based on previous studies from our groups due to its ability to mitigate heat stress and promote growth performance) and *Lactiplantibacillus plantarum* 1 x 10^6/egg led to early gut colonization by commensal bacteria (*Lactobacillus spp.* and *Bifidobacteria spp*.) in chickens thus conferring positive effects on cecal histomorphology, antioxidant activities, upregulation of immune-related genes suggesting a stable and healthy gut. Moreover, performance parameters together with the selected plasma metabolites were not impaired. In a nutshell, the *in ovo* stimulation of galactooligosaccharide 3.5mg/egg and 1 × 10^6 *Lactiplantibacillus plantarum*/egg can be used in poultry production to improve gut health, performance and overall welfare of broiler chickens.

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4. ENGLISH ABSTRACT

Impact of the selected bioactive substances delivered *in ovo* on gut health and production performance of broiler chickens.

Modou Mangan, MSc.

Keywords: Antioxidant, Gene expression, Gut microbiota, *In ovo*, Prebiotic, Probiotic

During the perinatal period, embryos are exposed to various microbes coming from the eggshells and their immediate environments and this could microbial imbalance and affect the gut health and production performance. Thus, the *in ovo* injection of bioactive compounds on day 12 of embryonic development could mitigate these negative factors by colonizing the gut microbiota with beneficial bacteria. Therefore, this PhD dissertation was performed to evaluate the efficiency of *in ovo* stimulation of galactooligosaccharide 3.5mg/egg and *Lactiplantibacillus plantarum* 1 x 10^6 on gut health, relative bacterial abundance, cecal histomorphology, gene expression of immune-related genes and antioxidant activities, and various plasma metabolites and production performance metrics. The *in vitro* study was performed to assess the kinetic growth of the bioactive compounds and select the ones with the best growth potentials for antioxidant assay. The 2,2-diphenyl-1-picrylhydrazyl in vitro assay was used to screen the bioactive compounds that demonstrated high free radical scavenging activities which is effective for evaluating bioactive substance antioxidant potential that can alleviate oxidative stress in chickens. Upon the *in vitro* study, an *in ovo* stimulation of the selected bioactive compounds (galactooligosaccharide and *Lactiplantibacillus plantarum*) was performed and an animal trial (*in vivo* study) to validate the impact and influence of the treatments on several key parameters related to chicken gut health and performance, chicken gut microbiome by analysis of the relative abundance of bacteria in feces and cecal content. Additionally, gene expression associated with the immune system and antioxidant activities was conducted on a range of tissues (cecal mucosa, spleen, breast muscle and liver), cecal histomorphology, production performance metrics (hatching rate, hatchling quality, body weight, feed efficiency, feed conversion ratio, meat quality and carcass traits). The results demonstrated that the selected probiotics exhibited good growth. Regarding the antioxidant assay, *Lactiplantibacillus plantarum* 1 x 10^6 exhibited

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BW than the control groups. The feed intake, FCR, meat quality and carcass traits were not impaired upon *in ovo* delivery of LP and GOS.

- The *in ovo* stimulation of LP and GOS showed a high abundance of *Lactobacillus spp.* in the excreta of chickens at different life stages (days 7, 21 and 35), with the highest amount of commensal bacteria (*Lactobacillus spp.)* prevailing in the GOS treatment group on day 35. In addition, upon *in ovo* stimulation, the presence of *Bifidobacterium spp*. was highest on day 35 in both of the treatment groups.
- Interestingly, both treatments (LP and GOS) increased the *Lactobacillus spp.* and *Bifidobacterium spp.* population in bird's cecal content.
- The cecal histomorphology study demonstrated that both GOS and LP positively influenced the measured parameters (villus width, villus height and crypt depth).
- The gene expression analysis performed in the cecal mucosa significantly increased the expression of numerous genes related to immune functions (*MUC6*, *AVBD1*, *IL-1β* and *CATHL2* while only LP upregulated both *FFAR2 and CLDN1*.
- Moreover, LP and GOS elevated the expression levels of both *IL4 and SOD1* in the chicken spleen *IL8* and *IL12p40* upregulated only in the GOS *in ovo*-treated chickens.
- Additionally, the *in ovo* stimulation of GOS and LP remarkably increased the expression of *SOD1* and *CAT* in chicken breast muscle with no changes in HO-1 and ZO-1 expression. Furthermore, both treatment groups upregulated both genes (*GPx*1 and *NRF2*) in chicken's liver.

From the findings obtained from this PhD project, the dissertation concludes that the *in ovo* stimulation of LP 1 x 10^6 and GOS 3.5mg/egg on 12 days of embryonic development modulated the gut microbiota from the perinatal period and throughout the chicken's life, improve the immune functions, cecal histomorphology parameters without impairing production parameters. In a nutshell, this suggests that both GOS and LP can play a significant role in improving the performance, immune system, and intestinal health of birds thus rendering more research using more advanced sequencing techniques needs to be carried out to have a better comprehension of the biological mechanism involve.

3.6 Summary

This PhD work was carried out to test the effects of the selected bioactive compounds (galactooligosaccharide and *Lactiplantibacillus plantarum*) on early gut colonization thereby improving production parameters, antioxidant status, intestinal health, and immunological responses of chickens upon *in ovo* stimulation on ED 12. The first step of this PhD project involved an *in vitro* study in which several bioactive compounds were tested based on their growth and antioxidant potentials (free radical scavenging abilities). Afterward, the probiotics that grew the best with the highest radical scavenging ability were selected for an *in vivo* study to validate their ability to improve chicken gut health, immune function, antioxidant status and productivity.

The primary findings of the aforementioned experiments are as follows:

The following Probiotics (*L. reuteri, L. casei, L. rhamnosus, and L. plantarum*) with different concentrations were tested:

- The DPPH assay showed that both *Lacticaseibacillus casei* 1.4 x10^6*,* *Lactiplantibacillus plantarum* 1.0 x10^6, and *Limosilactobacillus reuteri* 7.9 x 10^6 exhibiting high antioxidant potentials with *Lactiplantibacillus plantarum* 1.0 x10^6 demonstrating the highest free radical scavenging activity indicating its antioxidant potentials (**68.89%)** and ultimately enhancing the antioxidant defense mechanism.
- The probiotic *Limosilactobacillus reuteri* 1.9 x 10^6 and *Lacticaseibacillus rhamnosus* 2.7 x 10^7 exhibited relatively poor antioxidants (**20%** and **17.90%)** respectively. This suggests that their ability to mitigate heat or oxidative stress is low, therefore were not selected for further studies.
- The *in vivo* validation of the *in vitro* study performed showed that galactooligosaccharide 3.5mg/egg and *Lactiplantibacillus plantarum* 1 x10^6/egg injected *in ovo* had no adverse impacts on hatchability and chick quality. However, the BW of the newly hatched chicks was higher in GOS and LP, this trend continued in the bird's first week of life in chickens *in ovo*-treated with LP. Additionally, by the end of the trial phase, both treatment groups had numerically higher

3.5.6. Shortcomings of the protocol

Despite the numerous potential benefits reported in this PhD project, a few shortcomings might hinder its full realization. One of these might be its practice and adaptation in the commercial poultry sector. Other factors might be the preparation and handling of these bioactive substances, dose use and injection method, breed use, flock age and management practice. Furthermore, it is essential to use more advanced sequencing techniques to unravel and better understand the biological mechanism of these bioactive substances, and the functionality of the immune system and get a whole picture of all the bacteria communities present. Nonetheless, this PhD project highlights the potential benefits of the *in ovo* injection of LP and GOS to improve the production performance, immune functions and health of chicken and could be a basis for future research.

3.5.5. *In ovo* stimulation – indirect impact on production parameters

Taking into account all the positive outcomes realized in this PhD project, it is noteworthy to highlight that to ensure a successful poultry production cycle, high hatchability, good and healthy high-performing chicks are essential (Bednarczyk et al., 2016; Bilalissi et al., 2019; Slawinska et al., 2020; Duan et al., 2021; Wishna-Kadawarage et al., 2024). In this PhD project, the *in-ovo* injection of LP and GOS significantly increased the weight of one-day-old chicks without impairing hatchability. This could be attributed to the capability of GOS and LP to colonize the chicken gut microbiome and promote the development of the immune system and nutrient intake (Gao et al., 2024; Mangan et al., 2024b). With regards to chick length and quality (Pasgar score), there were no statistical differences. Therefore, the results from this PhD dissertation showed that the *in ovo* stimulation of either LP or GOS enhances chick quality parameters without negatively impacting hatchability and thus could have a long-lasting beneficial impact on chickens' growth performance and health.

From a general standpoint, the results reported in this dissertation highlight the potential benefits of the *in ovo* stimulation of LP and GOS in modulating the chicken gut microbiome and subsequently promoting the development of chicken’s immune system, gut health, and upregulation of immune and antioxidant-related genes. Interestingly, no significant changes were observed in the production performance (BW, FI, FCR, meat quality and carcass traits analysis) of chickens. However, no adverse effects were found on these parameters. This may be explained due to the similar housing conditions of the chickens, nutrition and most importantly, genetic factors, Ross 308 broiler chickens are selected due to their growth potential and efficiency.

Besides the positive impacts of the *in ovo* stimulation of LP and GOS presented in this PhD thesis, several approaches could be used to ameliorate heat and oxidative stress. As noted, heat stress adversely affects chicken's health and production performance while increasing the incidence of pathogen infections (Mangan and Siwek, 2024). Therefore, this PhD dissertation publication series, entails a systematic literature review that highlighted and suggested several potential strategies (proper housing design, good management practices, genetic selection, nutritional strategies and early-life heat conditioning to alleviate the harmful effects of high ambient temperatures in broiler chickens (Mangan and Siwek, 2024).

integrity (Kawabe et al. 2001). Additionally, *CATHL2* was upregulated by LP and therefore promoted gut barrier functions and modulated the immune system's inflammatory responses (Mangan et al., 2024a). Furthermore, the expression of *FFAR2* was also increased upon *in ovo* stimulation of GOS. Therefore, suggesting that *FFAR2* also influences and promotes metabolic functions and the recruitment of immune cells in chickens and eventually modulates chicken's gut microbiome (Corrêa-Oliveira et al., 2016; Slawinska et al., 2019; Schlatterer et al., 2021).

The results from the splenic tissue showed a significant upregulation of *IL12p40* and *IL4* and *IL8* while the expression levels of *CATHL2* and *IL2* genes remained unaffected. These immune-related genes (*IL12p40* and *IL4* and *IL8*) were highly expressed suggesting that they were activated and thus improved the health and immune functioning of the chickens. Although *IL8* expression is primarily known to occur in response to infection, it is also known to participate in regular immune system modulation, homeostasis and recruitment of heterophils to the spleen Yu et al., 2020; Pietrzak et al., 2020; Elnagar et al., 2021). Furthermore, the transcriptomic analysis in the liver of birds showed that both *IL1-β* and *Occludin* were upregulated upon *in ovo* injection of LP while GOS did not lead to any major changes.

In addition to the above findings in this PhD project, further transcriptomic analysis on the breast muscle and liver was performed to determine the presence of antioxidants in the same experimental groups. The results revealed that the *in ovo* injection of probiotic LP and prebiotic GOS both led to high expression of *SOD*, *NRF2, CAT* and *GPx1* in chicken's breast muscle and liver while *MnSOD* was upregulated in the GOS treatment group. These antioxidants serve as the major defense mechanism of chickens against oxidative stress; hence they regulate the oxidant/antioxidant balance by breaking down superoxide radicals to hydrogen peroxide (Surai et al., 2019; Karaca et al., 2022). In reference to the objectives of this dissertation, the *in ovo* supplementation of either LP or GOS elevated the antioxidant capacity of the chickens (Mangan et al 2024) suggesting that oxidative stress was ameliorated.

3.5.4. *In ovo* stimulation – indirect impact on blood parameters

Considering the results of the transcriptomic analysis, (gene expression), several plasma metabolites were also measured to gain more insight into the health and physiological status of the chickens. The blood biochemical analysis (PCA analysis) revealed no significant changes in most of the parameters measured suggesting that the physiology and status of the chickens were not compromised. Interestingly, the results displayed a higher LDL in the chickens treated with LP than those treated with PC and GOS. The increased levels of LDL could be attributed to the ability of LP to initiate compensatory mechanisms in lipid metabolism thus temporarily elevating lipid production (Trapani et al., 2012).

potential benefits of *in ovo* stimulation of GOS and LP by improving intestinal health, immune functions and performance (Liu et al., 2023; Mangan et al., 2024b) thus meeting the expectations of the main objectives of this PhD project.

3.5.2. *In ovo* stimulation – indirect impact on host gut histology

Besides the bacterial relative abundance, histomorphology analysis was performed on the ceca of chicken. The crypt depth, villus width, villus height and villus height-to-crypt depth ratio are important markers of the functional ability of chicken's intestine and gut health (Oladokun et al., 2023). The results obtained from the histology analysis demonstrated that LP and GOS administered *in ovo* had positive effects on villus width, villus height and crypt depth without any adverse effects on muscle membrane and villus surface area compared to the control group. The crypt depth is the main site of cell production and therefore participates in the renewal of cells (Sobolewska et al., 2017). In addition, GOS and LP positively influence the overall cecal histomorphology and therefore improve gut barrier function, the immune functioning of birds, and epithelial cell wall integrity via increased cell renewal and eventually decrease disease infection (Wiersema et al., 2021). These findings are in agreement with those of (Slawinska et al., 2019, 2020) who showed that the *in ovo* injection of GOS on day 12 of ED increase relative bacterial abundance in chicken's gut microbiota, promote immune and gut barrier functions and production performance metrics.

3.5.3. *In ovo* stimulation – indirect impact on host transcriptome

Furthermore, the transcriptomic analysis (mRNA gene expression) was performed and the immunomodulatory impacts of LP and GOS injected *in ovo* on the cecal tonsil, liver and spleen of the same chickens were investigated (Mangan et al., 2024a). The *in ovo* stimulation of LP and GOS caused a remarkable increase of *MUC6, AVBD1, IL1-β*, and *CATHL2* in chicken's cecal mucosa. The *MUC6* gene is essential for the synthesis and secretion of mucin thus improving gut barrier integrity and reducing pathogen infections (Forder et al., 2012); while the *AVBD1* is responsible for the secretion of avian β-defensin1 and therefore contributes a major role in the exclusion of pathogens in chickens (Zhang and Sunkara, 2014; Lyu et al., 2020). Despite the upregulation of *AVBD1* being a common feature during infection, SCFAs such as butyrate and acetate could affect and stimulate defensin production in epithelial cells without inducing gut dysbiosis or inflammation (Chen et al., 2020; Wishna-Kadawarage et al., 2024). *IL1-β* is crucial in proinflammatory cytokine production, inhibition of infectious diseases and eventually promote a healthy gut in chickens (Khosravi and Mazmanian, 2013; Slawinska et al., 2019). The *in ovo* administration of LP upregulated *CLDN1* which also plays a key role in maintaining the epithelial cell

Reflecting on the hypothesis and objectives of this PhD dissertation the *in ovo* stimulation of the selected bioactive substances will modulate the gut microbiota and subsequently improve gut health, and production performance while mitigating oxidative stress, the antioxidant properties and the efficacy of the selected bioactive substances were evaluated. The results of the antioxidant experiment (DPPH assay) suggest that *Lactiplantibacillus plantarum* possesses high antioxidant potential and could mitigate oxidative stress and improve chicken gut health and performance. The results of the relative bacterial abundance in chicken feces demonstrated the efficacy of *in ovo* delivery of LP and GOS on day 12 of egg incubation which was confirmed by the increase in *Lactobacillus* spp. throughout the rearing period (days 7, 21 and 35). Moreover, the presence of *Bifidobacteria* spp. increased remarkably in both GOS and LP in 5 weeks old chickens. Interestingly, the *in ovo* stimulation of LP and GOS further orchestrated a major increase in the relative abundance of both *Bifidobacterium* spp. and *Lactobacillus* spp. in the ceca of 5 weeks chickens. Similar findings demonstrated by (Dunislawska et al. 2017) that the supplementation of synbiotics (raffinose with (*Lactobacillus plantarum)* and galactooligosaccharides with *Lactobacillus salivarius*) increased the relative abundance of beneficial bacteria in the ileum of chickens while decreasing the *Bacteroides-Prevotella*, the *Eubacterium rectale* clusters, *Lactobacillus* spp. and *Clostridium leptum*, These bacteria produce butyric acid and could impact chicken intestinal health (Dunislawska et al., 2017).

Additionally, (Yang et al., 2024) claimed that *Lactobacillus plantarum* significantly increases the presence of commensal bacteria and *Ruminococcus* and *Lachnospiraceae* thereby improving the growth and health of broilers, and this may be explained due to the presence of short-chain fatty acid-producing bacteria and modulation of the chicken's gut microbiome. As claimed by (Duan et al., 2021), the *in ovo* injection of *Lactobacillus plantarum* with 2 mg/egg Astragalus polysaccharide and 1 x 10^6 CFU/egg *Lactobacillus plantarum* and 1 × 10^6 CFU/egg reduced Escherichia coli and increased the prevalence of Bifidobacterium and *Lactobacillus* thus colonizing the chicken cecum. Similarly, *Lactobacillus plantarum* PA01 increased the presence of *Lactobacillus*, *Firmicutes* and reduced the relative abundance of Salmonella, *Proteobacteria*, *Bacteroidota*, and *Actinobacteriota* in chicken's ceca. (Guo et al., 2023). The prebiotic (GOS) increased the abundance of *Bifidobacteria* in the chicken's caecum. Furthermore, the *in ovo* stimulation of GOS on day 12 of ED remarkably increased the *Bifidobacteria* spp. population in chicken's caecum while reducing the prevalence of *Lactobacillu*s spp. in chicken's ileum. This may be explained as a result of the bifidogenic effects of GOS leading to the so-called competitive exclusion of *Lactobacillus* spp. (Slawinska et al., 2019). Therefore, taking into account the significant increase of *Lactobacillus* spp. and *Bifidobacterium* spp. in the feces and ceca of chickens, this may explain the

subsequently improving the intestinal gut barrier and tight junctions while excluding pathogens (Slawinska et al., 2019). In addition, these bioactive substances modulate the gut microbiota and enhance embryonic development, hatching rate, quality of chicks, physiology, health, production performance and general welfare of birds which may subsequently translate to economic profit for the poultry industry (Mangan et al., 2024b).

3.5.1. *In ovo* stimulation – direct impact on bacteria abundance

It is reported that an appropriate *in ovo* stimulation of probiotics, prebiotics and synbiotics into the egg's air chamber on day 12 of ED stimulates and increases the presence of commensal bacteria in the gut microbiome of chickens therefore inhibiting harmful bacteria without impairing hatchability and chick quality and influence the health and future performance of chickens while reducing perinatal stresses (Siwek et al., 2018; Slawinska et al., 2020). The *in ovo* technology has been demonstrated to modulate the gut microbiota, improve the production performance (BW, FI, meat quality, carcass traits) and health conditions of birds (Tavaniello et al., 2023) without negatively impacting hatching parameters and chick quality (Akosile et al., 2023). In addition, the *in ovo* technology implores a strategy (*in ovo* feeding) that involves the *in ovo* delivery of bioactive compounds on day 17/18 of ED to ensure and facilitate chicks' adaptation to different nutrients (carbohydrates, proteins fats) after hatch which may subsequently increase enterocytes, improve the gut morphology, growth and development of chickens (Siwek et al., 2018; Duan et al., 2021). The probiotic (*Lactiplantibacillus plantarum*) selected in this PhD project is well known for its gut microbiota modulation, antibacterial and antipathogenic effects, improved immune function, increased nutrient absorption and environmental stress resistance (Arena et al., 2016; Behera et al., 2018) and antioxidant properties (Kachouri et al., 2015). Furthermore, the probiotic used in this study is commercially available and is supplemented in a poultry diet, thus it is reported to be safe and effective (Smialek et al., 2018). To my knowledge, this is the first study that has reported the use of this probiotic for *in ovo* administration on day 12 of egg incubation (Mangan et al., 2024b). Therefore, this makes the selected probiotic an excellent candidate for achieving the goals of this project.

The prebiotic (galactooligosaccharide) can selectively stimulate and promote the presence of commensal bacteria like *Lactobacillus* spp. and *Bifidobacteria* spp. in the gut microbiome of chickens (Slawinska et al., 2019). Moreover, this prebiotic has been proven to enhance immune functions, mitigate heat stress, and improve intestinal health, production performance and the general welfare of birds (Bertocchi et al., 2019; Slawinska et al., 2020).

3.5. Discussion

Over the years there has been a significant stride in the expansion of the poultry industry to meet the demand for food supply across the globe. However, despite the immense development in the poultry sector, this comes with numerous challenges that could negatively affect poultry health, and growth performance and subsequently lead to economic losses. Some of these challenges include disease infection, heat stress, and the ban of antibiotics without suitable substitutes. To curb this menace in the poultry production cycle, the poultry sector has adopted several intervention strategies such as genetic selection, robust biosecurity, good and proper housing designs, feeding strategies and nutritional management. Despite all these efforts, production performance and the health of chickens remain a major concern due to the prevalence of diseases, oxidative stress and other stressors such as heat stress.

To address this problem, a promising strategy (*in ovo* technology) allows the successful *in ovo* injection of bioactive compounds during egg incubation which could eventually colonize the gut by beneficial bacteria and improve a healthy gut, immune system development and overall growth performance of broiler chickens. Despite its numerous advantages, an optimized protocol (procedure for selecting the types of bioactive substances, dosage, time of injection and method of injection) is essential for its successful application. An appropriate *in ovo* procedure is crucial for overcoming challenges such as pathogen infection, nutrient deficiency, heat and oxidative stress. For instance, appropriate doses of bioactive substances injected *in ovo* ensure early gut colonization embryonic development, improve gut health and subsequently reinforce gut integrity and immune defense mechanisms. In chickens, the gut microbiota harbors various microorganisms, and these microbes could either be beneficial or harmful to the host and therefore have major effects on nutrition absorption, metabolism, immune function and gut health of chickens. Numerous factors such as environmental stressors, toxic substances, nutrient deficiencies and disease infection can disrupt the gut microbiota leading to leaky gut, inflammation, metabolic disorders and infections (Shehata et al., 2022). Identifying this gap and the problem faced by the poultry industry caused by poor gut health, disease infection, and reduction in production performances warranted this PhD project. Taking this into account, this PhD project aimed to select bioactive substances that when injected *in ovo* could address the above-mentioned problems. Recently, the supplementation of probiotics, prebiotics and synbiotics has been reported to prevent gut dysbiosis and disease infection thus improving chicken gut health and productivity. These bioactive substances cause dynamic changes in the gut microbiome by increasing the presence of beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacteria* spp. (Dunislawska et al., 2017; Slawinska et al., 2019) and

3.4.2.11. Analysis of plasma blood metabolite

The *in ovo* stimulation of LP and GOS had no major impact on most of the plasma metabolites measured. The results of the Principal Component Analysis (PCA), showed no statistical differences across the treatments suggesting no negative impact on chicken metabolism. Additionally, the PCA indicates no clear separation of the treatment groups (samples dot plot; Fig. 10A and B). Furthermore, except for GGT, cholesterol, glucose and HDL, the PCA analysis demonstrates that the majority of the parameters clustered together thus indicating their positive correlation. In summary, no statistical changes were found across the treatments

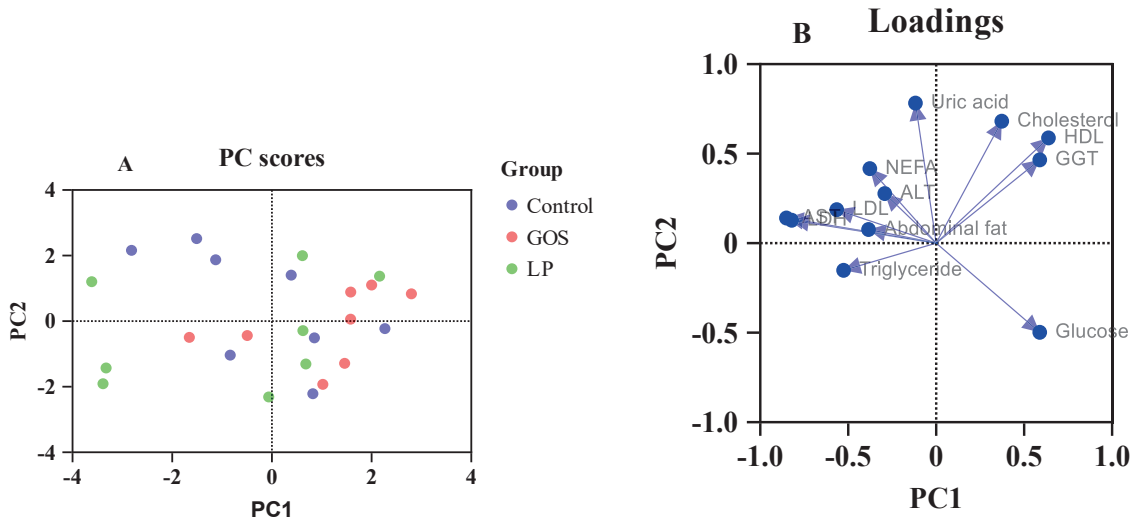

Figure 10: Shows the PC score (**A**) and variables/plasma metabolites (**B**) upon Principal component analysis (PCA). The variables are the parameters measured while the PC scores represent each sample per treatment. Blue: (C) control, Green: (LP) *Lactiplantibacillus plantarum*, and Orange: (GOS) galactooligosaccharide.

3.4.2.10. Relative gene expression in chicken's liver

The gene expression levels of *IL1β* and *Occludin* were highly expressed ($P < 0.05$) in the LP group and not in the GOS treatment group (Figure 9C and D). Interestingly, both GOS and LP demonstrated a significant upregulation of *GPx1* and *NRF2* in the breast muscles of chickens (Figure 9A and B). Surprisingly, no significant changes were observed in the expression levels of *HO-1* or *FFAR4* in the breast muscle of chickens in all the treatment groups.

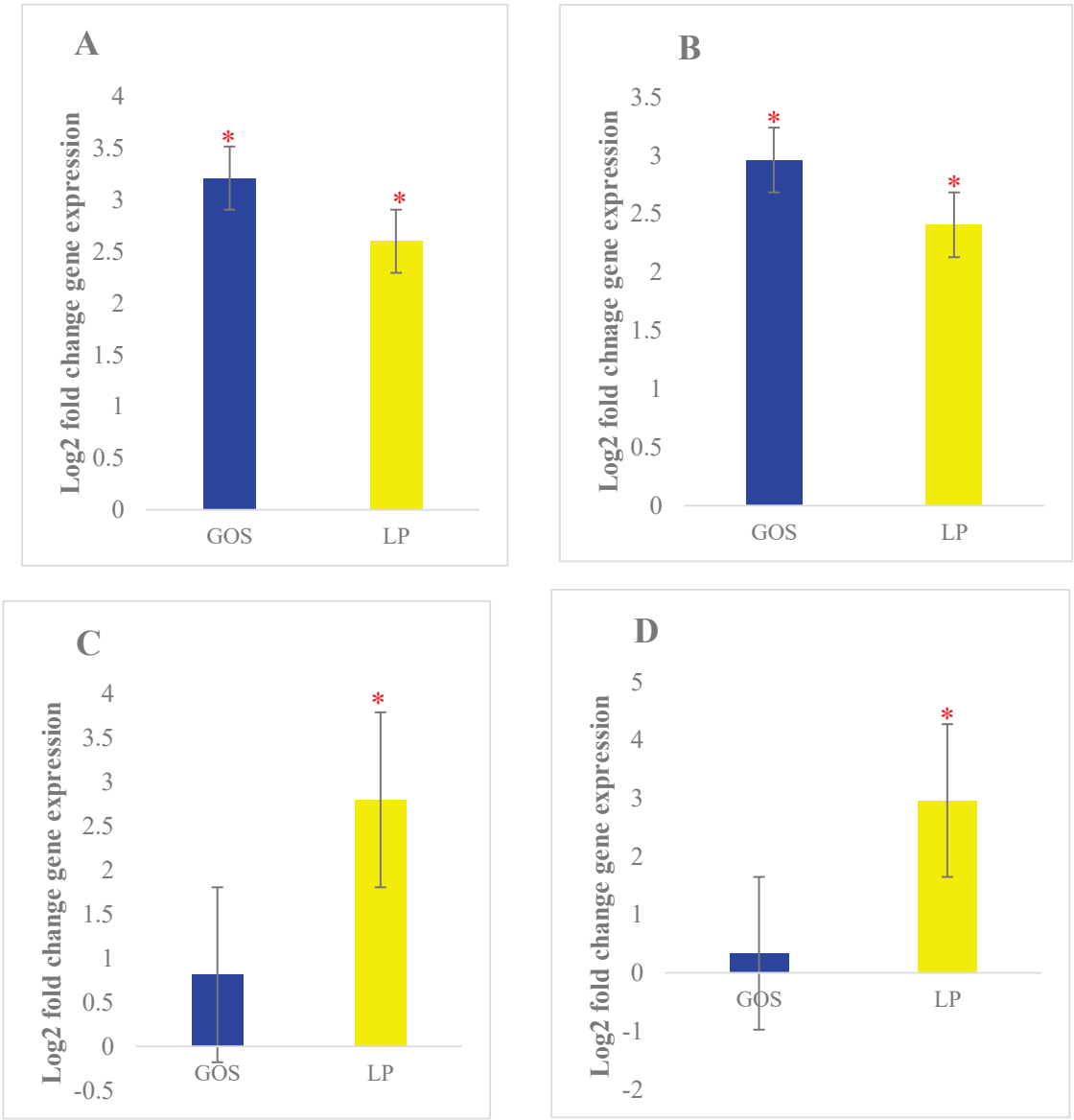

Figure 9: The gene expression patterns in chicken's liver upon *in ovo* administration of *Lactiplantibacillus plantarum* (LP) or galactooligosaccharide (GOS). (A) *GPx1*, (B) *NRF2*, (C) *IL1β*, and (D) *Occludin*. Error bars represent ± SE. Red asterisks (*) denote statistical differences ($P < 0.05$).

3.4.2.9. Gene expression in chicken breast muscle

The increased expression levels of *SOD1* and *CAT* indicate a significant statistical difference in chicken breast muscles upon *in ovo* delivery of LP or GOS (Figures 8A and D). Surprisingly, *MnSOD* and *NRF2* were upregulated only in the GOS *in ovo*-treated chickens (Figure 8B and C). However, the expression levels of *HO-1* and *ZO-1* in chicken breast muscle were not affected in all the experimental groups.

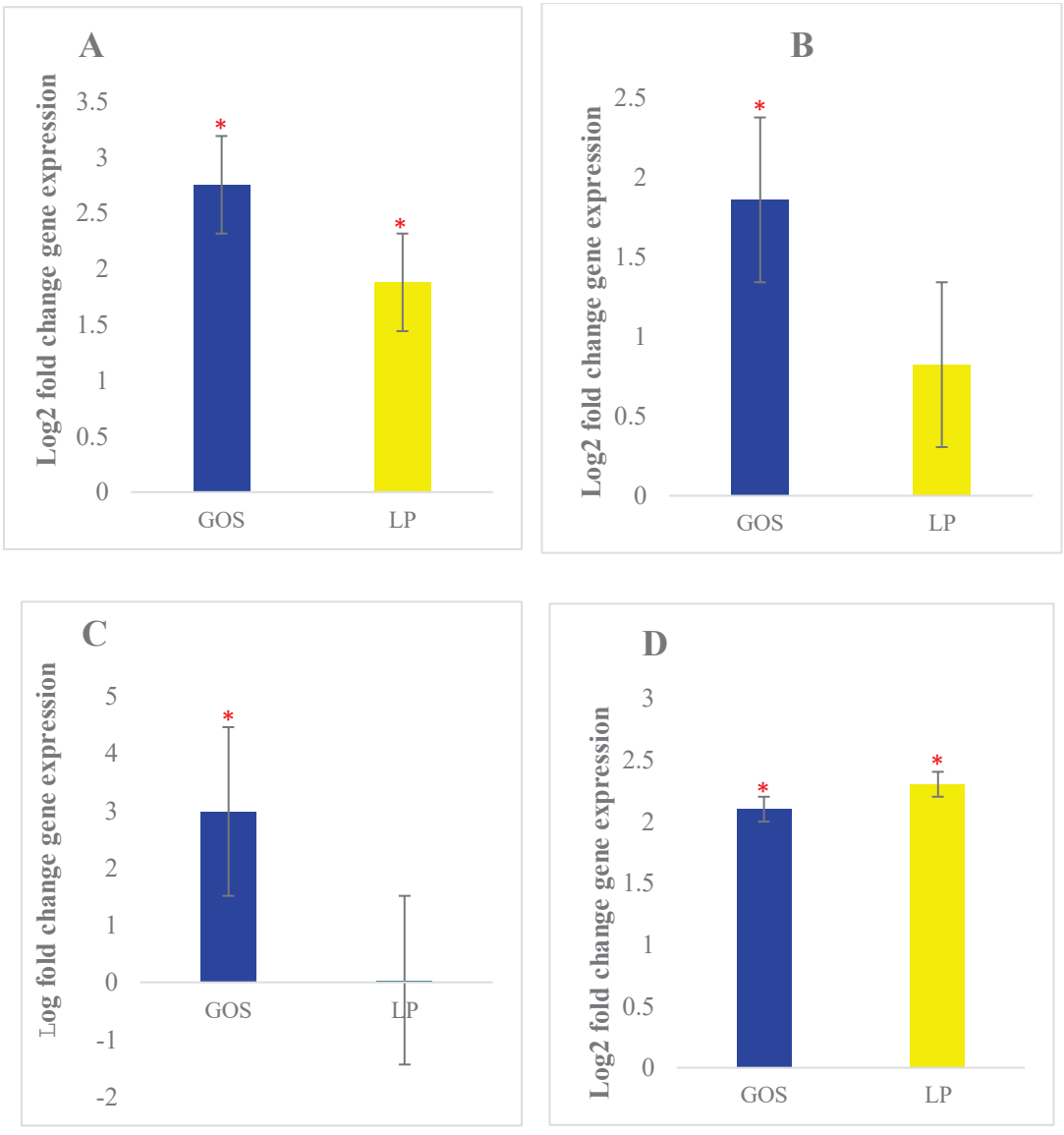

Figure 8: The gene expression pattern in chicken's spleen upon the administration of *Lactiplantibacillus plantarum* (LP) or galactooligosaccharide (GOS). (A) *SOD1*, (B) *MnSOD*, (C) *NRF2*, and (D) *CAT*. Error bars represent ± SE. Red asterisks (*) denote statistical differences ($P < 0.05$).

3.4.2.8. Gene expression analysis in chicken splenic tissue

The study of the gene expression revealed a statistical difference ($P < 0.05$) in the expression of *SOD1* and *IL4* in the chicken spleen tissue upon *in ovo* stimulation of GOS and LP (Figure 7A and C). Interestingly, *IL12p40 and IL8* (Figure 7B and D) were upregulated in the in *ovo* treated chickens with GOS and not in the LP and PC groups. Based on the expression levels of *CATHL2,* no statistical changes were observed across all the treatments.

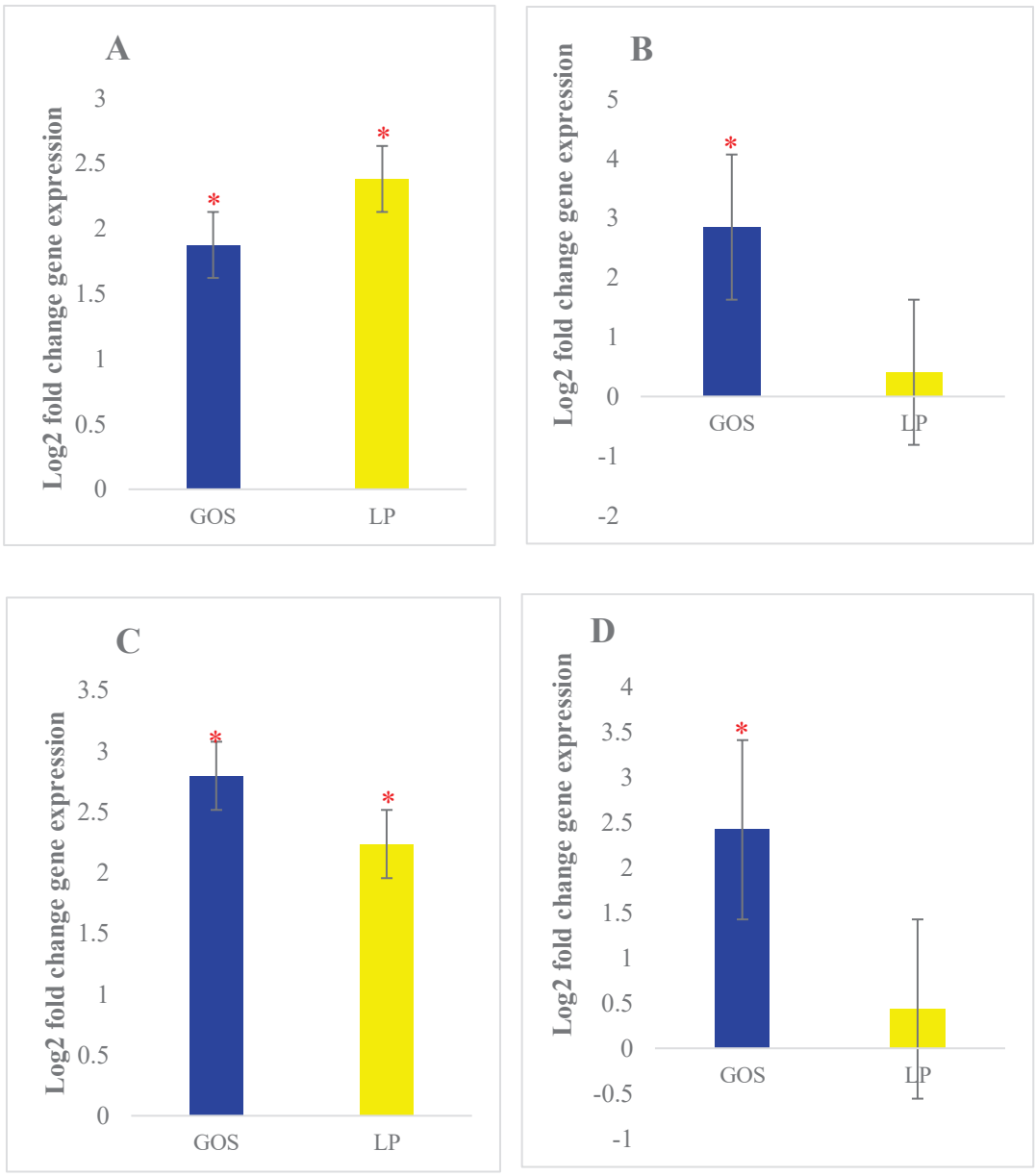

Figure 7: The pattern of gene expression in chicken's spleen upon the administration of *Lactiplantibacillus plantarum* (LP) and galactooligosaccharide (GOS). (A) *SOD*1, (B) *IL12p40*, (C) *IL4*, and (D) *IL8*. Error bars represent ± SE. Red asterisks (*) denote statistical differences ($P < 0.05$).

and *CATHL2* (Figure 6B, C, E and F) demonstrated a remarkable increase in their expression levels ($P < 0.05$) upon *in ovo* injection of LP or GOS. Additionally, a high expression level of *FFAR2* was observed upon *in ovo* stimulation of GOS while *in ovo* injection of LP led to high expression of *CLDN1* (Figure 6A).

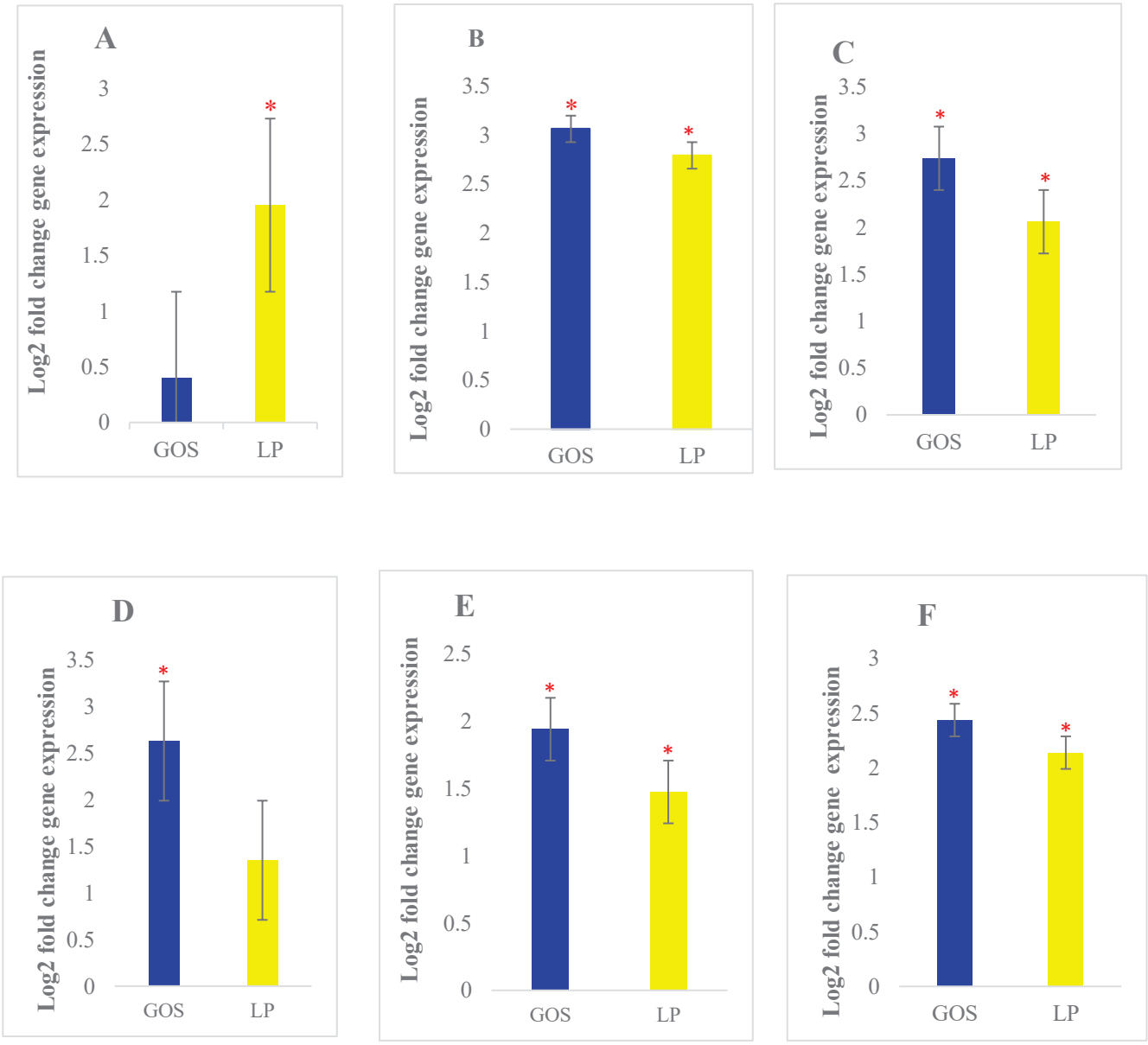

Figure 6: The pattern of gene expression in chicken's cecal mucosa upon *in ovo* delivery of *Lactiplantibacillus plantarum* (LP) and galactooligosaccharide (GOS) (A) *CLDN1*, (B) *MUC6*, (C) *AVBD1*, (D) *FFAR2*, (E) *IL-1β*, and (F) *CATHL2*. Error bars represent ± SE. Red asterisks (*) denote statistical differences ($P < 0.05$).

Table 8: Assessment of meat quality analysis.

Parameters	Treatments			
	PC	GOS	LP	Effect
Breast muscle				
pH_15 min	6.37 ± 0.17[b]	6.45 ± 0.16[a]	6.40 ± 0.15[a]	****
pH_24 h	5.94 ± 0.07	5.98 ± 0.09	6.03± 0.29	NS
L*	52.60 ± 16.68	56.66 ± 2.33	58.10 ± 1.50	NS
a*	9.88 ± 3.22	10.68 ± 0.71	10.24 ± 0.86	NS
b*	14.24 ± 4.71	15.05 ± 1.33	15.54 ± 1.13	NS
Drip losses 24 h (%)	0.93 ± 0.46	0.84 ± 0.23	1.00 ± 0.57	NS
Drip losses 48 h (%)	1.84 ± 0.79	1.75 ± 0.57	1.89 ± 0.92	NS
Thawing losses (%)	4.93 ± 1.99	3.55 ± 2.06	3.66 ± 2.23	NS
Cooking losses (%)	24.73 ± 8.39	31.13 ± 18.90	27.60 ± 3.06	NS
Shear force (N)	13.06 ± 5.78	13.00 ± 2.07	12. 58 ± 5.72	NS
Hardness	64.28± 23.05	73.20 ± 12.63	75.53 ± 13.90	NS
Springiness	0.32 ± 0.10	0.35 ± 0.03	0.35 ± 0.03	NS
Cohesiveness	0.38 ± 0.13	0.44 ± 0.04	0.44 ± 0.04	NS
Gumminess	26.87 ± 10.75	32.62 ± 7.82	33.37 ± 7.86	NS
Chewiness	9.40 ± 4.00	11.38 ± 3.08	11.50 ± 2.30	NS
Resilience	0.19 ± 0.06	0.23 ± 0.02	0.22 ± 0.02	NS
Adhesiveness	-0.06 ± 0.03	-0.05 ± 0.03	-0.06 ± 0.04	NS
Leg muscle				
pH15 min	6.38± 0.15[b]	6.43 ± 0.23[a]	6.62 ± 0.08[a]	****
pH24 h	6.24 ± 0.25	6.30 ± 0.08	6.34 ± 0.05	NS
L*	49.83 ± 1.99	49.71 ± 1.78	49.36 ± 1.88	NS
a*	15.23 ± 1.82	15.85 ± 0.60	15.31 ± 1.19	NS
b*	11.14 ± 0.92	11.30 ± 0.92	11.20 ± 0.90	NS
Drip losses 24 h (%)	0.57 ± 0.12	0.58 ± 0.08	0.58± 0.07	NS
Drip losses 48 h (%)	0.75 ± 0.15	0.80 ± 0.15	0.71 ± 0.08	NS
Thawing losses (%)	3.05 ± 1.00	2.95 ± 1.14	2.41 ± 0.95	NS
Cooking losses (%)	30.45 ± 2.55	28.27 ± 4.38	27.99 ± 1.83	NS

The data is shown as mean ± SD. Different letters (a, b) in the same row and means indicates statistical differences ($P < 0.05$) between the treatments, NC: Negative control, PC: Positive control, GOS: Galactooligosaccharide group, LP: *Lactiplantibacillus plantarum* group. The percentage refers to the proportion of each parameter in relation to meat quality. These percentages represent significant indicators of meat quality and nutritional content.

3.4.2.7. Gene expression analysis in chicken's cecal mucosa

Transcriptomic analysis was performed to reveal the impacts of GOS and LP on immune functions, gut health and antioxidant activities in chickens. Transcriptomic analysis revealed no statistical differences on the expression pattern of *TJAP1* and *IL10* in chicken's cecal mucosa. However, *MUC6*, *AVBD1*, *IL-1β*

Liver %	2.23 ± 0.30	2.25 ± 0.28	2.20 ± 0.17	NS
Gizzard %	0.96 ± 0.20	0.94 ± 0.13	0.93 ± 0.23	NS
Heart %	0.55 ± 0.08	0.54 ± 0.07	0.57 ± 0.07	NS
Leg bones %	3.98 ± 1.48	4.03 ± 0.38	4.15 ± 0.65	NS
Abdominal fat %	1.83 ± 0.30	1.90 ± 0.31	1.89 ± 0.32	NS
Breast muscles (g)	615.025 ± 50.32	606.18 ± 52.51	621.66 ± 68.82	NS
Leg muscles (g)	377.12 ± 42.78	366.22 ± 35.73	369.65 ± 38.61	NS
Giblets (g)	73.59 ± 10.15	74.008 ± 8.99	73.25 ± 5.82	NS
Liver (g)	43.91 ± 6.81	44.70 ± 7.01	43. 63 ± 4.51	NS
Gizzard (g)	18.88 ± 4.09	18.48 ± 2.56	18.35 ± 3.89	NS
Heart (g)	10.80 ± 1.95	10.84 ± 1.99	11.28 ± 1.51	NS
Leg bones (g)	78.18 ± 11.83	80.21 ± 11.65	82.73 ± 16.98	NS
Abdominal fat (g)	35.92 ± 6.11	37.61 ± 5.59	37.53 ± 7.29	NS

The results are presented as mean ± SD. Different letters (a, b) in the same row and means indicates statistical differences ($P < 0.05$) between the treatments, NC: Negative control, PC: Positive control, GOS: Galactooligosaccharide group, LP: *Lactiplantibacillus plantarum* group. The percentage refers to the proportion of each parameter in relation to the overall carcass weight. These percentages represent significant indicators of carcass composition, meat quality and nutritional content.

Regarding the meat quality analysis, several parameters such as meat color, pH value, water holding capacity, and texture are major indicators of chicken meat quality and are widely used for its assessment (Table 8). The results demonstrated a statistically significant effect on the pH at 15 minutes after slaughter on *in ovo* treated chicken with LP and GOS than that of the positive control group ($P < 0.05$). However, no statistical changes were recorded across all the treatments after the measurement of the pH at 24 hours post-mortem. Furthermore, there were no major differences in the other meat quality parameters (cooking loss, chewiness, meat color, drip loss, springiness, shear force, gumminess, thawing loss, etc.).

Table 6: Body weight (BW) development (means ± SD) from day 7 to day 35 of chickens from the three in ovo treatment groups. GOS: Galactooligosaccharides, LP: *Lactiplantibacillus plantarum*, PC: Positive Control, NC: Negative control. NS in the tables means Not Significant.

	Treatments				
BW (g)	**NC**	**PC**	**GOS**	**LP**	**Effect**
Day 7	180.50 ± 25.81^{b}	177.34 ± 23.04^{b}	179.60 ± 26.20^{b}	195.23 ±24.14^{a}	****
Day 14	480.20 ±71.50	490.81±58.22	485.93 ± 63.31	518.80 ± 66.13	NS
Day 21	1014.40 ± 143.10	1011.25 ±113.50	1017.70 ± 113.9	1044.30 ± 112.94	NS
Day 28	1681.50 ± 197.93	1663.40 ± 192.40	1655.40 ±168.33	1716. 24 ± 175.02	NS
Day 35	2437.50 ± 254.91	2433.60 ± 302.20	2526.90 ±276.01	2499.70 ± 225.09	NS

The results is reported as mean ± SD. Different letters (a, b) in the same row and means indicates statistical differences ($P < 0.05$) between the treatments.

3.4.2.6. Slaughter, carcass traits assessment and meat quality analysis

The carcass traits and the results of the meat (in leg muscles and breast muscles) are presented in Table 7 and 8. There were no major changes in most of the studied parameters on the slaughter and carcass traits of *in ovo*-treated chickens. However, regarding the cooling losses, the birds in the LP and GOS demonstrated significantly lower cooling losses than those of the PC group ($P < 0.05$).

Table 7: Assessment of slaughter and carcass traits of *in ovo* treated chickens.

Parameters	**Treatments**			
	PC	**GOS**	**LP**	**Effect**
Cooling losses (%)	1.79 ± 0.21^{a}	1.58 ± 0.40^{ab}	1.31 ± 0.37^{b}	****
Dressing percentage with giblets (%)	79.81 ± 1.14	80.19 ± 1.09	80.32 ± 1.08	NS
Dressing percentage without giblets (%)	76.83 ± 1.19	77.19 ± 1.15	77.35 ± 1.16	NS
Breast muscles %	31.35 ± 2.05	30.60 ± 1.70	31.34 ± 1.53	NS
Leg muscles %	19.19 ± 1.47	18.47 ± 1.14	18.70 ± 1.70	NS
Giblets %	3.75 ± 0.42	3.73 ± 0.34	3.70 ± 0.31	NS

Table 5: Histomorphology assessment of the cecal mucosa of *in ovo* treated chickens.

Traits	PC	GOS	LP
VH	296.31 µm ± 61.05^{b}	337.93 µm ± 48.82^{a}	326.12 µm ± 74.30^{a}
CD	39.38 µm ± 4.23^{b}	40.20 µm ± 7.50^{ab}	43.91 µm ± 5.06^{a}
VW	52.59 µm ± 12.51^{b}	69.48 µm ± 53.94^{a}	69.96 µm ± 28.41^{a}
VA	50260.61 µm^2 ± 24977.04^{a}	75128.22 µm^2 ± 66629.80^{b}	75349.80 µm^2 ± 50312.14^{b}
MM	149.51 µm ± 28.20	120.11 µm ± 24.58	148.05 µm ± 50.45
VH/CD	7.75 ± 1.10	6.80 ± 0.5	7.44 ± 0.55

The data is shown as mean ± SD. Different letters (a, b) in the same row and means indicates statistical differences ($P < 0.05$) between the treatments, NC: Negative control, PC: Positive control, GOS: Galactooligosaccharide group, LP: *Lactiplantibacillus plantarum* group, VH: Villus height VW: Villus width, CD: Crypt depth, VA: villus area; MM: muscle membrane, VH/CD: Villus height to crypt depth ratio.

3.4.2.5. Body weight, feed intake and feed conversion ratio

The results revealed a significant increase in BW on 7 days old ($P < 0.05$) chickens that were *in ovo* treated with LP when compared to the PC group. The LP and GOS treatments recorded a BW of 195.2 grams and 179.60 grams respectively. However, on days 14, 21, 28 and 35, no significant effect on BW was found (Table 6). Additionally, no statistical differences ($P > 0.05$) were recorded on chicken FI and FCR across all the treatments. However, by the end of the rearing period (day 35), the *in ovo* experimental groups had a slightly higher BW than those of the PC group. The findings demonstrated that GOS and LP enhanced the early growth performance of chicks (Table 6).

3.4.2.3. Relative bacterial abundance in the ceca

The results demonstrated a notable increase in the bacterial abundance of *Lactobacillus* spp., (Figure 5A) and *Bifidobacterium* spp. (Figure 5B) in the cecal content of both the GOS and LP group as compared to the PC group ($P < 0.05$). The results clearly showed that the prevalence of beneficial bacteria in chicken's ceca was highest in the LP treated chickens, then the chickens treated with GOS while it was lowest in the PC group. These findings suggest that LP and GOS modified the gut microbiome, improved chicken's immune system, gut health and performance.

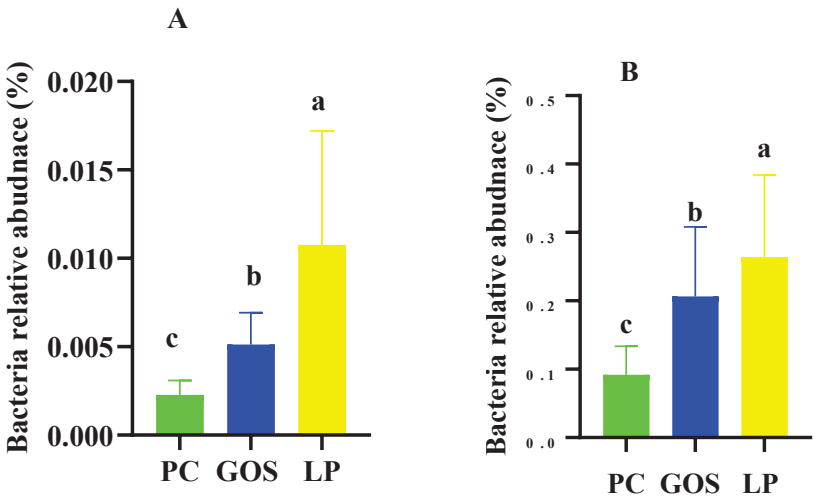

Figure 5. The prevalence of commensal bacteria in the ceca of *in ovo* treated chickens (A) *Lactobacillus* spp. (B) *Bifidobacterium* spp. Error bars: ± SE. a, b, c letters that are not similar indicate statistical differences across the treatments ($P< 0.05$) PC: positive control, GOS: Galactooligosaccharide, LP: *Lactiplantibacillus plantarum*.

3.4.2.4. Histomorphology measurements of the cecal mucosa on *in ovo* treated chickens

The results showed that the GOS and LP group's villus height and villus width of the chicken's cecal mucosa were statistically higher ($P < 0.05$) than those of the PC group (Table 5). Furthermore, no notable differences were found in the muscle membrane and villus height-to-crypt depth ratio among the groups.