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

POLITECHNIKA BYDGOSKA
im. Jana i Jędrzeja Śniadeckich

IMPACT OF SELECTED BIOACTIVE SUBSTANCES DELIVERED *IN OVO* ON GUT HEALTH AND PRODUCTION PERFORMANCE OF BROILER CHICKENS

by

Modou Mangan

DEPARTMENT OF ANIMAL BIOTECHNOLOGY AND GENETICS
FACULTY OF ANIMAL BREEDING AND BIOLOGY
BYDGOSZCZ UNIVERSITY OF SCIENCE AND TECHNOLOGY

Source of funding

This PhD project was funded by the European Union's Horizon 2020 research and innovation program under grant agreement N° 955374 under the MonoGutHealth project.

Acknowledgments

I thank the Almighty GOD for giving me the mental and physical ability to complete my PhD successfully. First of all, I would like to express my sincere and profound gratitude to my PhD supervisor (Professor Maria Siwek) and Co-supervisor (Professor Cornelia C. Metges) for their invaluable support, guidance, constructive criticism, suggestions and feedback. These have been instrumental in ensuring the successful completion of my doctoral studies. I would also like to extend my sincere appreciation to the academic staff, research staff, and auxiliary staff of the faculty of Animal Breeding and Biology, Department of Animal Biotechnology and Genetics, for their warm welcome and kindness and for allowing me to use the research facilities for my work throughout my time here. I would also like to thank the Bydgoszcz University of Science and Technology administration. I am grateful to Vetdiagnostica, Drobex-Agro in Poland, and the Research Institute for Farm Animal Biology in Germany for their invaluable support and training during my secondments with them and a special thank you to JHJ SP. Zoo for providing the probiotics used in this research work. Moreover, I would like to extend my profound gratitude to all the co-authors for their impactful contributions to my PhD publications. My sincere thanks to all the people, universities, companies/organizations that participated in the MonogutHealth project. A special thanks to all the supervisors, co-supervisors and my fellow Early Stage Researchers involved in the MonogutHealth project, I greatly appreciate your support, assistance, technical questions, suggestions, and feedback during our project meetings, this has improved my research skills and also helped me grow as a researcher. I would like to thank the European Union's Horizon 2020 research and innovation program for funding my PhD project. Furthermore, I would like to extend sincere gratitude to my labmates at the Department of Animal Biotechnology and Genetics for their unwavering support during laboratory experiments and other research activities. Finally, I would like to say a BIG thank you to my loving and caring wife (Mama B. Bah), daughter (Fatima Mangan), my affectionate parents, my friend (Ali Bah), my Master's degree supervisor (Professor Zsuzsanna Bősze), family members, and all well-wishers for their unflinching support, encouragement and always standing by my side, especially during difficult and challenging moments of my doctoral studies.

CONTENTS

1. Introduction

The gut microbiome entails all the microorganisms (mostly bacteria) present in a host organism (Bahrndorff et al., 2016). Most of these microbes present in chicken's gut microbiome consist of both harmful and beneficial bacteria, therefore a microbial shift can cause changes in the microbial population and consequently could either positively or negatively impact chicken's health, physiology, morphology, immune functions and production capabilities (Aruwa et al., 2021; Fathima et al., 2022). The commensal bacteria present in the chicken gut microbiome are crucial in shaping the health and productivity of chickens via pathogen inhibition, immune system development, gut health, aiding in nutrient absorption and alleviating the negative consequences of environmental stressors (Aruwa et al., 2021). Therefore, it has become a topic of discussion among researchers trying to find and ensure a stable gut microbiota in chickens.

The expansion and commercialization of the poultry sector is gaining momentum across the globe due to its short production cycle, and lower adverse effects to the environment compared to other livestock species. As reported, a 41% increase in the consumption of poultry products such as meat is expected in the next 8-10 years to feed the ever-increasing world population ("OECD-FAO Agricultural Outlook 2023-2032," 2023). In addition, broiler chickens are more affordable, well-accepted in many regions, cultures and religions and most importantly serve as an important source of food (Mottet and Tempio, 2017). According to (Liu et al., 2023; Ahmed et al., 2023), antibiotic supplementation in chicken feed helps to maintain and/or improve chicken health (reduce morbidity and mortality), production metrics (body weight (BW), feed intake (FI), feed conversion ratio (FCR), meat quality, etc.), and welfare and subsequently ensure profit. However, recently the utilization of antibiotics in poultry diet has faced immense scrutiny due to bacterial resistance, food safety concerns and human health issues, thus leading to its ban in the EU and other regions across the globe (Seal et al. 2013; Ahmed et al. 2023).

The chicken embryo depends solely on the nutrients in the egg throughout embryonic development (ED) and needs this in sufficient amounts to ensure continuous embryonic growth and successful hatching (Yang et al., 2021). In modern poultry practices, hatchlings are deprived of food for about 48–72 hours (hatch window) and this may retard gastrointestinal tract development and growth potentials (Kadam et al., 2013). Furthermore, this may cause dehydration (Christensen, 2009) and delay morphological and physiological maturation of the gut and development of the immune system (Willemsen et al., 2010; Noy and Uni, 2010; Leão et al., 2021; Shehata et al., 2021; Kpodo and Proszkowiec-Weglarz, 2023) and affects hatching and quality of chicks (Cheled-Shoval et al., 2011; Akosile et al., 2023).

The period of incubation (21 days) accounts for about 30-40% of broiler chicken's life. Therefore, any physical or physiological manipulation or changes during this time can affect embryonic growth, and overall health and production performance of poultry (Goel et al., 2023). Thus, this has warranted an exploration of new approaches from the scientific community and the poultry sector to find alternatives for antibiotics. This requires innovative and sustainable approaches to ensure robust intestinal gut health, improve performance, increase food safety (Oviedo-Rondón, 2019) while mitigating the adverse effects of high ambient temperatures and other environmental factors (Mangan and Siwek, 2024).

Significant advances have been realized therefore increasing the comprehension of the interaction of the host and the gut microbes thus numerous methods such as feeding regimes, restriction feeding, spray application during the first days post-hatching and supplementation of bioactive substances (probiotics, prebiotics, synbiotics, phytochemicals, postbiotics, minerals and vitamins) in the feed and/or water of chickens have been developed (Mangan and Siwek, 2024). During the prenatal period, the supplementation of appropriate nutrition supports the embryo's growth and development and may help minimize disease infection (Hou and Tako, 2018). Another innovative strategy is the *in ovo* technology (Bednarczyk et al., 2016; Das et al., 2021), which is said to have more beneficial effects and superior impact on chicken health, immune system development and post-hatching performance compared to the other approaches mentioned above. Several studies have confirmed the above claim (Dunislawska et al., 2017; Slawinska et al., 2020; Duan et al., 2021; Kpodo and Proszkowiec-Weglarz, 2023). This method was developed mainly to perform *in ovo* vaccination on day 18 of ED to protect the birds against infectious diseases (Williams and Zedek, 2010). However, in recent years, the *in ovo* technology has been used to administer bioactive compounds on day 17-18 (*in ovo* feeding) (Uni et al., 2005), day 12 (*in ovo* stimulation) (Kadam et al., 2013; Siwek et al., 2018), for *in ovo* sexing (Schijns et al., 2014) and epigenetic reprogramming to improve chicken's productivity, welfare and health (Bednarczyk et al., 2021). This technology has also been reported to avert heat stress in poultry birds (Ncho et al., 2021). The *in ovo* stimulation involves the *in ovo* administration of synbiotics, prebiotics and probiotics during day 12 of ED into the air sac and therefore stimulating the native microbiota in the developing embryo. During this period, the highly vascularized chorioallantoic membrane enables the prebiotic's passage from the egg air chamber and then enters the bloodstream and subsequently to the growing embryo and GIT which subsequently confers positive effects to chicken's gut microbiota (gut colonization by beneficial bacteria, improve immune development and health) (Siwek et al., 2018). Furthermore, probiotics injected *in ovo* enter the gastrointestinal tract (GIT) during the initial hatching phase (i.e. when the chick immediately breaks the inner membrane of the egg). At this point, these probiotics may act as the main bacteria

colonizing the gut, promoting the development of a stable gut microbiota and positively contributing to the modification of the chicken's intestinal milieu which could improve chicken's health and development (Pedroso et al., 2016). The aim of *in ovo* stimulation is to directly modulate gut microbiota and indirectly influence the entire host organism. Such bioactive substances can promote commensal microbial growth and positively influence chicken health and gastrointestinal tract development (Slawinska et al. 2019). Conversely, *in ovo* feeding involves the delivery of bioactive compounds such as carbohydrates, enzymes, minerals and vitamins into the amniotic sac from ED 14 to ED 18. The purpose of this *in ovo* strategy is to provide the required nutrition to chicks during pre-and post-hatch (Oladokun and Adewole, 2020; Das et al., 2021). To compare *in ovo* feeding and *in ovo* stimulation, two different studies were performed, the first by (Tako et al., 2014), in which prebiotics were delivered into the amniotic fluid on ED 17, and the other studies by (Villaluenga et al., 2004; Cheled-Shoval et al., 2011; Slawinska et al., 2019, 2020; Kpodo and Proszkowiec-Weglarz, 2023), in which *in ovo* stimulation led to the modulation of the gut microbiome and increased the amount of beneficial microbes thus ensuring immune system development, improve chick quality and overall health and production performance suggesting that it is safer than *in ovo* feeding and is the most optimal period for prebiotic delivery (Siwek et al., 2018). Therefore, ensuring long-term benefits throughout the chicken's life. Although, the *in ovo* stimulation of several bioactive substances during egg incubation has already been tested (Maiorano et al., 2012; Dankowiakowska et al., 2019; Slawinska et al., 2020; Yang et al., 2024), there is still a knowledge gap that has to be filled to understand the associated biological and molecular mechanism which makes *in ovo* administration beneficial for poultry health and performance.

Therefore, this PhD project was carried out to ensure optimal gut health, improve performance and ameliorate the potentially detrimental effects of life and environmental stressors by *in ovo* stimulation of LP and GOS on the 12^{th} day of egg incubation. Considering this, the first objective of the PhD dissertation was to assess and perform an *in vitro* study on numerous prebiotics and probiotics based on their growth and radical scavenging ability upon which the most promising were selected for further *in vivo* validation. The second aim was to study the impacts of the chosen bioactive substances on genes associated with gut health and immune system, gut histomorphology, blood parameters, antioxidant status and production performance on Ross 308 broiler chickens.

2. List of scientific articles constituting a series of publications series of a doctoral dissertation

1. Modou Mangan and Maria Siwek, Strategies to combat heat stress in poultry production-A review, Journal of Animal Physiology and Animal Nutrition (Wiley online library) 2024 December;108(3):576-595. doi: 10.1111/jpn.13916. MNiSW points $_{2024}$:100, Impact Factor $_{2024}$: 2.2

2. Modou Mangan, Patrycja Reszka, Katarzyna Połtowicz and Maria Siwek, Effects of *Lactiplantibacillus plantarum* and galactooligosaccharide administered *in ovo* on hatchability, chick quality, performance, cecal histomorphology and meat quality traits of broiler chickens, Journal of Animal Physiology and Animal Nutrition (Wiley online library) 2024 December. doi: 10.1111/jpn.14082. MNiSW $_{2024}$: 100, Impact Factor $_{2024}$: 2.2

3. Modou Mangan, Katarzyna Połtowicz, Cornelia C. Metges and Maria Siwek, Modulatory effects of *in ovo* delivery of galactooligosaccharide and *Lactiplantibacillus plantarum* on antioxidant capacity, gene expression and selected plasma metabolite parameters of broiler chickens, Journal of Applied Genetics (Springer) 2024 December. doi:10.1007/s13353-024-00931-7. MNiSW $_{2024}$: 140, Impact Factor $_{2024}$: 2.0

3. Justification of the thematic coherence of the doctoral dissertation publication cycle

3.1 List of abbreviations, symbols and units

AC: Absorbance of control

ACTB: Actin, beta

ADFI: Average daily feed intake

ALT: Alanine transaminase

ANOVA: Analysis of variance

AS: Absorbance of sample

AST: Aspartate aminotransferase

AVBD1: Avian beta-defensin 1

BW: Body weight

CAT: Catalase

CATHL2: Cathelicidin 2

CFU: Colony forming units

cDNA: Complementary DNA

CLDN1: Claudin 1

DNA: Deoxyribonucleic acid

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

EU: European Union

ED: Embryonic development

EC50: Half maximal effective concentration

FCR: Feed conversion ratio

FFAR2: Free fatty acid receptor 2

10:38773648

FFAR4: Free fatty acid receptor 4

FI: Feed intake

G6PDH: Glucose-6-Phosphate Dehydrogenase

GGT: Gamma-glutamyl transferase

GOS: Galactooligosaccharide

*GPx*1: Glutathione peroxidase 1

HDL: High-density lipoprotein

HO-1: Heme oxygenase 1

IL1-β: Interleukin 1 beta

IL2: Interleukin 2

IL4: Interleukin 4

IL8: Interleukin 8

IL10: Interleukin 10

IL12p40: Interleukin 12p40

LAB: Lactic acid bacteria

LC: *Lacticaseibacillus casei*

LDH: Lactose dehydrogenase

LDL: Low-density lipoprotein

LP: *Lactiplantibacillus plantarum*

LR: *Limosilactobacillus reuteri*

LRh: *Lacticaseibacillus rhamnosus*

mg*:* Milligram

MJ/kg: Mega Jules per Kilogram

mL: Milliliter

MM: Muscle membrane

mM: Millimolar

MnSOD: Manganese superoxide dismutase

MRS agar: De Man-Rogosa-Sharpe agar

MUC6: Mucin 6

NC: Negative control

NEFA: Non-esterified fatty acid

nm: Nanometer

NRF2: Nuclear factor erythroid 2-related factor 2

OD600: Optical density at 600nm

OECD: Organization for Economic Cooperation and Development

PAS: Periodic acid-Schiff

PC: Positive control

PCA: Principal Component Analysis

PCR: Polymerase chain reaction

qPCR: Quantitative polymerase chain reaction

RNA: Ribonucleic acid

rpm: revolutions per minute

SCFA: Short-chain fatty acids

SD: Standard deviation

SE: Standard error

SEM: Standard error of means

SOD1: Superoxide dismutase 1

TG: Triglyceride

TJAP1: Tight junction-associated protein 1

UA: Uric acid

µg/mL: Microgram/millilitre

µL: Microlitre

µM: Micromolar

VA: Villus surface area

VH: Villus height

VH/CD: Villus height to crypt depth ratio

VW: Villus width

ZO-1: Zonula Occludens 1

3.2. Hypothesis, objectives and scope of the research

Hypothesis

The *in ovo* stimulation of *Lactiplantibacillus plantarum* and galactooligosaccharide on day 12 of embryonic development will modulate chicken gut health, enhance intestinal development, immune functions and improve production performance.

Main objective:

The main objective of this PhD project was to improve chicken gut health and production performance using microbiome programming *in ovo*.

Secondary objectives:

1. Selection of the prebiotics and probiotics through in vitro screening tests that could enhance the growth, health status and performance of broiler chickens.

2. To optimize the dosage of bioactive substances for effective *in ovo* application in broiler chickens.

3. To evaluate the effects of bioactive substances applied *in ovo* on meat quality and carcass traits of broiler chickens.

Scope of the research

An essential sector of achieving global food self-sufficiency is the poultry industry. Poultry products such as meat and eggs provide food to humans in the form of protein and energy thus contributing to fulfilling the dietary needs and health of humans (Bist et al., 2024). Although it plays a tremendous role in ensuring food security, the poultry industry is faced with many challenges that pose risks to its sustainability as well as productivity (Mottet and Tempio, 2017). Some of these challenges include poor gut health, the inability to maintain a high hatchability rate, egg quality and good chick quality, and performance, which can be caused by disease, nutritional deficiencies or environmental stress such as heat stress. In the past, numerous strategies such as housing management, rearing methods, nutritional supplementation, breeding and genetics have been explored to mitigate these problems (Goel, 2021). However, the poultry industry is still affected. This dissertation consists of interrelated studies that highlight the importance of *in ovo* administration of bioactive compounds and thus contributing to the advancement of the poultry industry and possibly addressing some of the major obstacles impeding the poultry industry. The scope of this PhD dissertation specifically focuses on the impacts of *in ovo* stimulation of *Lactiplantibacillus plantarum*

(LP) and galactooligosaccharides (GOS) on Ross 308 broilers and explores the positive impacts of GOS and LP on the gut microbiota, gut morphology, immune functions, antioxidant capacity, growth performance, health and general welfare. The **first study of** this dissertation involved the selection of bioactive substances via an *in vitro* experiment. The growth ability of these bioactive substances were assessed and then followed by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was assessed. This assay is a common method for evaluating the radical scavenging activities and antioxidant levels of bioactive substances. Using this method (DPPH assay), the selected bioactive substances were screened to determine the ones that exhibited high radical scavenging activities. Bioactive compounds with strong antioxidant properties are essential in reducing oxidative stress, which could negatively impact immune responses, health conditions, productivity, and overall well-being of chickens. Thus, these bioactive compounds are crucial in mitigating the negative impacts that oxidative stress may induce.

The results obtained in the *in vitro* study are supported by the review paper, which reports that various strategies such as housing management, supplementation of feed with bioactive substances, improvement of breeding and genetics, *in ovo* technology, etc., could alleviate heat and oxidative stress in poultry and thereby improve immune functions, production performance, health and general well-being of broiler chickens Therefore, the primary goal of this experiment was to pre-screen and select bioactive substances that have high antioxidant potential. Based on the results of the *in vitro* (DPPH) assay, *Lactiplantibacillus plantarum* was chosen for *in ovo* application while galactooligosaccharide was selected based on its capability to alleviate heat stress, stimulate early gut colonization, improve performance, and health as reported by our research group (Siwek et al., 2018; Slawinska et al., 2020). The **second study** (*in ovo* application) was then carried out to validate the findings of the *in vitro* study.

The *in ovo* administration (i.e. *in ovo* stimulation) of the selected bioactive substances was injected in the egg air chamber on the 12th day ED. The *in ovo* stimulation with bioactive substances on ED 12 has several beneficial effects such as promoting early gut colonization by beneficial bacteria and immune system development (Siwek et al., 2018). With such positive impacts on the developing embryo, this could improve hatchability, and chick quality ensuring healthier and high-performing chicks. In the poultry industry, good quality and healthy chicks are of paramount importance in ensuring a high survival rate, health and production performance in realizing profit. The *in ovo* injection of LP or GOS on ED 12 aimed to increase the presence of beneficial bacteria in a chicken's gut microbiota and subsequently establish a healthy gut microbiome. Therefore, the relative bacterial abundance of beneficial bacteria (*Lactobacillus* spp. and *Bifidobacteria* spp.) was determined in the excreta of chickens (days 7, 21 and 35) and the cecal

content of chicken using qPCR. A stable gastrointestinal tract, resulting from *in ovo* stimulation of LP and GOS was expected to promote nutrient absorption, immune function, performance, and overall health in chickens. In addition, histological analysis was performed on the chicken's ceca to determine the status of the chicken gut. Having in mind the potential impacts of the *in ovo* technology (*in ovo* stimulation), the role played by the microbes inhabiting the gut and the function of the ceca on chicken health and production performance (BW, FI, FCR, carcass yields and meat quality), I evaluated the impacts of the *in ovo* stimulation of both LP and GOS on day 12 of ED on chicken production performance. Moreover, the production performance of chickens was further validated by investigating the mRNA gene expression (transcriptomic analysis) of relevant tissues (spleen, liver, breast muscle and cecal mucosa) associated with immune system development, antioxidant capacity and gut health using qPCR analysis. The *in ovo* stimulation of LP and GOS can positively impact the activation of key genes that participate in the regulation of the immune system and antioxidant defense mechanisms thereby improving chicken's health and performance. Furthermore, blood biochemical parameters (plasma metabolite) were evaluated upon *in ovo* stimulation of GOS and LP thereby providing a deeper understanding of how these bioactive substances could improve the physiology and health conditions of Ross 308 broilers.

This PhD project thus demonstrates the thematic consistency of this dissertation revolving around improving poultry health and productivity and resilience against oxidative stress. The first publication highlights a variety of strategies including the *in ovo* stimulation of bioactive compounds to alleviate heat stress in chickens while the other 2 publications address the secondary objectives of this PhD dissertation.

The results from this PhD dissertation contribute to a comprehensive understanding of the screening of potential bioactive substances for *in ovo* application (secondary objective 1), dosage optimization, *in ovo* procedure (secondary objective 2) and subsequently improving the production performance, immune functions, health and antioxidant capacity of birds (secondary objectives 1, 2 and 3). Taking into account the thematic consistency and the beneficial effects of the outcomes (results) obtained, this PhD dissertation will help to promote the performance and health of poultry and results be possibly applied practically in the poultry industry. In addition, this dissertation will contribute and provide new knowledge to the scientific research community by understanding the biological mechanisms and life-long positive impacts of the *in ovo* stimulation of LP and GOS in improving hatchability, chick quality, production metrics, health and antioxidant capacity of poultry and eventually increasing economic profitability in the poultry sector. In a nutshell, this dissertation presents a cohesive body of work that

bridges the gap between *in vitro* studies, *in ovo* application and *in vivo* to validate and to ensure a resilient and sustainable poultry industry, capable of meeting the demands of a growing world population.

3.3 Materials and Methods

3.3.1. Probiotic strains preparation and culture conditions (*in vitro* experiment 1)

The probiotics (Table 1) were kindly provided by JHJ Company, Nowa Wies, Gizałki, Poland. The probiotic cultures were preserved in 50% glycerol at −80°C. Using MRS broth, each probiotic was cultured at 37°C. Next, an IKA® RCT basic IKAMAGTM Safety Control Magnetic Stirrer was used to completely mix 6.82 g of MRS agar (de Man, Rogosa and Sharpe, Merck KGaA, Darmstadt) after it had been dissolved in 100 mL of distilled water. Afterward, each probiotic with 6.82 g of MRS agar dissolved in 100 Ml of distilled water was then vortexed for 10 s and autoclaved for 15 min at 121°C and then vortexed for 10 s. Probiotic strain stock cultures were cultured on agar plates and incubated at 37°C for 24 h. A 10 mL of DeMan, Rogosa, and Sharpe broth (MRS) (Merck KGaA, Darmstadt) were used to make a bacterial suspension for every strain. After that, 250 µL of MRS broth and 10 µL of each bacterial suspension were put to a 96-well microplate, which was then incubated for 48 hours at 37°C in an aerobic environment. For each probiotic, three repetitions were performed, with three replicates for each sample while MRS broth was used as the control (without probiotic culture). Next, a MultiskanTM FC Microplate Photometer and SkanIt software version 7.0 (Thermo Fisher Scientific, Waltham, MA) were used to determine bacterial growth (OD600) every 12 h. To ensure sample homogeneity, microtiter plates were shaken for 10 s before measurements.

Table 1: Probiotic concentrations used in the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) test.

Probiotic strains	**Probiotic concentrations**
	Lacticaseibacillus casei 1.4 x10^6
L. casei	*Lacticaseibacillus casei* 7.0 x10^5
	Lacticaseibacillus casei 3.5 x10^5
	Lactiplantibacillus plantarum 4.4 x10^6
L. plantarum	*Lactiplantibacillus plantarum* 2.1 x10^6
	Lactiplantibacillus plantarum 1.0 x10^6
	Limosilactobacillus reuteri 7.9 x 10^6
L. reuteri	*Limosilactobacillus reuteri* 3.9 x 10^6
	Limosilactobacillus reuteri 1.9 x 10^6
	Lacticaseibacillus rhamnosus 1.1 x 10^8
L. rhamnosus	*Lacticaseibacillus rhamnosus* 5.5 x 10^7
	Lacticaseibacillus rhamnosus 2.7 x 10^7

3.3.2. *In vitro* evaluation of the radical scavenging ability of the selected probiotics (Experiment 1)

All the probiotic strains used in this study are listed in Table 1. In order to pre-select probiotics for *in ovo* injection, I measured the free radical scavenging capabilities of these bioactive substances using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Sigma-Aldrich, St. Louis, MO). Briefly, 0.1 mM of DPPH was dissolved in 100 mL of ethanol. Afterward, the mixture was firmly shaken and left to react for 30 min in the dark at room temperature and on each day of analysis the mixture was freshly prepared to ensure accurate and reliable results.

To perform the DPPH analysis, serial dilution of the samples was done and then 10 µl of each sample (with an appropriate dilution) and 190 µl of the sample was pipetted in each well of a 96-well microtiter plate while the control (200 µl of DPPH ethanolic solution) was put in each well. The blank group contained MRS broth media and ethanol. All samples were measured in triplicate utilizing a MultiskanTM FC Microplate reader (520nm). The percentage of the free radical scavenging activities of the bioactive compounds was calculated using the formula below:

% scavenging activity = [(Ac-As)/Ac] × 100

where Ac is the absorbance of the control and As is the absorbance of the sample.

The results are reported as EC50 value (µg/mL), which is the minimal antioxidant level needed to decrease 50% of the initial DPPH reaction from the time the extract has reached stability. Based on the growth curve and the results obtained from the DPPH assay, the bioactive substances with the best growth and the highest free radical scavenging activity was selected for *in ovo* application. The rationale for selecting GOS for *in ovo* application and *in vivo* study was based on our research group's previous findings which reported that the *in ovo* stimulation of the prebiotic GOS enhanced embryonic development and improved health and performance of broiler chickens exposed to heat or oxidative stress (Slawinska et al. 2020b).

3.3.3. Experimental setup and *in ovo* injection protocol

Table 2: Experimental design for the *in-ovo* experiment

Groups	*In-ovo* injection treatments	Dose of bioactive /egg
Negative control (NC)	No injection	-
Positive control (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)

3.3.4. Bioactive substances preparation (GOS and LP)

On day 12 of egg incubation, prior to *in ovo* injections, an amount of 3.5 mg of GOS/egg required to inject 300 eggs was calculated and freshly prepared by dissolving 0.2 ml of physiological saline solution and injecting it *in ovo* into the air chamber (Slawinska et al., 2020).

The probiotic (*Lactiplantibacillus plantarum*), was cultured in MRS broth media for 15 h. According to the preliminary findings, after 15 h, the probiotics attained their maximum growth at 37˚C, where it was possible to acquire a sufficient number of viable and active cells (Wishna-Kadawarage et al., 2024). The probiotic (LP) cells were centrifuged at 4200 rpm for 20 min at 4˚C using a refrigerated centrifuge. Next, the cell pellets from each culture were resuspended in 0.9% saline solution after being washed twice with 0.9% physiological saline solution. Next, a microplate reader (Thermo Scientific Multiskan FC plate reader: Thermo Scientific, Poland) was used and the optical density was set at 600 nm (OD600) of the solution to achieve a cell density of 5×10^6 CFU/ml (based on the regression equation obtained from the preliminary study between the CFU/ml and OD600). Finally, 200 µl of this cell suspension was used for *in ovo* injection of each egg.

3.3.5. Egg incubation and *in ovo* administration procedure

A total of 300 fertile ROSS 308 broiler eggs were incubated in this experiment. All the eggs were incubated at standard incubation conditions with a temperature of 37.5°C and relative humidity of 65% and egg turning every hour using Midi series I, Fest Incubators, Poland during the first 18 days of egg incubation. On the 7^{th} day of egg incubation, all the eggs were candled and unfertilized and dead embryos were excluded from the experiment. The remaining viable eggs were randomly assigned to four treatment

groups (Table 2) and put back to the incubator. Subsequently, on day 12 of egg incubation, all eggs were disinfected using 70% ethanol. Next, a 20G needle was used to make a hole in the air sac of each egg. Except for the negative (NC) group, eggs from each group were manually injected (*in ovo*). The positive control (PC) group eggs received an injection of 0.2 mL of sterile 0.9% physiological saline solution, the LP group eggs received an injection of 10^6CFU of LP probiotic/egg suspended in 0.2 mL of physiological saline solution while the GOS group eggs received an injection of 3.5 mg of GOS/egg suspended in 0.2 mL of physiological saline. One drop of organic glue (Elmer's school glue, Elmer's Products Inc., USA) was used to seal the eggs immediately after injection, all eggs were returned to the incubator to continue incubation.

3.3.6. Hatchability

From day 18th of egg incubation, all the eggs in the setter trays were placed in hatching baskets and transferred to hatchers to continue incubation using Midi series I, Fest hatchers, Poland with a temperature of 37.5°C and relative humidity of 65%-70%. Upon hatching, the hatchability of each group was recorded and calculated by using the equation below:

Hatchability = (No. of chicks hatched/No. of hatching eggs)*100

3.3.7. Chick quality analysis

Immediately after hatching and recording of hatchability, the one-day-old chicks were subjected to chick quality assessment using the chick-hatchling weight, chick length and Pasgar score. A total of 25 chicks were randomly chosen from each treatment group and individually weighted using an electronic balance and the average BW was calculated. The same 25 chicks used for weight measurements, were used for chick length evaluation. The length of each chick was determined by placing the chick face down on a flat surface and straightening the right leg. The length (in cm) was measured from the tip of the beak to the tip of the middle toe using a ruler (Sozcu and Ipek, 2015). The Pasgar score is based on a 10 points-scoring method used to asses chicks based on the following traits: closing/opening of the navel, appearance of the belly, legs, beak, reflex and response to stimuli (Mukhtar et al., 2013). To assess the quality of the chicks (Pasgar score) for each treatment group, ten birds (out of the twenty-five randomly selected birds/group used for weight and length assessment) were selected.

3.3.8. Housing, animal rearing and sample collection

3.3.8.1 *In vivo* experiment

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

During the experiment, each bird from each treatment group, 25 birds/pens, was reared in separate pens under similar regulated environmental conditions. During the trial period, the birds had free access to feed and water. During the trial period, the birds were fed three different age-specific diets: starter (1 – 21 days), grower (22–28 days), and finisher (29–35 days). The starter diet included 22.3%, 20.2%, and 20.2% crude protein and 12.45, 13.01, and 13.01 MJ/kg of metabolizable energy, respectively. The dietary mixtures were fed to the chickens following the recommendation of the broiler chicken dietary requirements (Smuliikowska and Rutkowski, 2018). At the beginning of the trial period (first week), the temperature was 32–33°C and was gradually lowered by 2°C per week until reaching about 24°C at day 35 (the end of the rearing period).

3.3.8.2. Growth performance

The weekly assessment of individual chicken BW and FI of each group was carried out using a weighing scale. The (FCR) was recorded and computed as the proportion of feed consumed to weight gain.

3.3.8.3. Carcass traits and meat quality analysis

After the end of the experiment (35 days), 12 chickens per group (6 males and 6 females) were chosen based on their body weight (average body weight of each group) and after 12 hours of food deprivation, however, water was made freely accessible. After that, the birds were sacrificed by decapitation and left to bleed for roughly 90 s. After five min of bleeding, each bird was de-feathered and eviscerated. The carcasses with and without giblets were weighed and the carcass yield was recorded and computed as a percentage of the live weight. Moreover, organs and tissues such as the heart, liver, legbones, and gizzard, with abdominal fat, without abdominal fat, heart, breast muscles and leg muscles (thigh and drumstick) were excised and individually weighed. Next, the percentage of each tissue and

organ was reported as a percentage of the chilled carcass weight with giblets. After air chilling (at 4˚C) the carcasses, the breast muscle, and thigh muscles were used to evaluate the quality of meat. The pH was recorded using a portable CyberScan10 pH meter (Eutech Instruments Pte Ltd., Singapore) at 15 and 24 hours (pH15, pH24)). The color of the meat was assessed and recorded as lightness (L*), redness (a*) and yellowness (b*). Other factors determined included shear force, hardness, springiness, cohesiveness, gumminess, chewiness, resilience, adhesiveness, drip losses, cooking losses, and losses during thawing. The meat quality determination was conducted as described by (Połtowicz et al., 2015).

3.3.8.4. Relative bacterial abundance and cecal histomorphology analysis

During the experimental period, excreta samples (n=8) were taken from each group on days 7, 14, 21, 28 and 34 to evaluate the bacterial composition (selected bacteria) in the gut microbiota at different growth stages of the chickens. On day 35 (day of slaughter), 8 birds from each group were randomly chosen for histological examination, the middle portion of each chicken cecum was secured for histomorphology studies and thus was directly put into a Bouin's solution (HT101128, Sigma-Aldrich, Poland) until it was needed for further processing. The protocol for the histological analysis was performed as reported by (Bogucka et al., 2016) utilizing the paraffin technique. Samples of the caeca - ca. 2 cm long were collected from 8 birds/group. The cecal portions 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, United Kingdom), 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, United Kingdom) into 10 μm thick sections. Next, the slices were placed on a glass slide that had been previously coated with glycerin and egg white. The slides were then de-waxed and hydrated. This was followed by a Periodic Acid-Schiff (PAS) staining (Dubowitz and Brooke, 1973). Microscopic images of the caeca were captured using an Evolution 300 microscope (Delta Optical, Poland) fitted with a digital camera ToupCamTM (TP605100A, ToupTek, China) and saved 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 computed. The surface of the villi was determined using the formula described by (Sakamoto et al., 2000): $(2\pi) \times (VW / 2) \times (VH)$, where VW= villus width, and VH = villus height.

3.3.8.5. Collection of blood plasma and tissue sampling

Immediately after sacrificing the birds on day 35 of rearing (end of rearing period), chickens that were randomly selected for cecal histology analysis (8 birds/group) (n = 24) were utilized for blood plasma collection. Afterward, 2 mL of blood were drawn into K-EDTA tubes and were centrifuged for 15 min at 3,000 × g. Next, all samples were placed on dry ice and transferred to the laboratory and kept at -80°C for future analysis. Furthermore, liver, breast muscle, cecal mucosa and spleen samples from 8 from each group were obtained and preserved in fix RNA (E0280, EURx, Gdańsk, Poland). The samples were then transferred to the laboratory at room temperature, the fixed RNA was drained off, and the tissue samples in tubes were placed in a freezer (-80°C) until further analysis.

3.3.9. Bacterial DNA isolation

The GeneMATRIX Stool DNA Purification Kit (E3575, EURx, Gdańsk, Poland) was used to isolate DNA from the bird's cecal contents and fecal samples. Next, A NanoDrop 2000 spectrophotometer (ThermoScientific, Warsaw, Poland) was used to assess the quantity and quality of the isolated DNA. The integrity of the DNA was then ascertained by gel electrophoresis on a 2% agarose gel. All extracted DNA samples were preserved at -80°C. until they were needed for analysis.

3.3.10. RNA isolation, RT–PCR and qPCR transcriptomic analysis

For the RNA isolation process, tissues were homogenized using a TissueRuptor homogenizer (990890, Qiagen, Wrocław, Poland) and put into a tube filled with 1 mL of RNA extracol solution (E3700, EURx, Gdańsk, Poland). This was followed by centrifuging each sample with 0.2 mL of chloroform (112344305, Chempur, Piekary Śląskie, Poland). The remaining steps of the RNA extraction procedure were performed using a commercial kit (Universal RNA purification kit (E3598, EURx, Gdańsk, Poland). A NanoDrop 2000 spectrophotometer (Thermo Scientific, Warsaw, Poland) was used to measure the quality and quantity of the RNA, and a 2% agarose gel was used to assess the RNA integrity. The RT-PCR procedure was carried out using the smART First Strand cDNA Synthesis Kit (0804, EURx, Poland) following the recommendations of the manufacturer. After RNA extraction, the isolated cDNA of each sample was subsequently diluted to a concentration of 100 ng/µl. Next, 10 µL of total volume was used for RT–qPCR procedure. The reaction mixture contained Maxima SYBR Green qPCR Master Mix (0401, EURx, Gdańsk, Poland), 2 µl of diluted cDNA and 1 µM of each primer. Thermal cycling was performed using a LightCycler II 480 (Roche Diagnostics, Basel, Switzerland). Two technical duplicates of each RT-qPCR were performed on 96-well plates (4TI-0955, AZENTA, Genomed, Warszawa, Poland). The

transcriptomic analysis (qPCR) comprised of an initial denaturation at 95°C for 15 min, then 40 cycles of amplification consisting of denaturation at 95°C for 15 s, annealing at 58°C for 30 s and elongation at 72°C for 30 s. The target genes expression levels were evaluated through geometric means of *ACTB* and *G6PDH* (reference genes). All the genes and tissues analyzed are reported on Table 3. The ΔΔCt method was used for the determination of the expression levels of each gene in a specific tissue. Next, the ΔCt of the control group was deducted from the ΔCt of each of the treatment groups. The ddCT ($2^{-\Delta\Delta Ct}$) method was used to compute the relative fold gene expression of the target genes in each group against the control group.

Table 3: List of target genes used for qPCR gene expression analysis

Tissues	Gene	Primer Sequences (5′-3′)	References
Cecal mucosa	Claudin1 (*CLDN*1)	F:TCTTCATCATTGCAGGTCTGTC R: AACGGGTGTGAAAGGGTCAT	(Slawinska et al., 2019)
	Mucin 6 (*MUC*6)	F: TTCAACATTCAGTTCCGCCG R: TTGATGACACCGACACTCCT	(Slawinska et al., 2019)
	Avian beta- defensin 1 (*AVBD*1)	F: AAACCATTGTCAGCCCTGTG R: TTCCTAGAGCCTGGGAGGAT	(Slawinska et al., 2019)
	Free fatty acid receptor 2 (FFAR2)	F: GCTCGACCCCTTCATCTTCT R: ACACATTGTGCCCCGAATTG	(Slawinska et al., 2019)
	Tight junction-associated protein 1 (*TJAP*1)	F: AGGAAGCGATGAATCCCTGTT R: TCACTCAGATGCCAGATCCAA	(Slawinska et al., 2019)
	Interleukin 1 beta (*IL*1-*B*)	F: GGAGGTTTTTGAGCCCGTC R: TCGAAGATGTCGAAGGACTG	(Dunislawska et al., 2017)
	Interleukin 10 (*IL*10)	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG	(Rothwell et al., 2004)
	Cathelicidin 2 (*CATHL*2)	F: AGGAGAATGGGGTCATCAGG R: GGATCTTTCTCAGGAAGCGG	(Slawinska et al., 2019)
	Glutathione peroxidase-1 (*GPX*-1)	F: TTGTAAACATCAGGGGCAAA R: ATGGGCCAAGATCTTTCTGTAA	(Akbarian et al., 2014)
	Heme oxygenase 1 (*HO*-1)	F: CTCAAGGGCATTCATTCG R: ACCCTGTCTATGCTCCTGTT	(Wu et al., 2019b)
Liver	Nuclear factor erythroid 2-related factor 2 (*NRF*2)	F: ATCACCTCTTCTGCACCGAA R: GCTTTCTCCCGCTCTTTCTG	(Wu et al., 2019b)
	Interleukin 1 beta (*IL*1-*B*)	F: GGAGGTTTTTGAGCCCGTC TCGAAGATGTCGAAGGACTG	(Dunislawska et al., 2017)
	Occludin	F: TCATCCTGCTCTGCCTCATCT R: CATCCGCCACGTTCTTCAC	(Wu et al., 2019a)

Breast muscle	Free fatty acid receptor 4 (*FFAR*4)	F: AGTGTCACTGGTGAGGAGATT R:ACAGCAACAGCATAGGTCAC	(Slawinska et al., 2019)
	Superoxide dismutase 1 (*SOD*1)	F: AGGGGGTCATCCACTTCC R: CCCATTTGTGTTGTCTCCAA	(El-Deep et al., 2014)
	Catalase (*CAT*)	F: GGGGAGCTGTTTACTGCAAG R: CTTCCATTGGCTATGGCATT	(El-Deep et al., 2014)
	Nuclear factor erythroid 2-related factor 2 (*NRF*2)	F: ATCACCTCTTCTGCACCGAA R: GCTTTCTCCCGCTCTTTCTG	(Wu et al., 2019a)
	Manganese superoxide dismutase (MnSOD)	F: TTCCTGACCTGCCTTACGACTAT R: CCAGCGCCTCTTTGTATTTCT	(Li et al., 2011)
	Zonula Occludens 1 (*ZO*-1)	F:CTTCAGGTGTTTCTCTTCCTCCTC R:CTGTGG TTTCATGGCTGG ATC	(Chang et al., 2020)
	Cathelicidin 2 (*CATHL*2)	F: AGGAGAATGGGGTCATCAGG R: GGATCTTTCTCAGGAAGCGG	(Slawinska et al., 2019)
	Interleukin 4 (*IL*4)	F: GCTCTCAGTGCCGCTGATG R: GGAAACCTCTCCCTGGATGTC	(Sławinska et al., 2014)
Spleen	Interleukin 8 (*IL*8)	F: CCACTGCTCCCTGGGTACAG R:TCAGAATTGAGCTGAGCC TTG	(Sławinska et al., 2014)
	Interleukin 12p40 (*IL*12*p*40)	F: TTGCCGAAGAGCACCAGCCG R: CGGTGTGCTCCAGGTCTTGGG	(Brisbin et al., 2010)
Reference genes	Actin, beta *(ACTB*)	F: CACAGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	(Sevane et al., 2014)
	Glucose-6-phosphate dehydrogenase (G6PDH)	F: CGGGAACCAAATGCACTTCGT R: GGCTGCCGTAGAGGTATGGGA	(Sevane et al., 2014)

F: Forward primers, R: Reverse primers

Table 4: Primer sequences used for evaluating the bacteria relative abundance in fecal and cecal content using qPCR

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

F: forward primers, R: reverse primers

The bacteria relative abundance of *Bifidobacteria sp.,* and *Lactobacillus sp*. from excreta samples and cecal contents were subjected to qPCR transcriptomic analysis. All the bacteria were quantified in relation to the universal bacterial quantity in each sample.

A total volume of 12.5 µL was use for the reaction mixture, which included 10–20 ng of DNA, 1 µM of each primer (forward and reverse) (Sigma-Aldrich, Darmstadt, Germany), and 6.25 µl of SG qPCR Master Mix (2x) (0401, EURx, Gdańsk, Poland). The qPCR was carried out on a 96 well plates (4TI-0955, AZENTA, Genomed, Warsawa, Poland). In each sample 2 technical replicates were prepared, and the qPCR was conducted using a Light-Cycler 480 II (Roche-Diagnostics, Rotkreuz, Switzerland. The initial denaturation stage (95˚C) of the qPCR procedure lasted for 5 min and the next step was amplification (40 cycles) and a denaturation step lasting 10s at 95˚C for each amplification. The next step involved annealing for 15 s at 58˚C, and elongation for 30 s at 72˚C. The average Ct values of the two replicates from each sample were computed and used for statistical analysis. The standard curve relevant samples of all treatment groups were determined using 5 dilutions (1x, 0.5x, 0.25x, 0.125x, 0.0625x) of bacterial DNA pooled together from each treatment group. Next, the Light-Cycler 480 II software (Roche-Diagnostics) was used to assess the PCR primer efficiency as prescribed by (Slawinska et al., 2019 a; Wishna-Kadawarage et al., 2024):

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

E universal: the efficiency of qPCR with primers for all bacteria

Ct universal: the Ct values for reaction with primers for all bacteria

E target: the efficiency of qPCR with primers specific for *Bifidobacterium* spp. or *Lactobacillus* spp.

Ct target is the Ct values for reaction with primers for *Bifidobacterium* spp. or *Lactobacillus* spp.

3.3.11. Analysis of blood plasma metabolites

To analyze plasma metabolite concentrations and enzymatic activities from 35-day-old birds (8 bird/experimental), the plasma samples were placed in an automated enzymatic analyzer (Pentra C 400, Axon Lab AG, Germany) for analysis. The laboratory analysis was performed at the Institute of Nutritional Physiology at the Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany. The parameters analyzed were low-density lipoprotein (LDL): A11A01638, high-density lipoprotein (HDL): A11A01636, triglyceride (TG): Kit No. A11A01640 (Horiba ABX), total cholesterol: Kit No. A11A01634, aspartate aminotransferase (AST): Kit No. A11A01629; non-esterified fatty acid (NEFA): Kit No. 434-91795 (Wako Chemicals GmbH, Neuss, Germany), alanine aminotransferase (ALT): A11A01627, uric acid: Kit No. A11A01670, gamma-glutamyl transferase (GGT): Kit No. A11A01630 (Axon Lab AG, Reichenbach, Germany), lactose dehydrogenase (LDH): Kit No. A11A01871 and glucose: Kit No. A11A01667.

3.3.12. Data analysis

Using the Shapiro-Wilk and Levene's test, all datasets were subjected to a normality test to ensure the normal distribution of all data available. Next, a One-Way ANOVA in GraphPad Prism version 10.1.2 (324) was used to analyze the hatchability data, pasgar score, hatchling weight and length, and the in vitro results obtained from the DPPH assay. The body weight of chickens was analyzed using a repeated measures ANOVA taking into account repeated measures over time (7 day, 14 day, 21 day, 28 day and 35 day) in GraphPad Prism. In addition, other parameters such as FI, FCR, slaughter parameters, meat quality and bacterial abundance were analyzed with the aid of a one-way ANOVA analysis. To analyze plasma metabolites, I used principal component analysis (PCA). The Kruskal-Wallis test was used for the analysis of the datasets that did not meet the rule of normal distribution and afterward, the significant differences between the groups were determined. The means between the groups were compared using the Tukey's HSD to determine significant differences between groups ($P < 0.05$). GraphPad Prism version 10.1.2 (324) was used for graphing and visualization of the results. For transcriptomic analysis, the results from the ΔCt values of each group were compared with the control group using GraphPad Prism, while significant changes ($P < 0.05$) between the groups were determined using Student's t-test and Microsoft Excel was used for graphing. In the preparation of the content and drafting of the review article, a thorough literature search was performed in January 2022 using Google Scholar, PubMed, the Web of Science database and use of Google search engine. During the literature search, certain keywords such as heat stress, high

incubation temperature, high temperatures, and the impacts of heat stress effects on chicken production performance, gut health, development of the immune system and the quality of meat were used to select suitable scientific articles for this review. Upon identifying these manuscripts, all were read thoroughly and the papers that did not highlight heat stress effects on production performance, gut health, immune functions or meat quality in the title, abstract, keywords, or results were excluded. In addition, the search focused on broilers and layer chickens, ducks and turkeys.

3.4. Results

3.4.1. DPPH Antioxidant assay

The *in vitro* experiment was conducted to assess the growth rate, and the antioxidant potential of the selected bioactive substances. This served as the basis for subsequent *in vivo* experiments to test the potential benefits of GOS and LP on chicken performance and gut health. The *in vitro* study tested the different *Lactobacillus* species for their antioxidant activities (Table 1). The antioxidant potential of several Lactobacillus species: *Lacticaseibacillus casei* 1.4 x10^6, *Lactiplantibacillus plantarum* 1.0 x10^6, *Lactiplantibacillus plantarum* 1.0 x10^6 and *Limosilactobacillus reuteri* 7.9 x 10^6 are presented in Figure 8. Among the probiotic bacteria evaluated, *Lactiplantibacillus plantarum* 1.0 x 10^6, demonstrated the highest antioxidant potentials with **68.89%** radical scavenging activities ($P < 0.05$). *Limosilactobacillus reuteri* 1.9 x 10^6 and *Lacticaseibacillus rhamnosus* 2.7 x 10^7 exhibited the lowest antioxidant potentials, with radical scavenging activity of **20%** and **17.90%** respectively.

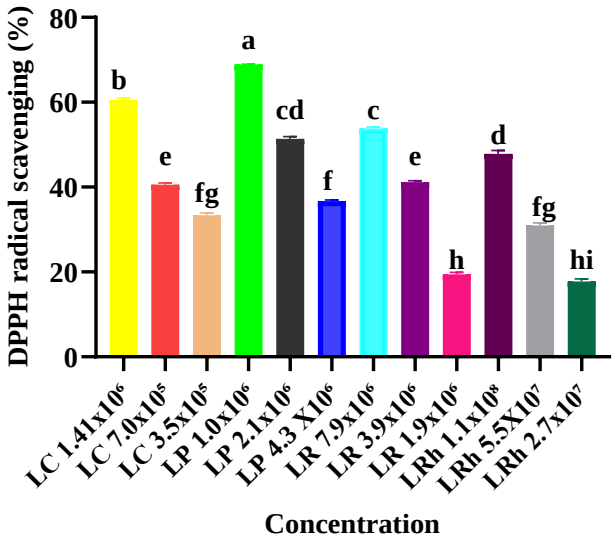

Figure 1: The free radical scavenging ability of each probiotic strain was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The outcomes from this study are presented as means ± SEM (n = 3), distinct letters (a–i) indicate significant differences between treatments ($P < 0.05$). LRh: *Lacticaseibacillus rhamnosus,* LP: *Lactiplantibacillus plantarum, casei* LR*: Limosilactobacillus,* LC*: Lacticaseibacillus*

Based on the results above, the probiotic *Lactiplantibacillus plantarum* 1 x10^6 was chosen for *in ovo* stimulation because of its high antioxidant capacity which could potentially mitigate oxidative stress and subsequently improve chicken gut health and production performance.

3.4.2. In vivo validation of the impact of *in ovo* stimulation of gaalctooligosaccharide and *Lactiplantibacillus plantarum* on gut health and production performance of broiler chickens.

3.4.2.1 Hatchability and chick quality analysis

The hatchability results demonstrated that the *in ovo* delivery of LP or GOS did not impair hatchability and no statistical changes were observed across the treatments. The NC group had the highest hatchability rate (92%). However, among the *in ovo*-injected treatment group, the GOS group *in ovo*-stimulated with 3.5 mg/egg had a numerically higher hatchability rate (90.67%) compared to PC and LP (86.92% and 85.53%) respectively. Statistically, no significant changes were found in Pasgar score and chick length. Surprisingly, the results reported statistical differences ($P < 0.05$) in the BW of the day-old chicks in the LP and GOS treatment groups (50 g and 47 g) as compared to those of the NC and PC (Figure 2).

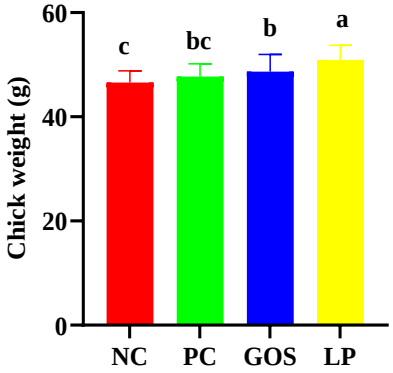

Figure 2. Bodyweight of hatchlings (day-old chicks) of the four *in ovo* treatment groups. Error bars: ± SD. Tukey HSD test ($P < 0.05$) was used to check for statistical differences with different letters a, b, c. NC: Negative control, PC: Positive Control, GOS: Galactooligosaccharides, LP: *Lactiplantibacillus plantarum*.

3.4.2.2. Relative bacterial abundance in excreta samples

The bacterial relative abundance in chicken excreta from different timepoints (Day 7, 21 and 34) was reported in this study. The results indicate a remarkable rise ($P < 0.001$) of the bacterial population of the *Lactobacillus spp.* from days 7, 21 and 34 in the GOS and LP treatments as compared to that of the PC treatment (Figure 3) with GOS having the highest relative bacterial abundance. In addition, no significant changes in the bacterial abundance of *Bifidobacteria* on days 7 and 21 were found across all the groups. However, a day before the termination of the trial period (D34), the relative bacterial

abundance of *Bifidobacteria* spp. was found to be statistically significant ($P < 0.001$) in the chickens *in ovo*-treated with GOS and LP as compared to those of the PC group (Figure 4). The GOS group had the highest *Bifidobacteria* population with the PC group recording the lowest.

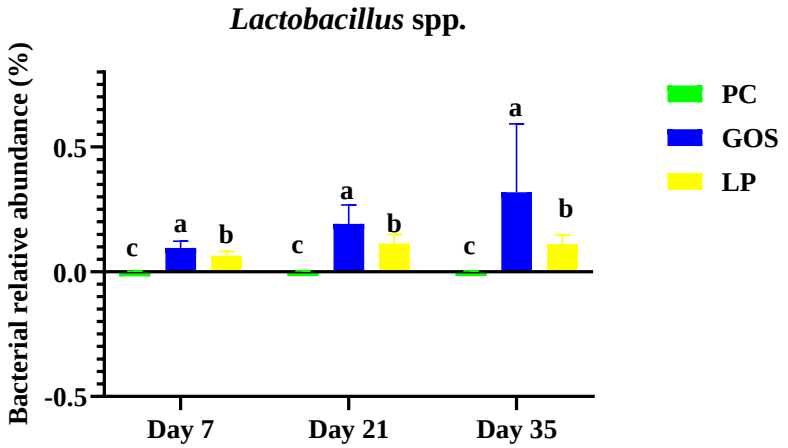

Figure 3. Relative abundance of *Lactobacillus* spp. in the excreta of *in ovo* treated chickens on days 7, 21 and 35. Error bars: ± SE. a, b, c letters that are not similar indicate statistical differences across the treatments ($P < 0.05$) PC: positive control, GOS: Galactooligosaccharide, LP: *Lactiplantibacillus plantarum*.

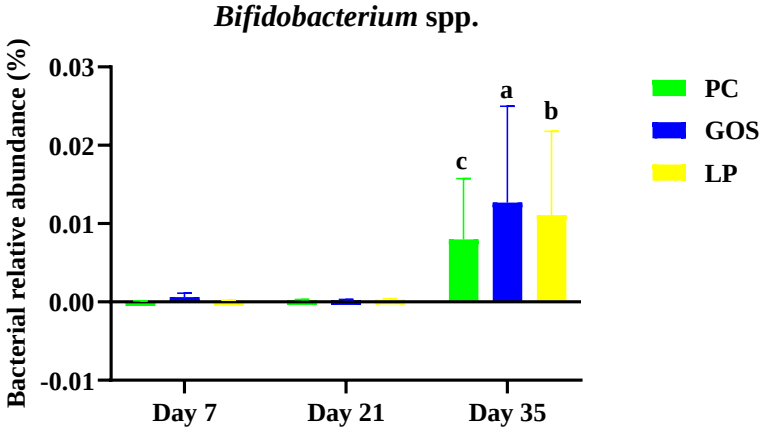

Figure 4. Relative abundance of *Bifidobacterium* spp. in the excreta of *in ovo* treated chickens on days 7, 21 and 35. Error bars: ± SE. a, b, c letters that are not similar indicate statistical differences across the treatments ($P < 0.05$) PC: positive control, GOS: Galactooligosaccharide, LP: *Lactiplantibacillus plantarum*.

3.4.2.3. 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.

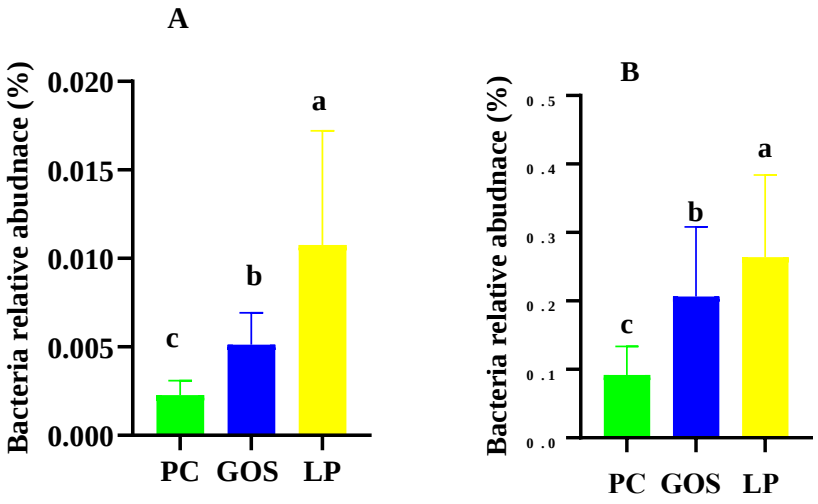

Figure 5. The prevalence of commensal bacteria in the ceca of *in ovo* treated chickens (A) *Lactobacillus* spp. (B) *Bifidobacterium* spp. Error bars: ± SE. a, b, c letters that are not similar indicate statistical differences across the treatments ($P< 0.05$) PC: positive control, GOS: Galactooligosaccharide,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

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).

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

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 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*

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).

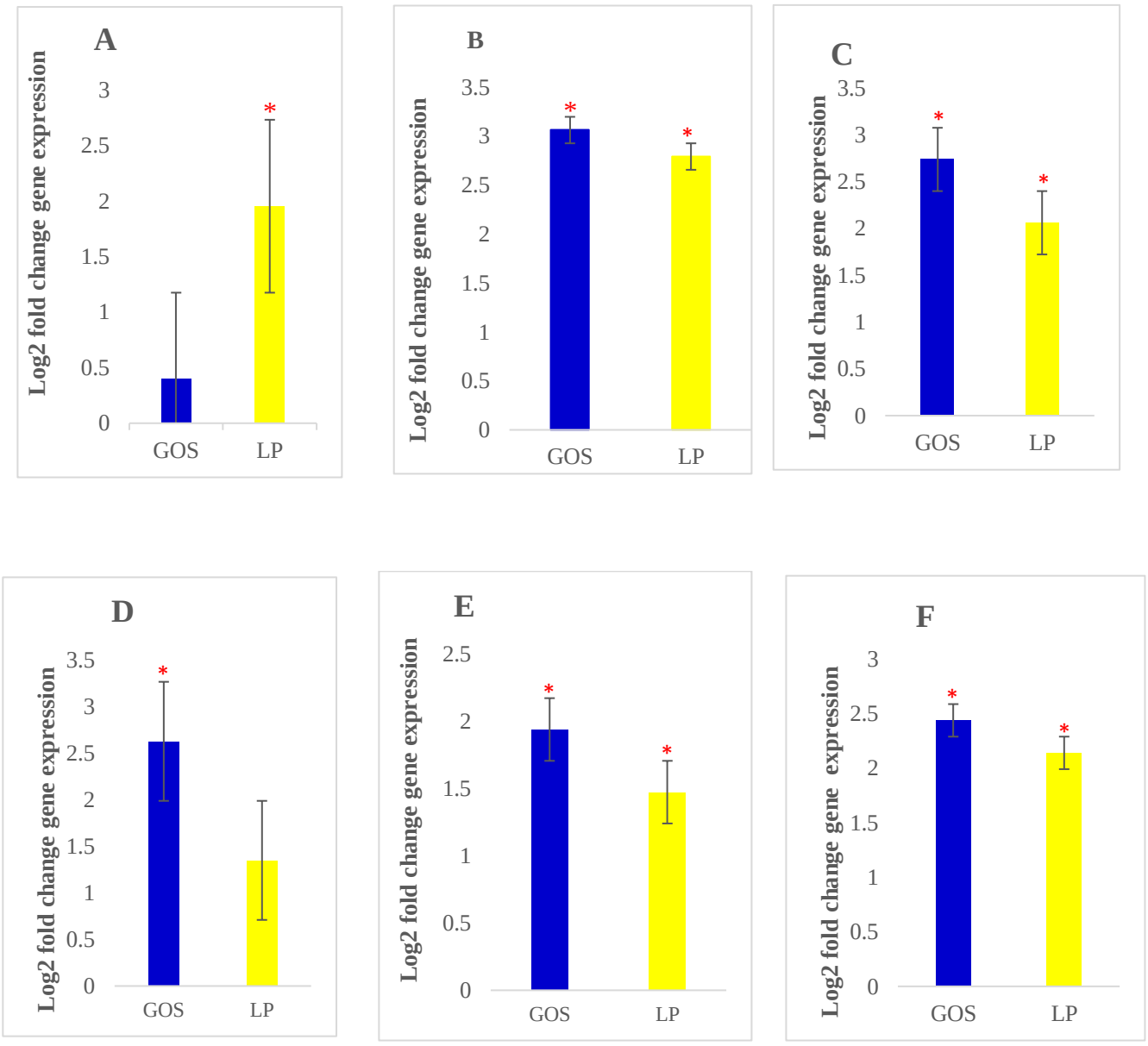

Figure 6: The pattern of gene expression in chicken's cecal mucosa upon *in ovo* delivery of *Lactiplantibacillus plantarum* (LP) and galactooligosaccharide (GOS) (A) *CLDN1*, (B) *MUC6*, (C) *AVBD1*, (D) *FFAR2*, (E) *IL-1β*, and (F) *CATHL2*. Error bars represent ± SE. Red asterisks (*) denote statistical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

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.

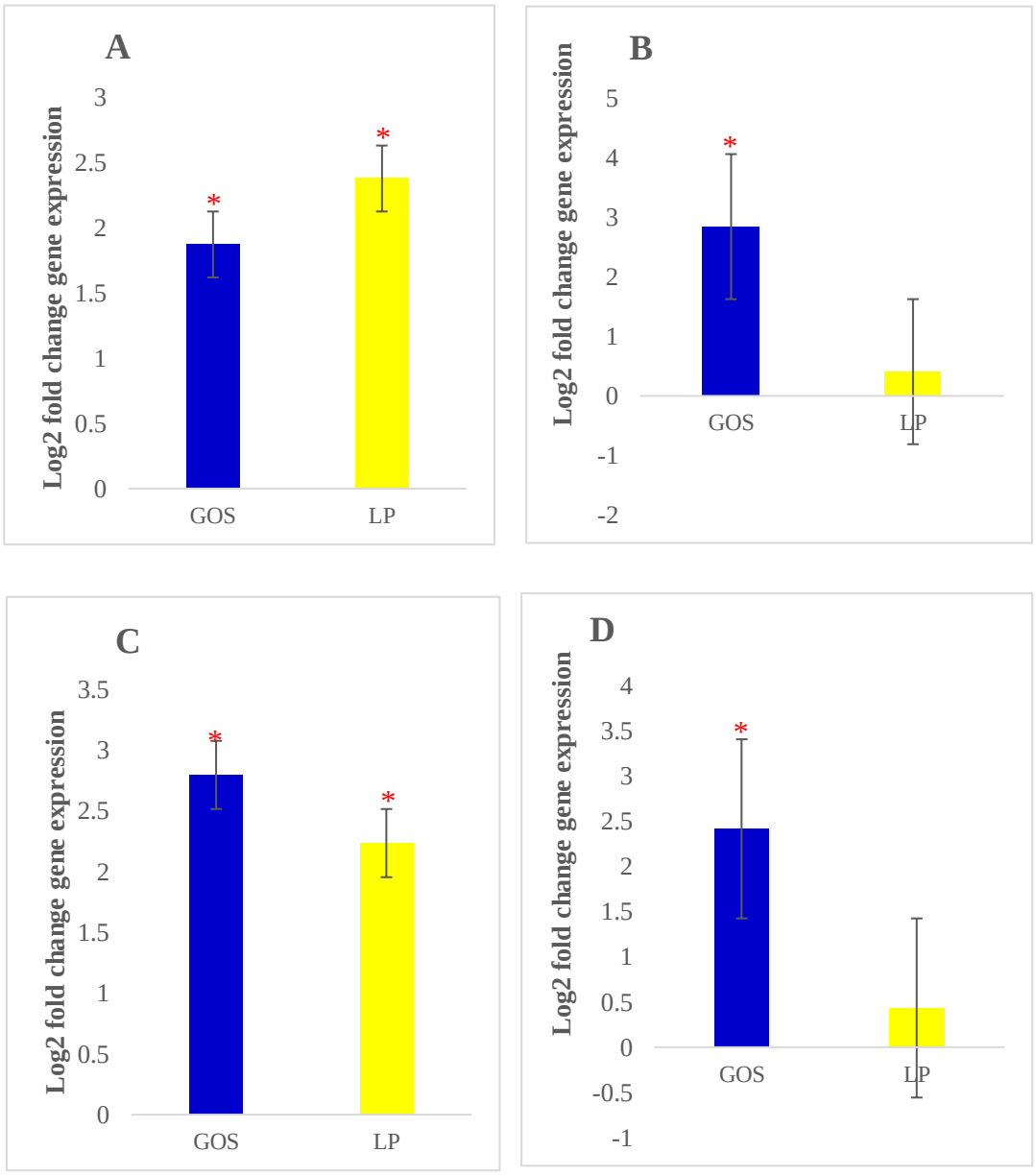

Figure 7: The pattern of gene expression in chicken's spleen upon the administration of *Lactiplantibacillus plantarum* (LP) and galactooligosaccharide (GOS). (A) *SOD*1, (B) *IL12p40*, (C) *IL4*, and (D) *IL8*. Error bars represent ± SE. Red asterisks (*) denote statistical differences ($P < 0.05$).

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.

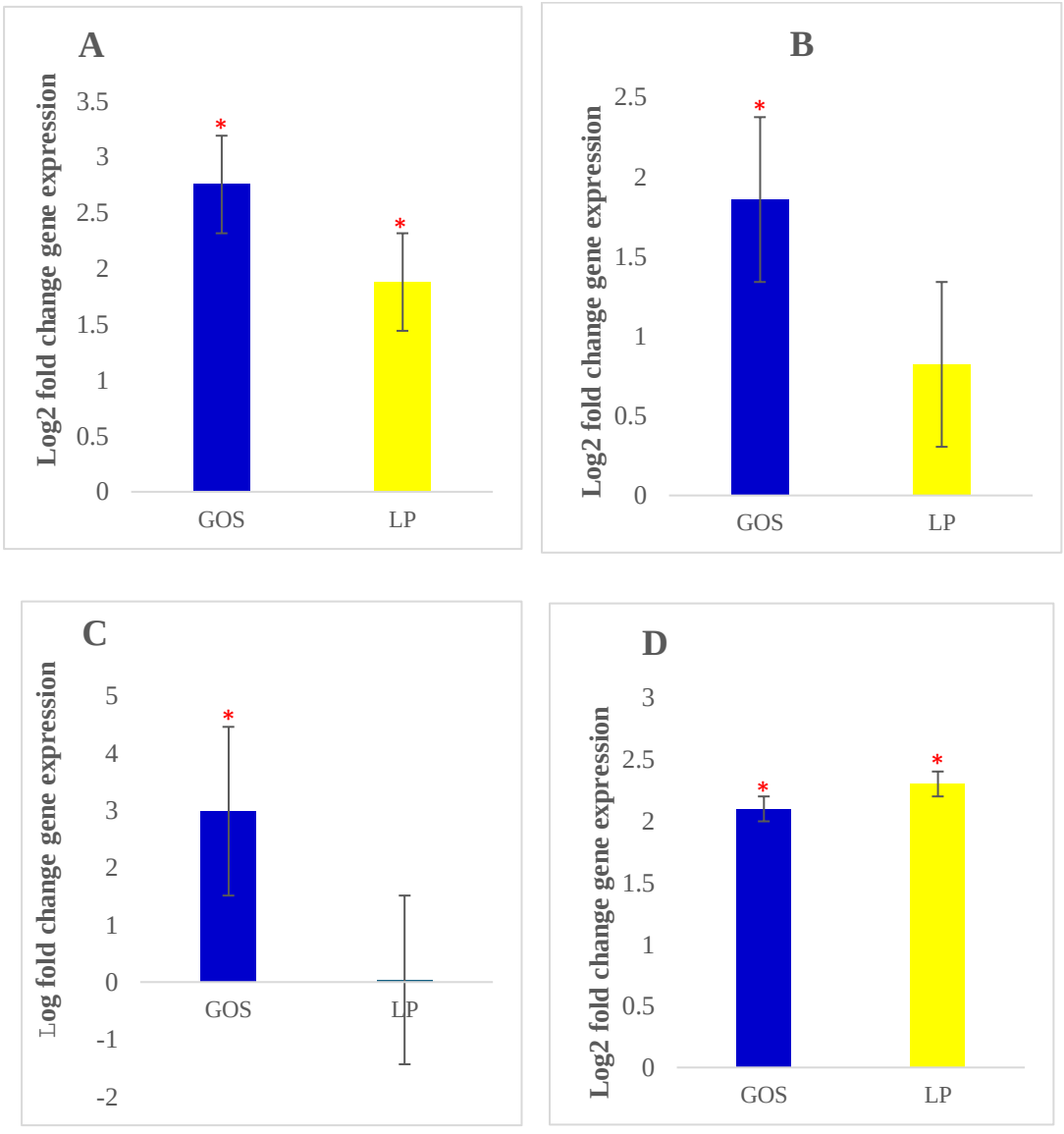

Figure 8: The gene expression pattern in chicken's spleen upon the administration of *Lactiplantibacillus plantarum* (LP) or galactooligosaccharide (GOS). (A) *SOD1*, (B) *MnSOD*, (C) *NRF2*, and (D) *CAT*. Error bars represent ± SE. Red asterisks (*) denote statistical differences ($P < 0.05$).

3.4.2.10. 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.

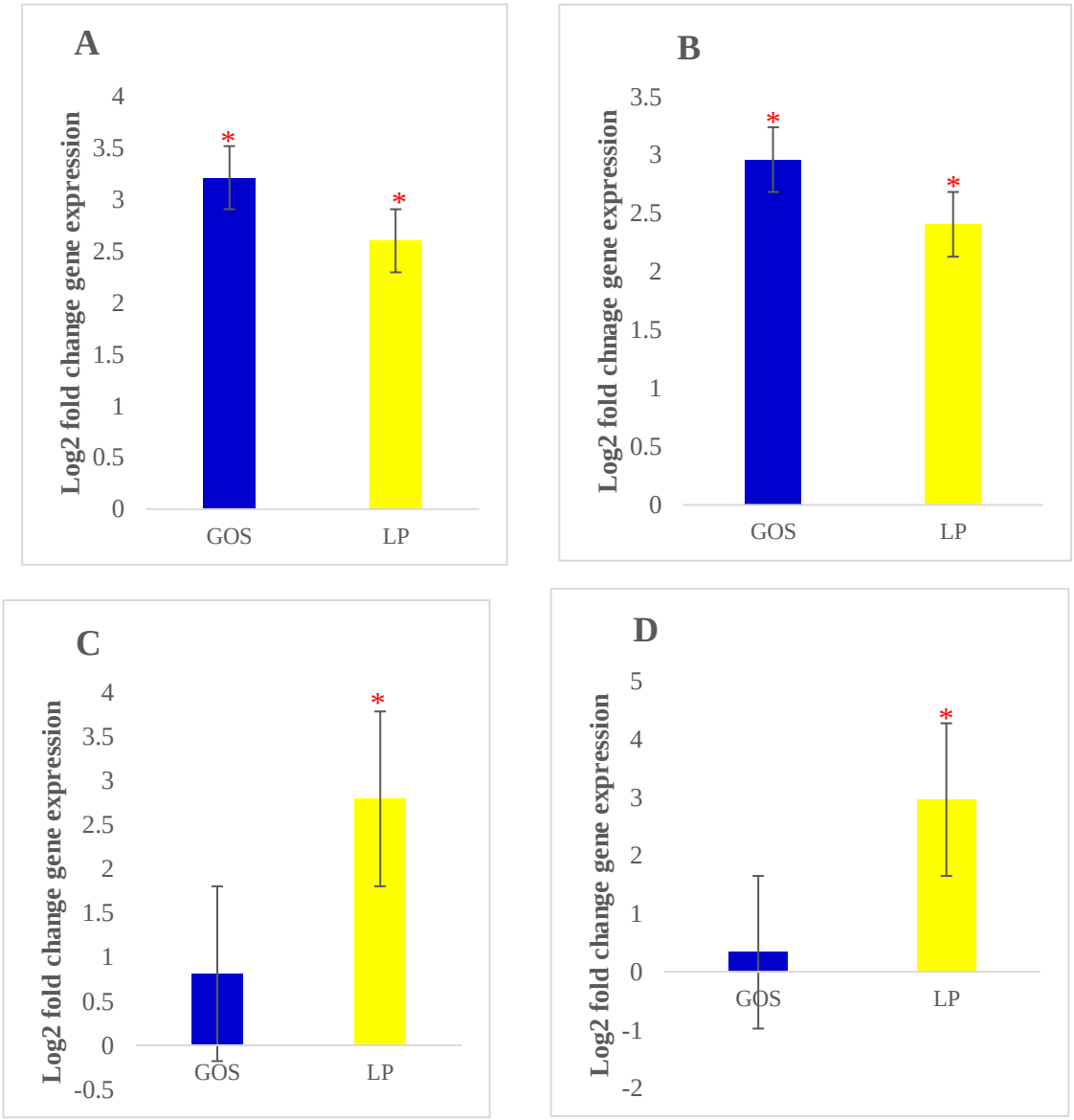

Figure 9: The gene expression patterns in chicken's liver upon *in ovo* administration of *Lactiplantibacillus plantarum* (LP) or galactooligosaccharide (GOS). (A) *GPx1*, (B) *NRF2*, (C) *IL1β*, and (D) *Occludin*. Error bars represent ± SE. Red asterisks (*) denote statistical differences (P < 0.05).

3.4.2.11. 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

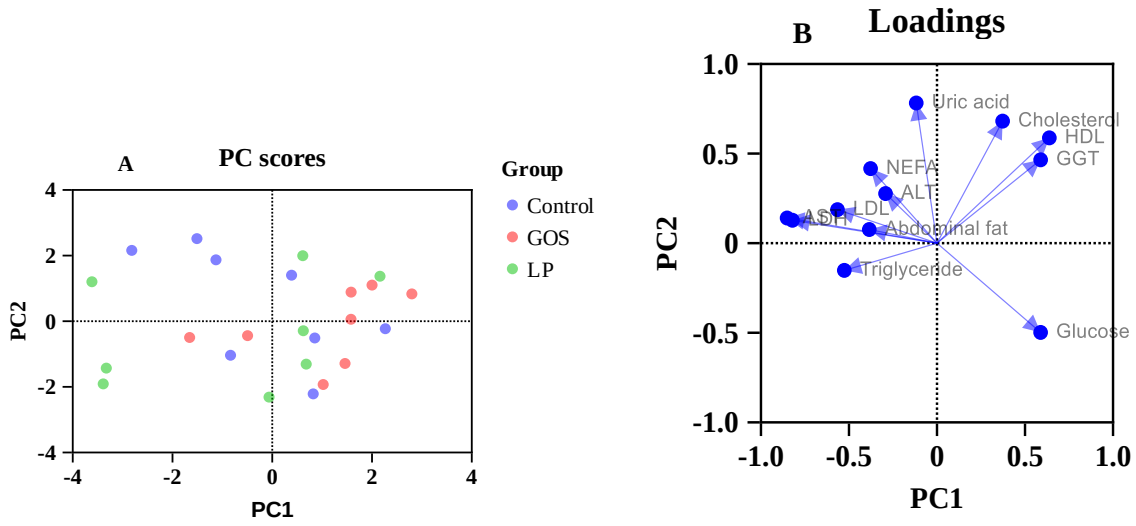

Figure 10: Shows the PC score (**A**) and variables/plasma metabolites (**B**) upon Principal component analysis (PCA). The variables are the parameters measured while the PC scores represent each sample per treatment. Blue: (C) control, Green: (LP) *Lactiplantibacillus plantarum*, and Orange: (GOS)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

3.5. 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

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).

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

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

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).

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).

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.

3.6 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

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.

3.7 References of the dissertation

Ahmed, M. M. N., Z. S. H. Ismail, I. Elwardany, J. Lohakare, and A. A. A. Abdel-Wareth. 2023. In Ovo Feeding Techniques of Green Nanoparticles of Silver and Probiotics: Evaluation of Performance, Physiological, and Microbiological Responses of Hatched One-Day-Old Broiler Chicks. Animals 13:3725.

Akbarian, A., J. Michiels, A. Golian, J. Buyse, Y. Wang, and S. De Smet. 2014. Gene expression of heat shock protein 70 and antioxidant enzymes, oxidative status, and meat oxidative stability of cyclically heat-challenged finishing broilers fedOriganum compactum andCurcuma xanthorrhiza essential oils. Poultry Science 93:1930–1941.

Akosile, O. A., F. O. Kehinde, A. I. Oni, and O. E. Oke. 2023. Potential Implication of *in ovo* Feeding of Phytogenics in Poultry Production. Translational Animal Science 7:txad094.

Arena, M. P., A. Silvain, G. Normanno, F. Grieco, D. Drider, G. Spano, and D. Fiocco. 2016. Use of Lactobacillus plantarum Strains as a Bio-Control Strategy against Food-Borne Pathogenic Microorganisms. Front. Microbiol. 7 Available at https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2016.00464/full (verified 23 November 2024).

Aruwa, C. E., C. Pillay, M. M. Nyaga, and S. Sabiu. 2021. Poultry gut health – microbiome functions, environmental impacts, microbiome engineering and advancements in characterization technologies. J Animal Sci Biotechnol 12 Available at https://jasbsci.biomedcentral.com/articles/10.1186/s40104-021-00640-9 (verified 17 September 2024).

Bahrndorff, S., T. Alemu, T. Alemneh, and J. Lund Nielsen. 2016. The Microbiome of Animals: Implications for Conservation Biology. International Journal of Genomics 2016:1–7.

Bednarczyk, M., A. Dunislawska, K. Stadnicka, and E. Grochowska. 2021. Chicken embryo as a model in epigenetic research. Poultry Science 100:101164.

Bednarczyk, M., K. Stadnicka, I. Kozłowska, C. Abiuso, S. Tavaniello, A. Dankowiakowska, A. Sławińska, and G. Maiorano. 2016. Influence of different prebiotics and mode of their administration on broiler chicken performance. Animal 10:1271–1279.

Behera, S. S., R. C. Ray, and N. Zdolec. 2018. Lactobacillus plantarum with Functional Properties: An Approach to Increase Safety and Shelf-Life of Fermented Foods. Biomed Res Int 2018:9361614.

Bertocchi, M., M. Zampiga, D. Luise, M. Vitali, F. Sirri, A. Slawinska, S. Tavaniello, O. Palumbo, I. Archetti, G. Maiorano, P. Bosi, and P. Trevisi. 2019. In ovo Injection of a Galacto-Oligosaccharide

Prebiotic in Broiler Chickens Submitted to Heat-Stress: Impact on Transcriptomic Profile and Plasma Immune Parameters. Animals 9:1067.

Bilalissi, O. N'nanle, D. Nideou, H. T. Meteyake, Y. A. E. Kouame, E. Decuypere, M. Gbeassor, O. Onagbessan, and K. Tona. 2019. The appropriate time to improve day-old chick production and post-hatch growth through Moringa oleifera leaf extract inoculation into the hatching egg. Europ.Poult.Sci. 83 Available at http://www.european-poultry-science.com/artikel.dll/ROJ_VIEWJUMP?DOI=10.1399/eps.2019.286 (verified 20 December 2023).

Bist, R. B., K. Bist, S. Poudel, D. Subedi, X. Yang, B. Paneru, S. Mani, D. Wang, and L. Chai. 2024. Sustainable poultry farming practices: a critical review of current strategies and future prospects. Poultry Science 103:104295.

Bogucka, J., A. Dankowiakowska, G. Elminowska-Wenda, A. Sobolewska, A. Szczerba, and M. Bednarczyk. 2016. Effects of Prebiotics and Synbiotics Delivered In Ovo on Broiler Small Intestine Histomorphology During the First Days After Hatching. Folia Biol (Krakow) 64:131–143.

Brisbin, J. T., J. Gong, P. Parvizi, and S. Sharif. 2010. Effects of Lactobacilli on Cytokine Expression by Chicken Spleen and Cecal Tonsil Cells. Clin Vaccine Immunol 17:1337–1343.

Chang, C. H., P. Y. Teng, T. T. Lee, and B. Yu. 2020. Effects of multi-strain probiotic supplementation on intestinal microbiota, tight junctions, and inflammation in young broiler chickens challenged with Salmonella enterica subsp. enterica. Asian-Australas J Anim Sci 33:1797–1808.

Cheled-Shoval, S. L., E. Amit-Romach, M. Barbakov, and Z. Uni. 2011. The effect of in ovo administration of mannan oligosaccharide on small intestine development during the pre- and posthatch periods in chickens. Poultry Science 90:2301–2310.

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

Christensen, V. L. 2009. Development during the First Seven Days Post-hatching. Avian Biology Research 2:27–33.

Corrêa-Oliveira, R., J. L. Fachi, A. Vieira, F. T. Sato, and M. A. R. Vinolo. 2016. Regulation of immune cell function by short-chain fatty acids. Clinical & Translational Immunology 5:e73.

Dankowiakowska, A., J. Bogucka, A. Sobolewska, S. Tavaniello, G. Maiorano, and M. Bednarczyk. 2019. Effects of in ovo injection of prebiotics and synbiotics on the productive performance and microstructural features of the superficial pectoral muscle in broiler chickens. Poult Sci 98:5157–5165.

Das, R., P. Mishra, and R. Jha. 2021. In ovo Feeding as a Tool for Improving Performance and Gut Health of Poultry: A Review. Front. Vet. Sci. 8:754246.

Dibner, J. J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poultry Science 84:634–643.

Duan, A., A. Ju, Y. Zhang, Y. Qin, L. Xue, X. Ma, W. Luan, and S. Yang. 2021. The Effects of In Ovo Injection of Synbiotics on the Early Growth Performance and Intestinal Health of Chicks. Front. Vet. Sci. 8:658301.

Dunislawska, A., A. Slawinska, K. Stadnicka, M. Bednarczyk, P. Gulewicz, D. Jozefiak, and M. Siwek. 2017. Synbiotics for Broiler Chickens—In Vitro Design and Evaluation of the Influence on Host and Selected Microbiota Populations following In Ovo Delivery (BA Wilson, Ed.). PLoS ONE 12:e0168587.

El-Deep, D. Ijiri, Y. Z. Eid, H. Yamanaka, and A. Ohtsuka. 2014. Effects of Dietary Supplementation with *Aspergillus Awamori* on Growth Performance and Antioxidative Status of Broiler Chickens Exposed to High Ambient Temperature. J. Poult. Sci. 51:281–288.

Elnagar, R., R. Elkenany, and G. Younis. 2021. Interleukin gene expression in broiler chickens infected by different Escherichia coli serotypes. Vet World:2727–2734.

Fathima, S., R. Shanmugasundaram, D. Adams, and R. K. Selvaraj. 2022. Gastrointestinal Microbiota and Their Manipulation for Improved Growth and Performance in Chickens. Foods 11:1401.

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

Gao, M., Y. Ren, S. Lu, R. Reddyvari, K. Venkitanarayanan, and M. A. Amalaradjou. 2024. In ovo probiotic supplementation supports hatchability and improves hatchling quality in broilers. Poultry Science 103:103624.

Goel, A. 2021. Heat stress management in poultry. Animal Physiology Nutrition 105:1136–1145.

Goel, A., C. M. Ncho, V. Gupta, and Y.-H. Choi. 2023. Embryonic modulation through thermal manipulation and in ovo feeding to develop heat tolerance in chickens. Animal Nutrition 13:150–159.

Guo, W., J. Zhou, Y. Liu, J. Bai, Y. Zhu, X. Yang, and X. Yang. 2023. Embryonic injection of Lactobacillus plantarum PA01 alters the microbial diversity in the gastrointestinal tract of the broilers before and after hatching. Poultry Science 102:102764.

Hou, T., and E. Tako. 2018. The In Ovo Feeding Administration (Gallus Gallus)—An Emerging In Vivo Approach to Assess Bioactive Compounds with Potential Nutritional Benefits. Nutrients 10:418.

Kachouri, F., H. Ksontini, M. Kraiem, K. Setti, M. Mechmeche, and M. Hamdi. 2015. Involvement of antioxidant activity of Lactobacillus plantarum on functional properties of olive phenolic compounds. J Food Sci Technol 52:7924–7933.

Kadam, M. M., M. R. Barekatain, S. K Bhanja, and P. A. Iji. 2013. Prospects of *in ovo* feeding and nutrient supplementation for poultry: the science and commercial applications—a review. J Sci Food Agric 93:3654–3661.

Karaca, B., M. Yilmaz, and U. K. Gursoy. 2022. Targeting Nrf2 with Probiotics and Postbiotics in the Treatment of Periodontitis. Biomolecules 12:729.

Khosravi, A., and S. K. Mazmanian. 2013. Disruption of the gut microbiome as a risk factor for microbial infections. Current Opinion in Microbiology 16:221–227.

Kpodo, K. R., and M. Proszkowiec-Weglarz. 2023. Physiological effects of in ovo delivery of bioactive substances in broiler chickens. Front. Vet. Sci. 10:1124007.

Leão, A. P. A., R. R. Alvarenga, and M. G. Zangeronimo. 2021. In ovo inoculation of probiotics for broiler chickens: Systematic review and meta-analysis. Animal Feed Science and Technology 280:115080.

Li, S., L. Lu, S. Hao, Y. Wang, L. Zhang, S. Liu, B. Liu, K. Li, and X. Luo. 2011. Dietary Manganese Modulates Expression of the Manganese-Containing Superoxide Dismutase Gene in Chickens. The Journal of Nutrition 141:189–194.

Liu, L., L. Li, C. Li, H. Wang, X. Zhang, Q. Ren, H. Zhang, N. Jin, C. Li, and C. Zhao. 2023. Effects of Lactiplantibacillus plantarum LPJZ-658 Supplementation on the Production, Meat Quality, Intestinal Morphology, and Cecal Microbiota of Broilers Chickens. Microorganisms 11:1549.

Lyu, W., L. Zhang, Y. Gong, X. Wen, Y. Xiao, and H. Yang. 2020. Developmental and Tissue Patterns of the Basal Expression of Chicken Avian β-Defensins (S Ahmad, Ed.). BioMed Research International 2020:1–12.

Maiorano, G., A. Sobolewska, D. Cianciullo, K. Walasik, G. Elminowska-Wenda, A. Slawinska, S. Tavaniello, J. Zylinska, J. Bardowski, and M. Bednarczyk. 2012. Influence of in ovo prebiotic and synbiotic administration on meat quality of broiler chickens. Poult Sci 91:2963–2969.

Mangan, M., K. Połtowicz, C. C. Metges, and M. Siwek. 2024a. Modulatory effects of in ovo delivery of galactooligosaccharide and Lactiplantibacillus plantarum on antioxidant capacity, gene expression, and selected plasma metabolite parameters of broiler chickens. J Appl Genetics Available at https://doi.org/10.1007/s13353-024-00931-7 (verified 12 December 2024).

Mangan, M., P. Reszka, K. Połtowicz, and M. Siwek. 2024b. Effects of *Lactiplantibacillus plantarum* and Galactooligosaccharide Administered In Ovo on Hatchability, Chick Quality, Performance, Caecal Histomorphology and Meat Quality Traits of Broiler Chickens. Animal Physiology Nutrition:jpn.14082.

Mangan, M., and M. Siwek. 2024. Strategies to combat heat stress in poultry production—A review. Journal of Animal Physiology and Animal Nutrition 108:576–595.

Mottet, A., and G. Tempio. 2017. Global poultry production: current state and future outlook and challenges. World's Poultry Science Journal 73:245–256.

Mukhtar, N., S. H. Khan, and M. S. Anjum. 2013. Hatchling length is a potential chick quality parameter in meat type chickens. World's Poultry Science Journal 69:889–896.

Ncho, C.-M., A. Goel, C.-M. Jeong, V. Gupta, and Y.-H. Choi. 2021. Effects of In Ovo Feeding of γ-Aminobutyric Acid on Growth Performances, Plasma Metabolites, and Antioxidant Status in Broilers Exposed to Cyclic Heat Stress. Sustainability 13:11032.

Noy, Y., and Z. Uni. 2010. Early nutritional strategies. World's Poultry Science Journal 66:639–646.

OECD-FAO Agricultural Outlook 2023-2032. 2023. OECD Available at https://www.oecd.org/en/publications/2023/07/oecd-fao-agricultural-outlook-2023-2032_859ba0c2.html (verified 18 September 2024).

Oladokun, S., and D. I. Adewole. 2020. In ovo delivery of bioactive substances: an alternative to the use of antibiotic growth promoters in poultry production—a review. Journal of Applied Poultry Research 29:744–763.

Oladokun, S., S. Dridi, and D. Adewole. 2023. An evaluation of the thermoregulatory potential of in ovo delivered bioactive substances (probiotic, folic acid, and essential oil) in broiler chickens. Poultry Science 102:102602.

Oviedo-Rondón, E. O. 2019. Holistic view of intestinal health in poultry. Animal Feed Science and Technology 250:1–8.

Pedroso, A. A., A. B. Batal, and M. D. Lee. 2016. Effect of in ovo administration of an adult-derived microbiota on establishment of the intestinal microbiome in chickens. ajvr 77:514–526.

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

Pietrzak, E., A. Dunislawska, M. Siwek, M. Zampiga, F. Sirri, A. Meluzzi, S. Tavaniello, G. Maiorano, and A. Slawinska. 2020. Splenic Gene Expression Signatures in Slow-Growing Chickens Stimulated in Ovo with Galactooligosaccharides and Challenged with Heat. Animals 10:474.

Połtowicz, K., J. Nowak, and D. Wojtysiak. 2015. Effect of Feed Restriction on Performance, Carcass Composition and Physicochemical Properties of the M. Pectoralis Superficialis of Broiler Chickens. Annals of Animal Science 15:1019–1029.

Rothwell, L., J. R. Young, R. Zoorob, C. A. Whittaker, P. Hesketh, A. Archer, A. L. Smith, and P. Kaiser. 2004. Cloning and Characterization of Chicken IL-10 and Its Role in the Immune Response to *Eimeria maxima*. The Journal of Immunology 173:2675–2682.

Sakamoto, K., H. Hirose, A. Onizuka, M. Hayashi, N. Futamura, Y. Kawamura, and T. Ezaki. 2000. Quantitative Study of Changes in Intestinal Morphology and Mucus Gel on Total Parenteral Nutrition in Rats. Journal of Surgical Research 94:99–106.

Schijns, V. E. J. C., S. Van De Zande, B. Lupiani, and S. M. Reddy. 2014. Practical Aspects of Poultry Vaccination.Pages 345–362 in Avian Immunology. Elsevier.

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

Seal, B. S., H. S. Lillehoj, D. M. Donovan, and C. G. Gay. 2013. Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. Anim. Health. Res. Rev. 14:78–87.

Sevane, N., F. Bialade, S. Velasco, A. Rebolé, M. L. Rodríguez, L. T. Ortiz, J. Cañón, and S. Dunner. 2014. Dietary Inulin Supplementation Modifies Significantly the Liver Transcriptomic Profile of Broiler Chickens (MFw Te Pas, Ed.). PLoS ONE 9:e98942.

Shehata, A. M., V. K. Paswan, Y. A. Attia, A.-M. E. Abdel-Moneim, M. Sh. Abougabal, M. Sharaf, R. Elmazoudy, W. T. Alghafari, M. A. Osman, M. R. Farag, and M. Alagawany. 2021. Managing Gut Microbiota through In Ovo Nutrition Influences Early-Life Programming in Broiler Chickens. Animals 11:3491.

Shehata, A. A., S. Yalçın, J. D. Latorre, S. Basiouni, Y. A. Attia, A. Abd El-Wahab, C. Visscher, H. R. El-Seedi, C. Huber, H. M. Hafez, W. Eisenreich, and G. Tellez-Isaias. 2022. Probiotics, Prebiotics, and Phytogenic Substances for Optimizing Gut Health in Poultry. Microorganisms 10:395.

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

Slawinska, A., A. Dunislawska, A. Plowiec, M. Radomska, J. Lachmanska, M. Siwek, S. Tavaniello, and G. Maiorano. 2019. Modulation of microbial communities and mucosal gene expression in chicken intestines after galactooligosaccharides delivery In Ovo (S-B Wu, Ed.). PLoS ONE 14:e0212318.

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

Slawinska, A., M. Zampiga, F. Sirri, A. Meluzzi, M. Bertocchi, S. Tavaniello, and G. Maiorano. 2020. Impact of galactooligosaccharides delivered in ovo on mitigating negative effects of heat stress on performance and welfare of broilers. Poultry Science 99:407–415.

Smialek, M., E. Kaczorek, E. Szczucińska, S. Burchardt, J. Kowalczyk, B. Tykałowski, and A. Koncicki. 2018. Evaluation of Lactobacillus spp. and yeast based probiotic (Lavipan) supplementation for the reduction of Salmonella Enteritidis after infection of broiler chickens. Polish Journal of Veterinary Sciences:5–10.

Smuliikowska and Rutkowski. 2018. Recommended allowances and nutritive... - Google Scholar. Available at https://scholar.google.com/scholar_lookup?title=Recommended%20Allowances%20and%20Nutritive%20Value%20of%20Feedstuffs%20for%20Poultry&publication_year=2018&author=S.%20Smulikowska&author=A.%20Rutkowski (verified 16 October 2024).

Sobolewska, A., G. Elminowska-Wenda, J. Bogucka, A. Dankowiakowska, A. Kułakowska, A. Szczerba, K. Stadnicka, M. Szpinda, and M. Bednarczyk. 2017. The influence of in ovo injection with the prebiotic DiNovo® on the development of histomorphological parameters of the duodenum, body mass and productivity in large-scale poultry production conditions. J Animal Sci Biotechnol 8:45.

Sozcu, A., and A. Ipek. 2015. Quality assessment chicks from different hatcher temperatures with different scoring methods and prediction of broiler growth performance. Journal of Applied Animal Research 43:409–416.

Surai, Kochish, Fisinin, and Kidd. 2019. Antioxidant Defence Systems and Oxidative Stress in Poultry Biology: An Update. Antioxidants 8:235.

Tako, E., R. P. Glahn, M. Knez, and J. C. Stangoulis. 2014. The effect of wheat prebiotics on the gut bacterial population and iron status of iron deficient broiler chickens. Nutr J 13:58.

Tannock, G. W., A. Tilsala-Timisjarvi, S. Rodtong, J. Ng, K. Munro, and T. Alatossava. 1999. Identification of *Lactobacillus* Isolates from the Gastrointestinal Tract, Silage, and Yoghurt by 16S-23S rRNA Gene Intergenic Spacer Region Sequence Comparisons. Appl Environ Microbiol 65:4264–4267.

Tavaniello, S., D. De Marzo, M. Bednarczyk, M. Palazzo, S. Zejnelhoxha, M. Wu, M. Peng, K. Stadnicka, and G. Maiorano. 2023. Influence of a Commercial Synbiotic Administered In Ovo and In-Water on Broiler Chicken Performance and Meat Quality. Foods 12:2470.

Trapani, L., M. Segatto, and V. Pallottini. 2012. Regulation and deregulation of cholesterol homeostasis: The liver as a metabolic "power station." World Journal of Hepatology 4:184.

Uni, Z., P. R. Ferket, E. Tako, and O. Kedar. 2005. In ovo feeding improves energy status of late-term chicken embryos. Poultry Science 84:764–770.

Villaluenga, C. M., M. Wardeńska, R. Pilarski, M. Bednarczyk, and K. Gulewicz. 2004. Utilization of the chicken embryo model for assessment of biological activity of different oligosaccharides. folia biol (krakow) 52:135–142.

Wiersema, M. L., L. R. Koester, S. Schmitz-Esser, and D. A. Koltes. 2021. Comparison of intestinal permeability, morphology, and ileal microbial communities of commercial hens housed in conventional cages and cage-free housing systems. Poultry Science 100:1178–1191.

Willemsen, H., M. Debonne, Q. Swennen, N. Everaert, C. Careghi, H. Han, V. Bruggeman, K. Tona, and E. Decuypere. 2010. Delay in feed access and spread of hatch: importance of early nutrition. World's Poultry Science Journal 66:177–188.

Williams, C. J., and A. S. Zedek. 2010. Comparative field evaluations of in ovo applied technology. Poultry Science 89:189–193.

Wishna-Kadawarage, R. N., K. Połtowicz, A. Dankowiakowska, R. M. Hickey, and M. Siwek. 2024. Prophybiotics for in-ovo stimulation; validation of effects on gut health and production of broiler chickens. Poultry Science 103:103512.

Wu, Y., B. Wang, Z. Zeng, R. Liu, L. Tang, L. Gong, and W. Li. 2019a. Effects of probiotics Lactobacillus plantarum 16 and Paenibacillus polymyxa 10 on intestinal barrier function, antioxidative capacity, apoptosis, immune response, and biochemical parameters in broilers. Poultry Science 98:5028–5039.

Wu, B., Y. Wu, and W. Tang. 2019b. Heme Catabolic Pathway in Inflammation and Immune Disorders. Front. Pharmacol. 10:825.

Yang, S., Y. Qin, X. Ma, W. Luan, P. Sun, A. Ju, A. Duan, Y. Zhang, and D. Zhao. 2021. Effects of in ovo Injection of Astragalus Polysaccharide on the Intestinal Development and Mucosal Immunity in Broiler Chickens. Front. Vet. Sci. 8:738816.

Yang, C., S. Wang, Q. Li, R. Zhang, Y. Xu, and J. Feng. 2024. Effects of Probiotic Lactiplantibacillus plantarum HJLP-1 on Growth Performance, Selected Antioxidant Capacity, Immune Function Indices in the Serum, and Cecal Microbiota in Broiler Chicken. Animals 14:668.

Yu, H., W. Zou, X. Wang, G. Dai, T. Zhang, G. Zhang, K. Xie, J. Wang, and H. Shi. 2020. Research Note: Correlation analysis of interleukin-6, interleukin-8, and C-C motif chemokine ligand 2 gene expression in chicken spleen and cecal tissues after Eimeria tenella infection in vivo. Poultry Science 99:1326–1331.

Zhang, G., and L. Sunkara. 2014. Avian Antimicrobial Host Defense Peptides: From Biology to Therapeutic Applications. Pharmaceuticals 7:220–247.

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

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

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.

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

odpornościowym. Dodatkowo, parametry wydajnościowe, takie jak wskaźnik wylęgowości, masa ciała, konwersja paszy oraz jakość mięsa, utrzymały się na stabilnym poziomie.

Podsumowując, stymulacja *in ovo* galaktooligosacharydem (3,5 mg/jajo) oraz *Lactiplantibacillus plantarum* (1×10^6/jajo) może być skuteczną metodą wspierania zdrowia jelit, poprawy wydajności produkcyjnej oraz ogólnego dobrostanu kurcząt brojlerów w nowoczesnej hodowli drobiu.

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

6. ATTACHMENTS (MANDATORY)

6.1. Copies of scientific articles constituting a series of scientific publications of a doctoral dissertation

Received: 16 June 2023 | Accepted: 12 December 2023

DOI: 10.1111/jpn.13916

REVIEW

Poultry

Journal of Animal Physiology and Animal Nutrition

WILEY

Strategies to combat heat stress in poultry production—A review

Modou Mangan | Maria Siwek

Department of Animal Biotechnology and Genetics, Bydgoszcz University of Science and Technology, Bydgoszcz, Poland

Correspondence

Modou Mangan

Email: Modou.Mangan@pbs.edu.pl

Funding information

This work was supported and funded by the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement [Number 955374] MonoGutHealth project.

Abstract

The effects of heat stress (HS) caused by high temperatures continue to be a global concern in poultry production. Poultry birds are homoeothermic, however, modern-day chickens are highly susceptible to HS due to their inefficiency in dissipating heat from their body due to the lack of sweat glands. During HS, the heat load is higher than the chickens' ability to regulate it. This can disturb normal physiological functioning, affect metabolism and cause behavioural changes, respiratory alkalosis and immune dysregulation in birds. These adverse effects cause gut dysbiosis and, therefore, reduce nutrient absorption and energy metabolism. This consequently reduces production performances and causes economic losses. Several strategies have been explored to combat the effects of HS. These include environmentally controlled houses, provision of clean cold water, low stocking density, supplementation of appropriate feed additives, dual and restricted feeding regimes, early heat conditioning and genetic selection of poultry lines to produce heat-resistant birds. Despite all these efforts, HS still remains a challenge in the poultry sector. Therefore, there is a need to explore effective strategies to address this long-lasting problem. The most recent strategy to ameliorate HS in poultry is early perinatal programming using the in ovo technology. Such an approach seems particularly justified in broilers because chick embryo development (21 days) equals half of the chickens' posthatch lifespan (42 days). As such, this strategy is expected to be more efficient and cost-effective to mitigate the effects of HS on poultry and improve the performance and health of birds. Therefore, this review discusses the impact of HS on poultry, the advantages and limitations of the different strategies. Finally recommend a promising strategy that could be efficient in ameliorating the adverse effects of HS in poultry.

KEYWORDS

bioactive substance, heat stress, high temperature, mitigation, poultry production

1 | INTRODUCTION

The continuous increase in environmental temperatures and global warming presents a major threat to the success of the poultry industry (Chang et al., 2020; Lara & Rostagno, 2013; Sohail et al., 2012; Wasti et al., 2021). The optimum temperature range (thermo-neutral zone) to enhance broiler performance and health are 33–32°C for the 1st, 32–28°C for the 2nd, 28–26°C for the 3rd, 26–24°C for the 4th, 18–24°C for the 5th and 18–24°C for 6th weeks of age (Cassuce et al., 2013). Heat stress (HS) is a condition in which animals are unable to dissipate excess heat in their bodies to the surrounding environment resulting in an increase in body temperature (Sugiharto, 2020). HS in poultry production can be described as acute (short and sudden periods of extremely high temperatures) or chronic (extended periods of increased environmental temperatures) (Kpomasse et al., 2021). Both types of HS can subsequently lead to serious physiological problems, immune suppression and gut microbial imbalance (Attia et al., 2018; Chang et al., 2020; Lara & Rostagno, 2013; Sohail et al., 2012; Wasti et al., 2021). Since birds lack sweat glands, they resort to reducing their physical and behavioural activities (less walking, resting, staying near shady areas, wing spreading, dustbathing), feed intake to reduce heat production, as well as increasing their panting and water consumption to dissipate heat loss by evaporation (Renaudeau et al., 2012). As a result, the detrimental effects of HS greatly reduce production performance, health and food safety. This subsequently causes high morbidity, mortality and consequently leading to economic losses in the poultry sector (He et al., 2018; Lara & Rostagno, 2013; Pawar et al., 2016; Tavaniello et al., 2020). It is reported that in the United States, an amount of $128–165 million is lost from the poultry industry annually due to HS effects (He et al., 2018; Kim et al., 2021; St-Pierre et al., 2003), therefore, it is essential to find mitigatory strategies that will be able to ameliorate the detrimental effects of HS.

Over the years, numerous researchers have reported several intervention strategies to attenuate the negative effects of HS (Attia & Hassan, 2017; Goel, 2021; Nawab et al., 2018; Pawar et al., 2016). In other to alleviate the detrimental effects of HS, a holistic and multifactorial strategies are required. These strategies includes housing (Nawab et al., 2018; Oloyo & Ojerinde, 2020; Pawar et al., 2016), management practices (Saeed, Abbas, et al., 2019), genetic selection (Tóth et al., 2021), feeding and nutrition, thermal manipulation (Al-Zghoul & El-Bahr, 2019; Goel et al., 2023; Ncho, Goel, Jeong, Youssouf, et al., 2021; Sgavioli et al., 2019), reduce stocking density (Shakeri et al., 2014), embryonic manipulation (Elnesr et al., 2019; Han et al., 2018), early heat conditioning (EHC). As discussed in this review (see subchapter Strategies to mitigate HS in poultry), these strategies offer several advantages to mitigate the adverse effects of HS in poultry however they comes with consequences and limitations. As a result, in recent years, the in ovo administration of bioactive substances for alleviating the adverse effects of HS has gain a lot of attention among researchers (Bednarczyk et al., 2016; Das et al., 2021; Goel, 2021; Sgavioli et al., 2019). This is due to the fact that when bioactive substances are administered in ovo, they can lead to early gut colonization by increasing the number of beneficial bacteria while reducing the harmful ones, improving production performance, physiological and immune functions, as well as improving heat tolerant from embryonic development (ED) to the final age of birds (Attia et al., 2018; Das et al., 2021; Ncho, Goel, Jeong, Gupta, et al., 2021; Pietrzak et al., 2020; Slawinska et al., 2019, 2020).

Thanks to the progress in knowledge of genetic selection and nutrition, the market age of broilers continuous to reduce. Chickens spends about 30%–40% of their overall lifespan inside the egg (Hulet et al., 2007). Therefore the importance of the embryonic life stage has gathered significant importance. The reprogramming of the developing embryo is reported to positively influence the development of the embryo and chickens later in life. In light of the above information, in ovo administration of bioactive substances have been practiced over the past few decades (Das et al., 2021; Ncho, Goel, Jeong, Youssouf, et al., 2021; Slawinska et al., 2020). Several researchers reported that the supplementation of nutrients inside the eggs during ED has been demonstrated to improve performance, health and immunity (Attia et al., 2018; Goel et al., 2017; Kim et al., 2021; Pietrzak et al., 2020). Recently, in ovo administration of bioactive substances has been explored to relieve the adverse effects of HS in chickens (Han et al., 2018; Ncho, Goel, Jeong, Gupta, et al., 2021, 2022; Slawinska et al., 2020).

Therefore in this present review, we aim to assess the effects of HS on poultry performance and health, as well as to evaluate different intervention strategies to mitigate the deleterious effects HS on poultry. Further, this review will discuss the advantages and limitations of each strategy and suggest the strategy that will be the most suitable approach to alleviate HS in poultry production.

2 | WHAT ARE THE PHYSIOLOGICAL, BIOLOGICAL AND BEHAVIOURAL MECHANISMS CHICKENS USE TO ADAPT TO HS?

In poultry, especially broilers, physiological responses are the immediate reactions to external stimuli. Therefore chickens utilize physiological responses as the first-line defense mechanisms to combat HS. To cope with HS, chickens are involved in different activities to regulate their body temperature. Their featherless body parts often act as thermal windows used as a thermoregulator to dissipate heat (Shakeri & Le, 2022; Yalcin et al., 1997). Besides physiological responses, chickens use several adaptive behaviours to cope with HS. To regulate their body temperature, chickens spread their wing, keep their wings away from the body, ruffling their feathers, dustbathing and less movement. Chickens tend to drink more and increase resting time while reducing feed intake (Nawab et al., 2018; Shakeri & Le, 2022). Due to their high sensitivity to HS, chickens will seek cooler parts of the house to avoid the high ambient temperatures. While these early responses are effective in assisting chickens to maintain their body temperature within a comfortable range, they may prove insufficient during extremely high

environmental temperatures. With regard to these, other biological mechanisms are necessary to regulate their body temperature (Shakeri & Le, 2022).

Due to the lack of sweat glands, chickens cannot cool their bodies down like humans. However, chickens have a unique respiratory system that involves air sacs connected to their lungs. The air sacs are not directly part of the lungs but are associated structures. These air sacs are linked to pneumatic bones through diverticula, enhancing the bird's efficient respiratory process. The presence of air sacs allows for a continuous and unidirectional flow of air through the respiratory system, facilitating efficient gas exchange and supporting the high metabolic demands of birds (Onagbesan et al., 2023; Wu et al., 2023). It is important to point out that the increased levels of peripheral blood flow reduce excessive produced heat, and increased panting facilitates body cooling through evaporation (Attia et al., 2018; Goel, 2021; Pawar et al., 2016). Chickens use these two biological responses to cope and withstand HS. However, this requires a commensurate reduction of blood flow from other areas which is primarily obtained from the chicken core body. When chickens are exposed to HS, they use these mechanisms to reduce their body temperature. However, there are negative effects of these mechanisms as they tend to have adverse effects on poultry and poultry products (Goel, 2021; Shakeri et al., 2019, 2020; Wasti et al., 2020).

3 | DETRIMENTAL EFFECTS OF HS

The daily increase of environmental temperature around the globe and the continuous expansion of the poultry industry in the arid, tropical and subtropical areas poses a major threat in the poultry industry (Attia et al., 2017, 2018; Nawab et al., 2018; Pawar et al., 2016). As shown in Figure 1, HS disturb normal physiological function, suppress immune system, causes respiratory alkalosis, decreases feed intake and consequently decreased body weight and quantity and quality of eggs (Attia et al., 2018; Awad et al., 2020; Barrett et al., 2019; Saeed, Abbas, et al., 2019). In addition, gut integrity comprised of enterocytes and tight junctions is usually negatively influenced by HS (Zhang et al., 2017). The initiation of immune inflammation takes place due to the penetration of microbes arising from challenged gut health leading to disease infections and eventually raising food safety issues (Galarza-Seeber et al., 2016; Pawar et al., 2016; Zhang et al., 2017). Furthermore, HS causes undesirable meat characteristics and subsequently leads to poor meat quality. These detrimental effects of HS on chickens are discussed below (Nawab et al., 2018; Shakeri et al., 2020).

4 | HS EFFECTS ON CHICKEN PHYSIOLOGICAL RESPONSE

As shown in Figure 1, the physiological functions of chickens are negatively affected by increase temperatures at any stage of their life which in the ends affects their performance. The physiological behaviour has an impact on the growth rate and the production costs (Neves et al., 2014). It is reported that 1-day-old chicks have a fast metabolism and growth rate, which makes its difficult to adapt to the continuous increase of environmental temperatures (Nawab et al., 2018). As mentioned earlier, chicken lacks sudoriferous glands and this makes them to be very sensitive to HS (Goel, 2021). When chickens are exposed to HS the initial response involves the activation

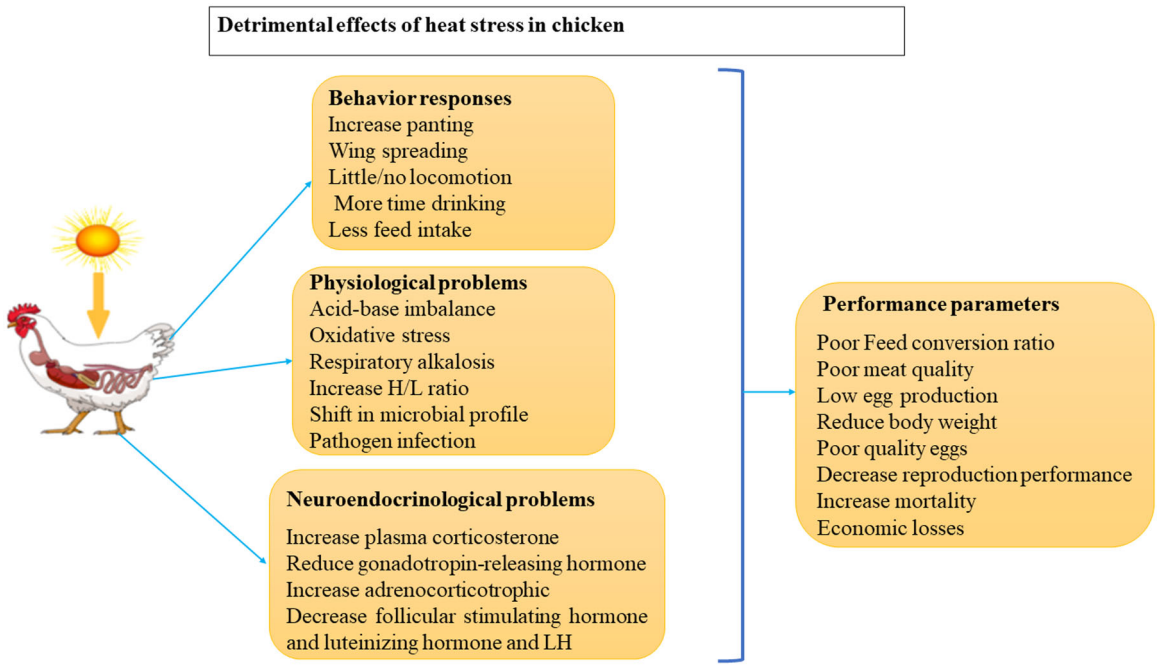

FIGURE 1 The response of chickens to heat stress and how heat stress adversely affects chickens. [Color figure can be viewed at wileyonlinelibrary.com]

doi:10.5941483

of the neurogenic system (Løtvedt et al., 2017). During stress, the neurogenic system triggers the elevation of blood sugar levels, increase respiration, muscle tone and nerve sensitivity, such a response and activation of the neurogenic system leads to the secretion of two key hormones which are mediated by epinephrine and norepinephrine. In addition to the neurogenic response, high heat waves affect the hypothalamic–pituitary–adrenal cortical system and eventually release corticotrophins-releasing factor (Løtvedt et al., 2017). This hormone acts as a messenger and send signal to the pituitary gland to release an adrenocorticotropic hormone (ACTH) (Løtvedt et al., 2017). ACTH is released from the pituitary while corticosteroid is secreted from the adrenal cortical tissue. Increased levels of corticosteroids in the bloodstream can have a major effect on several physiological processes which includes glucose synthesis, mineral metabolism and this can result to hypercholesterolemia, changes in immune functions, cardiovascular diseases, gastrointestinal (GI) lesions (Binsiya et al., 2017). In other to regulate homoeostasis during HS, animals use several mechanisms such as a conduction, convection and evaporative heat loss which involve vasodilatation and perspiration (Pawar et al., 2016). HS alters the metabolic function of chickens leading to an increase production of glucose to maintain homeostasis in organisms that are under HS condition. During HS, the air sacs play an important role in facilitating gaseous exchange by enhancing air circulation toward the body surface and subsequently heat is dissipated via evaporation (Pawar et al., 2016). It is important to note that increased panting which is a common response by chicken under HS leads to exhalation of more carbon dioxide. As a result, this causes the elevation of blood pH, a condition known as respiratory alkalosis (Borges et al., 2004). This changes in the blood chemistry disrupt the availability of bicarbonate and free calcium which are vital for the mineralization of the eggshell. This phenomenon is important in the layer industry as they need to produce good-quality eggs (El-Tarabany, 2016).

5 | DECREASE PRODUCTION PERFORMANCE

Several studies have reported that HS not only affects the physiology, health and immune functions of chickens but also negatively affects their production performances. During high waves, the priority of chickens is to survive instead of growth (Nawaz et al., 2021). It is reported that broilers at 42 days of age exposed to chronic heat stress (CHS) had a 32.6% body weight gain, 16.4% reduced feed intake and a 25.6% higher feed consumption ratio (Sohail et al., 2012). Prolonged HS (CHS) in broilers can cause acid-base imbalance and activation of lipid peroxidation, consequently leading to adverse effects on fat metabolism, muscle growth, reduce meat quality and shift in blood chemical profile (Babinszky et al., 2011; Kim et al., 2017; Nawab et al., 2018). In addition, high heat waves decrease protein content and increase fat deposits in chickens (Ayo et al., 2011).

In another study on broilers, it is showed that both CHS and continuous HS significantly affect the growth rate by decreasing protein digestibility up to 9.7% (Nawaz et al., 2021). In yet another study, it is shown that broilers exposed to HS (32°C) had increased metabolizable energy intake (20.3%) and heat production (35.5%), and reduced energy retention (20.9%) and energy efficiency (32.4%) when compared to the control group (Souza et al., 2016).

It is demonstrated that HS decreased chicken daily feed intake by 28.58 g and 28.8% egg production in laying hens in a 12-day trial (Zhang et al., 2017). Overall, HS reduced whole egg weight, eggshell thickness, only eggshell weight and eggshell by percent 3.24%, 2%, 9.93%, 0.66% respectively (Ebeid et al., 2012; Mack et al., 2013).

Another study reported that when layer hens were exposed to 5 weeks of CHS (35°C), weight gain reduced to 1.233 kg as compared to the control group (1.528 kg body weight). The significant reduction in weight could be attributed to decreased feed intake as birds under HS consumed less feed as compared to the control group (Mashaly et al., 2004). Therefore, they found that the decreased in feed intake and nutrient digestibility negatively affected production performance and egg quality (Song & King, 2015; Song et al., 2022; Zaboli et al., 2019). The cause of the differences may be as a result of duration of HS, heat intensity, bird's age or the genetic and physiological status of the chicken's (Nawab et al., 2018). Furthermore, it is reported that at 28–35 days of rearing, chickens feed 0%, 1% and 2% shredded, steam-exploded pine particles were exposed to CHS from 9:00 AM at 21°C the temperature was gradually increase by 2°C/h until 31°C and no mortality was recorded during the HS period of the entire experiment and all birds were healthy (Goel et al., 2022). Another study showed that the health and livability of birds were not affected by CHS birds when fed on high protein diet with ME (22% CP 13.8 MJ ME/kg) and high protein diet (22% CP and 13.2 MJ ME/kg) (Attia & Hassan, 2017).

6 | EFFECTS OF HS ON POULTRY GUT HEALTH AND IMMUNE RESPONSE

Gut health plays a vital role in ensuring efficient digestion and absorption of feed, water and electrolyte balance as well as in immune system development (Rostagno, 2020). HS can alter the gut microbiota and, therefore, leads to gut dysbiosis and subsequently affects gut barrier functions (Brugaletta et al., 2022). Numerous studies have demonstrated the effects of HS on gut microbiota composition and health of birds (Ringseis & Eder, 2022). In one study, it was reported that HS reduced the viable counts of *Lactobacillus* and elevated the viable counts of harmful bacteria such as *Escherichia coli* in caecal contents of Ross 308 broilers exposed to cyclic HS (33°C for 10 h/d) from 21 to 42 days of age, causing disruption of intestinal morphology and a decreased jejunal transepithelial electrical resistance in the broilers (Song et al., 2013). In another study from the same research group, they found decreased viable counts of *Lactobacillus* and *Bifidobacterium* in Ross 308 broilers exposed to CHS (33°C for 10 h/d) from 22 to 42 days of age while an increase of pathogenic bacteria such as *Clostridium*, *Coliform* in the small intestine of raised at thermoneutral temperature (22°C) was observed (Song et al., 2014). Both studies strongly suggested that HS alters the microbial profile and subsequently affects the gut

barrier functions in broilers. Yet another study reported a decrease in *Lactobacillus* and *Bifidobacterium* and increased *E. coli*, *Salmonella* and *Clostridium* in broilers exposed to CHS (33°C for 10 h/d) from 21 to 42 days of age compared to broilers raised under thermoneutral conditions (22°C). Furthermore, the adverse effects of HS were reported to reduce the expression of tight junction proteins (CLDN1, OCLN, ZO1, E-cadherin) and mucins (MUC2) while elevating the intestinal permeability (serum D-lactic acid concentration and diamine oxidase activity) in broilers exposed to high ambient temperatures and subsequently impaired the intestinal barrier functions in broilers exposed to HS (Zhang et al., 2017). The alteration of the gut microbiota profile was also reported in Arbor Acres broilers exposed to CHS (32°C for 10 h/d) from 22 to 35 days of age (Liu et al., 2020). In this study, the researchers found an increase *Parabacteroides*, *Saccharimonas*, *Romboutsia* and *Weissellain* the ceca, impaired intestinal morphology while bacteria such as *Anaerofustis*, *Pseudonocardia*, *Rikenella* and *Tyzzerella* were reduced in HS broilers (Liu et al., 2020).

High temperatures also affect the immune status of chickens (Attia et al., 2018; Nawab et al., 2018; Slawinska et al., 2019). The initiation of immune inflammation takes place due to the penetration of microbes arising from challenged gut health and loosening of enterocytes (Galarza-Seeber et al., 2016; Zhang et al., 2017). HS causes decrease in chickens' thymus, spleen, bursa of Fabricius, liver and lymphoid organ weights and causes immune dysregulation. In a study, it is reported that broilers exposed to thermal stress had a reduced ratio of circulating antibodies such as IgG and IgM, and decreased systemic humoral responses (Lara & Rostagno, 2013). High ambient temperatures is known to decreased the intraepithelial lymphocytes, IgA-secreting cells antibody titre in laying hens and also negatively affect the macrophages performance of phagocytosis in broilers (Lara & Rostagno, 2013; Sugiharto, 2020). Furthermore, HS reduces the phagocytic ability of macrophages and reduces macrophage basal and oxidative burst in broilers (Gomes et al., 2014). In addition, high temperature can alter the ratio of circulating cells and increase the ratio of heterophil to lymphocyte, due to lower lymphocytes and higher number of heterophils in circulation (Lara & Rostagno, 2013; Zhang et al., 2023).

7 | EFFECTS OF HS ON MEAT QUALITY AND FOOD SAFETY

In recent times, meat quality and food safety have become a hot topic and in the poultry sector across the globe (Lara & Rostagno, 2013).

When chickens are exposed to high temperatures during rearing, it can lead to poor meat quality. It is reported that the occurrence of glycogen conversion to increase lactic acid production cause a rapid drop in pH during and after slaughter. On the other hand, the denaturation of sarcoplasmic proteins and low pH leads to pale, soft and exudative meat as a result of low water-holding capacity of muscle. Furthermore, long panting period during heat exposure causes metabolic acidosis in skeletal muscle, and the release of body heat increase the exhalation of carbon dioxide, high release of corticosteroid hormones and impair the structure and functions of the enzymes that regulate sarcoplasmic calcium levels in muscles (Zaboli et al., 2019). A research group from Italy reported that HS causes an increase in fat content while a dramatic alteration of the carcass composition of broiler chickens was observe by lowering the lean tissue proportion, especially the breast yield (Zampiga et al., 2021).

Numerous studies have reported evidences that when chickens are reared under high heat waves, it can negatively affect food safety (Pawar et al., 2016). One study highlighted that pathogen shedding in chicken farms could be due to stress conditions, especially HS (Lara & Rostagno, 2013; Pawar et al., 2016). HS produces undesirable meat characteristics and quality loss in broilers (Kim et al., 2017). In addition, meat quality losses were also described during the transportation of broiler chickens from farms to processing units under hot climatic conditions (Lara & Rostagno, 2013). High ambient temperatures is reported to lower egg production and decreased quality of eggs in layer chickens (Ebeid et al., 2012; Pawar et al., 2016).

8 | STRATEGIES TO MITIGATE HS IN CHICKEN

In the past years, numerous strategies have been used to reduce the negative impacts of HS in chickens with little success. These strategies include environment and housing, reduced stocking density, early life heat conditioning, genetic selection, dietary manipulation, in ovo administration of bioactive substances, and so on. Each of these strategies is discussed below.

9 | ENVIRONMENT AND HOUSING

The environment and the shape of the house are pivotal in reducing HS in poultry during high heat waves. As a result, it is important to make sure easy flow of air into and out of the poultry house. By ensuring this (easy flow of air into and outside of the house), the negative effects of HS could be minimized (Nawab et al., 2018; Pawar et al., 2016). In hot and humid environments, open-style houses with proper shading, enough air circulation and water intake are crucial. The house should be oriented in the east-west direction (Oloyo & Ojerinde, 2020). The width of such housing should not exceed 12 m, while the length of the building can depend upon convenience. In the case of long buildings, doors should be placed at an interval of 15–30 m. It is recommended to have a side-wall height of at least 2.1 m along with curtails that can be raised or lowered easily (Pawar et al., 2016). Ventilation should be adequate as the air movement facilitates the removal of build-up ammonia, carbon dioxide and moisture from the poultry sheds (Nawab et al., 2018; Pawar et al., 2016). Stress-associated health problems can be minimized via ventilation technologies (Nawab et al., 2018; Pawar et al., 2016). Ventilation equipment should be installed and maintained correctly. It is also important to have stand-by generators and additional ventilation fans in case of an emergency situation (power cut-off)

(Kapetanov et al., 2016). Also grass cover on the grounds surrounding the poultry house will reduce the reflection of sunlight into the house. Continuous trimming of vegetation should be practice to ensure air movement. Shade trees should be located where they do not affect air circulation (Lin et al., 2006; Nawab et al., 2018). Another important element to be considered to prevent heat build-up is the condition of the roofing. The roofs of poultry houses should be clean and dust-free. A shiny roof surface is more efficient to reflect solar radiation than a dark or rusty roof. To increase the reflection in the roof, metallic zinc paint or aluminum roof can be used. Evaporative panels are used in broilers to alleviate the negative effects of HS (Çaylı et al., 2021). These panels help to reduce the ambient temperature through the evaporation of water, and thus creating a more favourable temperature to birds. Furthermore, evaporative cooling of the ventral regions of the skin in laying hens has been developed as a new approach to cooling, based on wetting the skin and promoting water evaporation, demonstrating the potential of this method in alleviating HS in poultry (Wolfenson et al., 2001). Stocking density is one of the critical factors when temperatures are high (Kapetanov et al., 2016). Therefore, the number of chickens raised in a poultry house should be taken into consideration. The evaporative heat dissipation depends on temperature and humidity as it increases with temperature and decreases with increasing humidity (Lara & Rostagno, 2013; Lin et al., 2006). In recent years, dark housing systems (DHS) in poultry production have been used to alleviate HS through its ability to provide precision climate control, thereby creating an environment that mitigates the adverse effects of HS on poultry. This system involves controlling light exposure to the birds and utilizes automated sensors and controls to dynamically adjust temperature, humidity and ventilation in the poultry house (Carvalho et al., 2015). According to (Carvalho et al., 2015), the feed conversion ratio for the birds reared on the DHS was 3.8% and 2.7% lower than those for the conventional housing system, average daily gain under the DHS was 11.4% and 9.3% higher, and body weight at 46 days was 11.4% and 9.3% higher. Therefore, the use of DHS in poultry production could be an alternative in mitigating the adverse effects of HS. However, such houses are expensive to build and maintain in developing nations (Glatz, 2013), and, therefore, dietary manipulations and other intervention strategies will be more efficient and appropriate are more appropriate (Wasti et al., 2020).

10 | STOCKING DENSITY

In a poultry house, stocking density is the number of birds in a particular area, for example, birds per square metre. The primary objective of increasing the number of chickens per unit of area is to maximize production. However, in commercial settings, it is possible to compromise with (Shakeri et al., 2014) slightly decreased growth rates in exchange for a reasonable economic profit. Nonetheless, it is important to note that crowding more birds into a limited area comes with consequences. Overcrowding (high stocking density) has negative effects on animals' well-being by decreasing the overall environmental quality and increasing competition among birds for available resources such as feed which may eventually lead to feather pecking and cannibalism (Shakeri et al., 2014). In general, high stocking density has deleterious effects on production performance, health and moisture of litter feed efficiency and may eventually lead to low economic return (Pawar et al., 2016; Shakeri et al., 2014). However, lowering stocking density to mitigate HS might also reduce the number of birds that could be raised in a given house/farm. Therefore, it is necessary to explore other strategies to alleviate HS while the stocking density can be maintained while not negatively affecting birds' performance and health.

11 | EARLY-LIFE HEAT CONDITIONING

The practice of EHC has been reported to be a promising approach for mitigating HS in the later life of chickens (Kang & Shim, 2021; Madkour et al., 2022). EHC is a method in which broiler chicks are introduced to high ambient temperature (36°C) for 24 h at 3–5 days of age (Lin et al., 2006). This method is inexpensive, making its adaptation to various farms across the globe much easier (Arjona et al., 1988; Kang & Shim, 2021; Yahav & McMurtry, 2001). When chicks are exposed to HS at an early age of life, it can induce heat resistance in later stages of development (Zhou et al., 1997). When young chicks are exposed to EHC (5-day-old chick at 40°C), they exhibit lower body temperature due to metabolic status changes (De Basilio et al., 2001, 2003). EHC provides thermotolerance to broiler chickens. Immediately after EHC (24 h heat exposure), young chicks experience a slow growth rate but growth performance increases subsequently resulting in higher body weight gain compared to the nonconditioned chickens (Yahav & McMurtry, 2001). It is also reported that early heat exposure (3-, 4- or 6 days old), reduced weight gain in the first week of life early but the growth accelerated to achieve higher marketing weights at the end of the rearing period (42 days). For example, at 42 days of age, 2.195 and 2.189 g body weight was recorded for the control group and heat expose group, respectively, indicating no significant difference (Arjona et al., 1988; Yahav & McMurtry, 2001). The mortality rate was also decreased, and the feed efficiency improved (Arjona et al., 1988).

In another study, they observed that EHC (35–37.8°C, 24 h, on Day 5 posthatch) prevented the negative effects caused by late CHS in broiler chicks (Yalçin et al., 2003; Zaboli et al., 2017). However, inducing EHC did not have a significant difference in mortality, weight gain or feed efficiency when compared to broilers raised under CHS (Chung et al., 2005). During HS, heat shock proteins in the liver and spleen are expressed and thus preventing cell and tissue damage by repair and protein unfolding (Goel, 2021). It is reported that when chickens under HS were supplemented with an increased (600 mg) of resveratrol per kg in the diet, it resulted to an increased expression of HSP27, HSP70 and HSP90 level in the Bursa of Fabricius and spleen (Liu et al., 2014). In addition, those of HSP27 and HSP90 in the thymus were decreased under HS compared with

no HS. The supplementation of resveratrol attenuated the HS-induced overexpression of HSP27, HSP70 and HSP90 in the bursa of Fabricius and spleen and elevated the low expression of HSP27 and HSP90 in thymus upon HS and liver (Liu et al., 2014). Despite it is reported effects in alleviating the negative effects of HS, EHC can lead to poor chick quality, and early mortality which may affect overall performance and economic returns (Kang & Shim, 2021; Madkour et al., 2022).

Aside from EHC, both embryonic manipulation and thermal manipulation have also been explored extensively as a strategy to provide thermotolerance to birds and withstand the adverse effects of HS after hatch and later in life (Al-Zghoul et al., 2019; Piestun et al., 2008; Saleh et al., 2020). Several findings showed that embryonic manipulation and thermal manipulation have numerous positive effects by increasing hatchability, hatchlings weight and rectal temperature at hatch, improve (Al-Zghoul et al., 2019; Goel et al., 2017; Piestun et al., 2008). Embryonic manipulation involving in ovo supplementation has been reported to afford thermotolerance to chickens (Elnesr et al., 2019; Han et al., 2018). On the other hand, thermal manipulation from the 7th to 16th ED at 39.5°C and 65% relative humidity (RH) for 12 h a day helps in increasing thermotolerance for later stages of chicken's life (Piestun et al., 2008). In a different study, it is shown that thermal manipulation from the 10th to 18th ED at 39.0°C and 65% RH for 18 h a day is ideal for thermotolerant chickens (Al-Zghoul & El-Bahr, 2019). In addition, thermal manipulation enhances heat tolerance capacity by modulating the antioxidant enzyme-related genes such as nicotinamide adenine dinucleotide phosphate oxidase 4, superoxide dismutase (SOD) and Catalase (CAT) and enhancing the HSP70 and immunity-related genes (IL-6, IL-1β, TNF-α, NFκB50) in heat-stressed chickens (Al-Zghoul et al., 2019; Saleh et al., 2020). To sum up, both embryonic manipulation and thermal manipulation exerts positive effects on improving chicken's thermotolerance, however, there are some drawback to these techniques that needs attention. During embryonic manipulation, it is important to be cautious with the age of the embryo, as this may impair hatchability and chick quality and subsequently reduce growth performance. Thermal manipulation at early age can also cause some drawbacks such as high mortality of 1–3 days old chicks when exposed to HS (Goel, 2021).

12 | GENETIC SELECTION

Genetic selection involves the choosing of high-quality birds to produce the next generation. Over the years, several parameters such as growth, immunity and so on have been used to select the best birds for production. However, one of the main drawbacks of broilers has been associated with low feed intake during high heat waves (Awad et al., 2020). In the past years, continuous genetic selection concentrated on achieving rapid growth in broilers. However, this certainly comes with consequences, as such birds have a lower heat tolerance in comparison to slow-growing broilers (Deeb & Cahaner, 2002). The main criterion for selecting laying hens is their ability to produce a high number of eggs with good quality. HS negatively impacts the production performance of layer hens by decreasing egg production and egg quality (Barrett et al., 2019). In light of the negative effects of HS in the poultry industry, a new genetic selection approach is needed to address these problems.

There are various genes that are associated with heat tolerance have been studied. Some of these genes include the dominant gene for naked neck (Na), which is linked with decreasing feather cover (Tóth et al., 2021), while others, such as the sex-linked recessive gene for dwarfism, reduce body size and thereby reduce metabolic heat output. The frizzle (F) gene causes the contour feathers to curve outward away from the body (Deeb & Cahaner, 2002). Furthermore, it is reported that the F gene (as Ff) reduced the featherweight of broilers in addition to the reduction caused by the Na gene (Deeb & Cahaner, 2002). The slow feathering (K) gene has been extensively used to 'auto-sex' strain and breed crosses have an effect on increased heat loss during early growth, all of which may assist the bird in resisting HS (Tixier-Boichard et al., 1989). Chickens with such genetic mutations due to their high production performance, adaptability, survivability and hatchability under HS (Fathi et al., 2022).

13 | DIETARY MANIPULATION TO ALLEVIATE HS IN POULTRY

13.1 | Feeding strategies

Feed restriction involves the withdrawal of feed over a period of time, in general from 8 AM to 5 PM Feed withdrawal (feed restriction) aids decreasing the metabolic rate of birds, decrease rectal temperature, reduce mortality (Uzum & Toplu, 2013) and reduce abdominal fat (Mohamed et al., 2019) in heat-stressed broilers. In another study, it is reported that feed deprivation for 8 h a day during the high heat waves in broilers improved feed efficiency and shortened tonic immobility (Uzum & Toplu, 2013). In addition, feed restriction in broilers was reported to decrease heat production by 23% (MacLeod & Hocking, 1993). However, this practice is not popularly practice in the poultry industry, as it results in decreasing growth rate and delayed marketing age of chickens (Francis et al., 1991; Uzum & Toplu, 2013; Wiernusz & Teeter, 1996). Besides feed restriction, there are other feeding programme that have been used to reduce HS in birds.

Dual feeding programmes involve offering birds a protein-rich portion of diet during the cooler part of the day and an energy-rich portion of diet during the warmer phase of the day (Teyssier et al., 2022). Studies have found that by feeding a protein-rich diet from 4 PM to 9 AM and an energy-rich diet from 9 AM to 4 PM during the high heat waves was reported to reduce the body temperature (De Basilio et al., 2001; Lozano et al., 2006) and mortality in heat-stressed broilers (De Basilio et al., 2001). While a dual-feeding approach might be feasible in tropical areas and less-intensive production systems, it is unsuitable for most commercial production operations due to cost and logistical constraints (Iyasere et al., 2021). This practice was not also able to improve growth performance and feed efficiency in broiler chickens exposed to HS (Lozano et al., 2006).

Wet feeding enhances digestion and increase nutrient uptake from the GI tract and ensure fast action of digestive enzymes on feed (Syafwan et al., 2011). High amount of water is lost by chickens during high ambient temperature. Therefore, providing cool water in the feed helps increase water intake and decreases viscosity in the gut and thus accelerate passage of feed. In broilers, wet feeding improved the feed intake, body weight and weight of the GI tract (Wasti et al., 2020). Studies have shown that providing wet feed to laying hens during HS increased egg production, egg weight and dry matter intake (Lin et al., 2006). This strategy has been found to demonstrate positive effects in chicken exposed to HS, however, it is not a prominent strategy as it may promote fungal growth in feed and eventually causing mycotoxicosis in chicken (Wasti et al., 2020).

Fat supplementation in the diet is another approach that has been shown to help bird cope with HS. During metabolism, fat produces lower heat increment as compared to protein and carbohydrates (Wasti et al., 2020). Adding fat in bird's diet increase nutrient utilization in the GI tract by lowering the rate of food (Attia et al., 2018). Furthermore, it is demonstrated that 5% fat added to a diet significantly improve performance of broilers (Ghazalah et al., 2008).Yet another study revealed that increasing oil supplementation in higher protein concentration diet attenuated the adverse effects of CHS on broiler performance, meat lipids, physiological and immunological traits (Attia et al., 2017).

The size and quality of the feed (feed form) is also crucial in increasing feed intake, digestibility feed efficiency and growth performance (Abdel-Moneim et al., 2021). It is reported that pelleting increased apparent metabolizable energy in cereal grains and protein sources (Khalil et al., 2021). Beside the positive effects of fine feed, studies have also demonstrated that providing coarse diets (large particle size) increased water retention in body of bird's which can be used for evaporative cooling and thus decreased body temperature. Coarse feed also increased the GI tract development and reduced heat generation and attenuated HS (Syafwan et al., 2011). Therefore, this practice could be a promising approach to reduce the negative effects of HS.

13.2 | Supplementation of vitamins, electrolytes, phytochemicals and osmolytes in the diet

The importance of the supplementation of various vitamins, minerals electrolytes, phytochemicals, resveratrol and so on have been widely reported (Abdel-Moneim et al., 2021; Attia & Hassan, 2017; Goel, 2021). Their effects on mitigating HS and improving poultry performance and health are summarized below.

Vitamin E (VE) is a fat-soluble vitamin that has antioxidant activity and helps to scavenge free radicals produced inside the cell (Dalólio et al., 2015). Studies have found that the supplementation of VE in the diet of laying hens reared under HS improved egg production, egg weight, eggshell thickness, egg specific gravity and Haugh unit (Khan et al., 2011). It is reported that adding 250 mg VE/kg of feed in the diet of layers relieved the negative impacts of HS (Sahin & Kucuk, 2001). Furthermore, a mixture of VE (100 mg/kg of feed), vitamin C (200 mg/kg of feed), probiotics (Saccharomyces cerevisiae and *Lactobacillus acidophilus* at 2 g/kg of feed) were reported to attenuate the deleterious effects of CHS in broilers (Attia et al., 2017).

The addition of Vitamin A (6000 and 9000 IU/kg of feed) was found to increase egg weight in layer hen reared under high temperatures (Lin et al., 2002). In another study, the supplementation of vitamin A (15000/kg of feed) was reported to increased body weight gain, improved feed efficiency and reduced malonaldehyde (MDA) concentration of broilers exposed to HS (Kucuk et al., 2003).

Vitamin C is a water-soluble antioxidant that can prevent oxidative stress by scavenging for reactive oxygen species. Although poultry can synthesize vitamin C, the amount is limited during HS conditions (Khan et al., 2012). Thus, dietary supplementation of vitamin C is an effective strategy to reduce the harmful effects of HS in poultry. Supplementation of vitamin C (250 mg/kg of feed) improved growth rate, nutrient utilization, egg production and quality, immune response and antioxidant status in heat-stressed birds (Khan et al., 2012). Dietary supplementation of vitamin C lowered the serum concentration of MDA, homocysteine and adrenal corticotropin hormone in heat-stressed Japanese quail (Sahin & Kucuk, 2003). In broilers, dietary supplementation of 200 mg ascorbic acid per kg of feed improved body weight gain and feed conversion ratio (FCR) (Njoku, 1986).

During HS, the addition of sodium selenite at 0.1 or 0.2 mg/kg of feed improved the carcass quality and performance of heat-stressed quails (Sahin & Kucuk, 2001). It has also been found that supplementation of 0.15 and 0.30 mg/kg of feed sodium selenite or selenomethionine during HS period increased feed intake, body weight and egg production and improvement in feed efficiency (Sahin et al., 2008).

Long period of panting during high heat waves causes alteration in the acid–base balance in blood plasma and eventually leads to respiratory alkalosis. This acid–base imbalance can be recovered by supplementation of electrolytes such as NH4Cl, NaHCO3 and KCl. During respiratory alkalosis, birds excrete a higher amount of bicarbonate ions from the kidney to restore normal blood pH. A high levels of dietary electrolyte balance 200–300 mEq/kg, has been suggested to be effective in alleviating the adverse effects of HS in poultry (Mushtaq et al., 2013). Moreover, supplementation of NaHCO3 in heat-stressed laying hens is also found to improve eggshell quality (Balnave & Muheereza, 1997). The supplementation of NaHCO3 (up to 0.5%) to broiler diets also improved the performance of broilers reared under high temperatures (Benton et al., 1998). Yet another study revealed that inclusion of 1.5%–2.0% K from KCl in poultry diet were effective in ameliorating CHS and ultimately improved FCR (Smith & Teeter, 1987). Besides supplementation of 0.2% NH4Cl or 0.15% KCl, 0.6% KCl, 0.2% NaHCO3 and carbonated water in drinking water also improved the performance in the heat-stressed broiler chickens (Lin et al., 2006).

Lycopene is a type of carotenoid pigment which is found in numerous plants, fruits and vegetables such as tomatoes, carrot, pink guava, watermelon, apricots and so on. Lycopene has been reported to have positive effects on the physiology of chickens, particularly mitigating heat-induced oxidative stress. The mechanism of action of lycopene involves various activities that help maintain oxidative

balance in birds. These activities include serving as a free radical scavenger, inhibiting signalling pathways and activating host antioxidant enzymes such as SOD, glutathione peroxidase (GSH-Px) and CAT (Arain et al., 2018). Furthermore, lycopene has been reported to downregulate lymphocyte proliferation and improve antioxidant status, immune function and lipid metabolism in broiler chickens, improved growth performance in broiler chickens (Arain et al., 2018; Sahin et al., 2016). Therefore, studies have shown the positive effects of lycopene in mitigate HS in poultry. For example, lycopene supplementation (200 or 400 mg/kg of feed) in heat-stressed broilers improved the feed intake, body weight and FCR (Sahin et al., 2016). In laying hens, dietary supplementation of lycopene improved oxidative status (Arain et al., 2018).

Resveratrol is a natural bioactive polyphenol predominantly found in peanuts, grapes, berries and turmeric. In recent years, it has garnered attention due to its potential to combat HS in poultry. The mechanism of action of resveratrol in the physiology of poultry involves inducing antioxidant and heat shock protein mRNA expression, promoting fatty acid oxidation and modulating the immune response. These actions contribute to its potential in mitigating HS and maintaining the physiological balance in poultry (Hu et al., 2019). The supplementation of resveratrol (400 mg/kg of feed) enhanced the antioxidant capacity in the broilers during HS (Hu et al., 2019). Supplementation of resveratrol at 300 or 500 mg/kg of feed improved the average daily gain, decreased the rectal temperature, lowered the level of corticosterone, adrenocorticotropin hormone, cholesterol and MDA in yellow-feather broilers under HS (He et al., 2019). In laying hens, supplementation of 200 mg resveratrol/kg of feed improved the egg production.

Epigallocatechin gallate (EGCG) is a polyphenol derived from green tea which have high antioxidant and anti-inflammatory properties (Wasti et al., 2020). Different doses of EGCG in the feed (0, 300 and 600 mg/kg) of heat-stressed broiler birds was found to increase body weight, feed intake and level of serum total protein, glucose and alkaline phosphatase (Luo et al., 2018). The addition of 200 or 400 mg of EGCG/kg of feed in female quails exposed to HS increased intake, egg production, hepatic SOD, CAT and GSH-Px activity and resulted in a linear decrease of hepatic MDA level (Sahin et al., 2010).

Curcumin is a polyphenol which is extracted from turmeric and has the potential to mitigate HS in poultry through various mechanisms such as reducing oxidative stress, and inflammation (Hu et al., 2019). Previous results have shown that feed with curcumin improves the growth performance of heat-stressed broiler birds (Attia et al., 2017; Zhang et al., 2018). Studies have shown that the inclusion of curcumin at 100 mg/kg of feed significantly improved the final body weigh in broilers under HS conditions (Zhang et al., 2018). In laying hens, supplementation of 150 mg/kg of feed with curcumin improved the laying performance, egg quality, antioxidant enzyme activity and immune function during HS (Liu et al., 2020). During HS, supplementation of betaine levels from 0.05% to 0.20% improved the feed intake, carcass trait and egg production parameters in broilers, layers and ducks (Ratriyanto & Mosenthin, 2018). In laying hens, supplementing betaine (1000 mg/kg of feed) along with vitamin C (200 mg/kg of feed) improved laying performance during CHS (Attia et al., 2016).

13.3 | Probiotics, prebiotics and synbiotics supplementation in poultry diet

Probiotics play a vital role in mitigating the adverse effects of HS in poultry by modulating the microbiota–gut–brain axis and reducing stress-related inflammation and abnormal behaviours. Furthermore, the addition of probiotics to poultry diet have been associated with improvements in gut morphology, intestinal mucosal immunity, increase nutrient absorption and egg production in laying hens subjected to HS conditions (Cao et al., 2021; Wang et al., 2018). According to Deng et al. (2012), the supplementation of the probiotic (*Bacillus licheniformis* 0, 10^6 and 10^7 CFU) to the diets of laying hens exposed to HS (34°C) increased egg production and feed intake, restored intestinal health by improving deteriorated villus structure and reduced the adverse effects of HS. Probiotics decreased the HS-related negative effects and improved the performance, body weight, feed intake, feed conversion ratio and many blood parameters (Hasan et al., 2015).

Another study revealed that the addition of 1.5 g/kg of probiotic mixture (*B. licheniformis* 1.0×10^7 CFU/g, *Bacillus subtilis* 1.0×10^7 CFU/g and *Lactobacillus plantarum* 1.0×10^8 CFU/g) partially ameliorating intestinal barrier function of heat-stressed birds (Song et al., 2014).

Prebiotics have also been used to attenuate the negatives effects of HS, for example, supplementation of a mixture of chicory root, seaweed and Enterococcus faecium to the diets of broilers exposed to HS increased the ileum villus length, crypt depth, improved body weight, feed conversion ratio and carcass yield (Awad et al., 2008). The dietary addition of yeast (2%) and prebiotic (0.15%) in broilers exposed to HS increased body weight and feed efficiency (Silva et al., 2010). Furthermore, it is found that the supplementation of 0.5% prebiotic (mannan oligosaccharide) and 1% probiotic (Lactobacillus based) to the diet of heat-stressed broilers decreased serum cortisol and cholesterol concentrations, increased thyroxine concentrations and improved the humoral immunity, improved body weight, feed efficiency, villus length and crypt depth in broilers exposed to HS (35°C) (Sohail et al., 2010). Another study suggested that the addition of 5% level of prebiotic (mannan oligosaccharides) and/or 1% level of probiotic to the diets of broilers exposed to HS (35°C) decreased the negative effects (increases the villus length in ileum and jejunum, surface area and crypt depth) of HS (Ashraf et al., 2013; Awad et al., 2021). A study demonstrated that the inclusion synbiotic at 1500 mg/kg for starter diet and 750 mg/kg grower in the diet was effective in improving growth performance and meat quality of broilers reared in high ambient temperatures (Abdel-Wareth et al., 2019). It is suggested that the supplementation of the synbiotic a prebiotic (fructo-oligosaccharides), a probiotic mixture of (*Lactobacillus reuteri, Enterococcus faecium animalis and Pediococcus acidilactici*) could reduce the adverse effects of HS in broilers (Mohammed et al., 2018). In addition, it

has been found that the synbiotic (PoultryStar) could alleviate the detrimental the effects of HS by regulating stress reactions, and improving antioxidant status of broilers (Mohammed et al., 2019). Although the addition of probiotics, prebiotics or the two (synbiotic) in the diet of poultry helps to attenuate HS in poultry, it does not afford thermotolerant during egg incubation (embryonic manipulation). The other approaches such as in-feed or in-water of bioactive substances might not be efficient due to the possibility of negative interaction with other feed additives and potential water quality issues (Oladokun & Adewole, 2020). Other practices such as feed restriction, dual feeding, EHC does not offer the possibility of providing with thermotolerant during embryo development (Oladokun & Adewole, 2020). The in ovo delivery of bioactive substances offers the opportunity to overcome all these identified challenges. The in ovo administration of bioactive substances may address these challenges and subsequently mitigate the adverse effects of HS (Oladokun & Adewole, 2020, 2022; Oladokun et al., 2021).

14 | DEFINITION AND APPLICATION OF THE IN OVO TECHNOLOGY

14.1 | In ovo administration of prebiotics, probiotics

The development and the use of in ovo technology is gaining success in the poultry industry. Among the first pioneers of this technology were Sharma and Burmester (1982). In their studies in 1982, they reported that vaccines can be successfully delivered in ovo either in the amnion or embryonic body and subsequently provide protection to birds against Marek disease (Sharma & Burmester, 1982). This technology has been successfully used for the delivery of coccidiosis, Newcastle disease and infectious bronchitis vaccines to protect birds against these diseases. The in ovo technology can be defined as the administration of bioactive substances into the developing embryo to improve immunity, production performance and healthy gut microbiota (Sharma & Burmester, 1982). The delivery of such bioactive substances should be successfully done without disturbing the development of the embryo (Siwek et al., 2018). The in ovo technology remains to be the only available technique that when bioactive substances are successfully delivered can improve the immunity and oxidative status of the developing embryos and young chicks after hatch (Slawinska et al., 2019).

Due to its high efficiency, this technology has been transitioning from manual injection to an automation system (Johnston et al., 1997). With the use of automated machines, the in ovo technology give us the opportunity to deliver vaccine to high number of eggs within a short period of time while reducing errors during in ovo injection (Peebles, 2018; Ricks et al., 2003). Apart from vaccine administration, the in ovo technology has been used to deliver prebiotics, probiotics, amino acids, vitamin and other bioactive substances to improve bird's immunity, gut health, production performances and environmental stressors such as HS (Slawinska et al., 2020). In the past few years, it has been demonstrated that the in ovo administration of bioactive substances might be effective to mitigate HS in poultry. However, due to continuous rise in temperatures, global warming, HS continuous to be a challenging the poultry industry (Elnesr et al., 2019; Goel, 2021; Mack et al., 2013; Madkour et al., 2022; Sharifi et al., 2010). In order to remedy the negative effects of HS, an incubator parameters (temperature, RH, egg turning and ventilation) must be set correctly, overall hygiene in hatcheries must be improved (Williams & Zedek, 2010), dosage of bioactive substances (McGruder et al., 2011), time and site of injection and most importantly the timepoint must be defined. All these factors contribute to the efficiency of the in ovo administration of pre-and/or probiotics to chicken's embryo (Goel, 2021).

14.2 | Probiotics

Probiotics are live microbial feed supplements, that beneficially affects the host animal by improving its intestinal microbial balance (Oladokun & Adewole, 2020; Sohail et al., 2012) Many studies have reported that probiotics delivered in ovo can improve production performance and health while ameliorating HS (Abdel-Moneim et al., 2021). Knowing the effects of HS in broiler production, several studies have reported that the supplementation of probiotics could mitigate HS conditions. In the same studies, the authors reported a longer villus height (8.32 μm) in broilers fed with probiotic mixture as compared to the control (7.23 μm) (Song et al., 2013). Another study reported that the probiotic *B. licheniformis* alleviated HS-induced impairment of gut morphology in laying hens (Deng et al., 2012). Further studies reported that the use of probiotic *B. subtilis* was able to maintain blood serum, increase villus height, improve gut microflora and growth performance of broiler chickens reared under HS (Abdelqader et al., 2020).

14.3 | Prebiotics

Numerous definitions have been used to describe prebiotics but the one by The International Scientific Association of Probiotics and Prebiotics stands out. They defined prebiotics as 'selectively fermented ingredients that results in specific changes in the composition and/or activity of the GI microbiota, thus conferring benefits upon host health' (Oladokun & Adewole, 2020). Prebiotics should have high resistance to acidity and hydrolysis thus improving gut health, immunity, growth performance and reduce environmental stressors, for example, HS (Roberfroid, 2007). The prebiotics widely use in HS mitigation are galactooligosaccharide (GOS), fructooliogosaccharide cellooligosaccharide chitosan oligosaccharide (Li et al., 2007; Mohammed et al., 2019; Slawinska et al., 2020; Song et al., 2013).

When prebiotics are successfully delivered in ovo at the right time and optimal dose, they can restore stable gut microbiota, ensure quality chicks after hatch and eventually positive and improve

production performance. With this knowledge about prebiotics, several studies have been conducted to deliver prebiotics with different doses in different time and site of injection. GOS delivered in ovo slightly decreased the total polyunsaturated fatty acid concentration but had no effect on total n-3 concentration in heat-stressed broiler chickens. The reason for such contrary results is the diet used and the production level of short-fatty acid chains (Tavaniello et al., 2020). In the same study, it is reported that when GOS (3.5 mg GOS/egg) was inoculated in ovo it reduced HS in broilers (Slawinska et al., 2020). Several studies have been conducted in prebiotic supplementation via in-feed and or in-water as compared to in ovo (Bednarczyk et al., 2016; Oladokun & Adewole, 2020; Slawinska et al., 2019; Song et al., 2013). Several studies confirmed that in ovo delivery seems to be more effective because the prebiotics is administered at an early stage of ED and thus stimulating the immune system and colonization of the gut by beneficial bacteria (Pietrzak et al., 2020; Siwek et al., 2018; Slawinska et al., 2020). The supplementation of synbiotics have synergistic effects on gut health and subsequently improve growth performance and inhibit the negative effects of HS (Abdel-Wareth et al., 2019; Mohammed et al., 2019).

14.4 | Different timepoints for in ovo administration of bioactive substances to mitigate HS

The administration of bioactive substances during ED can improve broiler chickens thermotolerance as shown in (Table 1), gut microflora, performance indices, immunity (Goel, 2021), antioxidant status level (Elnesr et al., 2019). However, for probiotics, prebiotics and synbiotics to exert positive effects on the host, several factors such as injection time, site of injection, dosage of prebiotic or probiotic use and hatchery hygiene should be met (Bednarczyk et al., 2016; Oladokun & Adewole, 2020). Different timepoints for the administration of bioactive substances have been explored since the development of in ovo technology to improve poultry health, performance and thermotolerance to HS, thereby reducing economic losses (Elnesr et al., 2019; Goel et al., 2022; Ncho, Goel, Jeong, Youssouf, et al., 2021; Villaluenga et al., 2004). Among these timepoints, ED 12, 17 and 18 are widely regarded as the ideal times for the administration of bioactive substances (Das et al., 2021; Saeed, Babazadeh, et al., 2019). According to several authors, Day 12 of ED is the ideal time for in ovo administration of prebiotics, probiotics and synbiotics and should be delivered in the air chamber (Bednarczyk et al., 2016; Oladokun & Adewole, 2020; Villaluenga et al., 2004). During this time, the bioactive substances can easily maneuver and go into the GI canal of the developing embryo which is situated in the highly vascularized chorioallantoic membrane. The in ovo delivery of bioactive substances on 12 ED is prescribed as in ovo stimulation. When GOS was delivered on Day 12, ED in ovo it mitigated the negative effects of HS and improved the performance and welfare of broiler chickens (Slawinska et al., 2020). Another study suggested that GOS delivered in ovo on Day 12 of egg incubation, provided slow-growing chickens to resist acute heat and were tolerant to CHS (Pietrzak et al., 2020).

Another timepoint that is widely studied is the in ovo delivery of bioactive substances is ED 17 or 18 which is known as in ovo feeding (Das et al., 2021; Gupta et al., 2022; Ncho, Goel, Jeong, Youssouf, et al., 2021). This is usually the time in which vaccines are delivered in ovo. At Days 17–18 ED, bioactive substances or vaccines are often delivered in the amnion (Majidi-Mosleh et al., 2017). At this timepoint, it is important to ensure that the bioactive substances are delivered into the amnion before Day 19, because this is the time the embryo absorbs the amniotic fluid (Beck et al., 2019; Majidi-Mosleh et al., 2017). The optimum timepoint for in ovo feeding of bioactive substances is a crucial step as it has a long-lasting effect on ED, hatchability, chick quality and overall birds' health and performance (Siwek et al., 2018). Increasing incubation temperatures to 39.6°C for 6 h per day from the 10th to 18th ED and in ovo injection of 0.6 mL of 10% gamma-aminobutyric acid on 17.5 ED is shown to improved body weight of broiler chicks at hatch and greatly regulates stress-related gene expression while alleviate HS after hatch (Goel, 2021). This was also confirmed that the in ovo injection of methionine plus cysteine at Day 17.5 of incubation in broiler eggs under high temperature (39.6°C for 6 h daily) between 10th and 18th ED was important to protect the tissues of new chicks from oxidative damage, and may prepare chicks to resist future HS challenges (Elnesr et al., 2019). In a different study, it is reported that in ovo injection of L-Leu on 7th ED promoted the recovery of antioxidative status in broiler chickens after exposure to HS (Han et al., 2018). The use of nano curcumin (NC) and VE was also explored and it was shown that in ovo delivery of NC and VE solution into the amniotic sac at 17.5 ED improved antioxidant status of the hatched chicks (Heidary et al., 2020). Another timepoint that was applied for in ovo injection is 18 ED. In this study, it was found that in ovo injection of ascorbic acid at 3 mg/egg on 18 ED enhanced antioxidant defense system and immune system of hatchlings (El-Senousey et al., 2018). Respiratory alkalosis occurs when birds excrete a higher amount of bicarbonate ions from the kidney to restore normal blood pH. These bicarbonates ions are further coupled with Na+ and K+ ions before being excreted through the kidney. Ultimately, the loss of ions results in an acid-base imbalance (Ahmad et al., 2008). It has been demonstrated that in ovo injection of ascorbic acid on Day 18 of egg incubation could modulate the immune system of broiler chickens, and that high incubation temperatures provided broiler chickens ability to withstand high temperatures during rearing and, therefore, minimizing or avoiding the occurrence of respiratory alkalosis (Sgavioli et al., 2019). In another study, in ovo administration of betaine was performed at ED 17.5 and afterward chicks were exposed to elevated temperatures 4°C above optimum from 7 to 28 days of age for 4 h per day. However, they did not observed any effect of in ovo feeding of betaine on performance, immunity parameters, liver activity, blood cation-anion balance and bone parameters (Maddahian et al., 2021). The in ovo injection of 6 mg of black cumin at ED 17.5 yielded positive effects on broiler chickens when exposed to a high temperature (39.6°C) for 6 h daily from Day

TABLE 1 An overview of research results of bioactive substances use in combating the adverse effects HS in chicken.

Bioactive substance	Amount of dose used	Species	Injection time	Injection site	Time and duration of HS	Findings	References
Prebiotic	3.5 mg GOS/egg, 0.2 mL/egg	Slow-growing crossbred Hubbard chickens	Day 12	Air chamber	During rearing 30°C from 36 to 50 days posthatch	GOS delivered in ovo improves intestinal microbiota composition and mitigate the negative effects of HS in broiler chicken	Pietrzak et al. (2020)
	3.5 mg GOS/egg suspended in 0.2 mL of physiological saline	Ross 308	Day 12	Air chamber	During rearing on 32– 42 days of posthatch, 30°C	GOS injection in ovo modulates responses to the short-term heat in poultry, GOS prebiotic delivered in ovo dampened heat-induced immune- and stress-related gene expression signatures in spleen. GOS improve broiler's thermotolerance	Slawinska et al. (2019)
	3.5 mg GOS/egg suspended in 0.2 mL of physiological saline (Bi2tos, Clasado Biosciences) (*Bifidobacterium bifidum* 41171)	Ross 308	Day 12	Air chamber	During rearing on 32–42 days of posthatch, 30°C	In ovo treatment had no effect on PM weight, pH, water-holding capacity and shear force. GOS and S birds had lighter PM than C group, cholesterol and intramuscular collagen properties were not affected by treatment. In ovo injection of GOS could mitigate the detrimental effect of HS on some meat quality traits	Tavaniello et al. (2020)
GABA	0.6 mL of 10% in ovo (GABA)	Arbor Acres	Day 17.5	Amnion	During incubation 39.6°C from the 10th to 18th ED (6 h/day)	High temperatures (39.6◦C) and 10% GABA independently improve the BW of broiler chicks at hatch. High temperatures (39.6°C) strongly regulate stress-related gene expression and suggests that both High temperatures (39.6°C) and 10% GABA during incubation can improve performance and alleviate HS after hatch	Goel et al. (2022)
	Group 1: 5% Group 2: 10% Group 3: 20%	Arbor Acres	Day 17.5	Amniotic fluid	At 10th day, during rearing 32°C, ± 1 for 3 h per day	10% GABA significantly increased total antioxidant capacity and reduced malondialdehyde levels, downregulating the expression of HSP70 5% GABA increase CWEWR	Ncho, Goel, Jeong, Youssouf, et al., (2021)
Ascorbic acid	6 μg AA/100 μL water/egg	Cobb 500	Day 18	Albumen	During incubation 39°C, During rearing 32°C from the 1st to 6th week	Injection of ascorbic acid before incubation may serve as a long-term stimulator and modulator of the broiler immune system, and that high incubation temperatures induce adaptations in the electrolytic balance, minimizing respiratory alkalosis under hot rearing temperature	Sgavioli et al. (2019)

(Continues)

TABLE 1 (Continued)

Bioactive substance	Amount of dose used	Species	Injection time	Injection site	Time and duration of HS	Findings	References
BC	2 mg BC (TB), 4 mg BC (FB), 6 mg BC (SB) and 8 mg BC (EB)	Marshall broilers	Day 17.5	Air chamber	During incubation 39.6°C) for 6 h daily from 10th to 18th day	6 mg black cumin extract improved the antioxidant status and posthatch performance of thermally challenged broiler chickens	Oke et al. (2021)
NC and VE	0.01, 0.03 and 0.05 mL/egg of NC and 0.03, 0.06 and 0.09 mL/egg VE solution	Ross 308	Day 17.5	Amniotic cavity	During rearing HS applied from 24 days of age (8 h daily at 35°C)	In ovo administration of NC and VE had no significant effects on growth performance of broiler chickens during 1–10 days of age; but decreased feed intake during 1–24 days without affecting body weight gain and feed conversion ratio. Hatchability in 0.03 and 0.05 mL/egg NC treatments were decreased. In general, in ovo administration of NC improved antioxidant status of the hatchlings	Heidary et al. (2020)
L-Leucine	34.5 µmol/500 µL/egg L-Leucine	Chunky broiler eggs	Day 7	Yolk sac	During rearing acute HS 29 or 30 days old (30 ± 1°C for 120 mins) CHS (over 30 ± 1°C; 15–44 days old)	in ovo feeding of L-Leu significantly increased daily BW gain compared with that of the control group under CHS. Moreover, some essential amino acids, including Leu and isoleucine, were significantly increased in the liver and decreased in the plasma by L-Leu in ovo feeding under acute HS. L-Leu in ovo feeding afforded thermotolerance to broilers under acute HS	Han et al. (2018)
	L-Leu (69 µmol/0.5 mL/egg)	Ross 308	Day 7	Yolk sac	During rearing 21–39 days of age, chicks were exposed to natural summer heat waves	L-Leu in ovo feeding promoted the recovery of antioxidative status after CHS in broiler chickens. In ovo feeding of L-Leu did not affect the body weight gain or relative weight of organs under CHS; however, the serum GSH-Px was significantly increased and serum malondialdehyde was significantly decreased by L-Leu at 39 days of age	Han et al. (2018)
SAA	5.90 mg L-methionine plus 3.40 mg L-cysteine (sulphur amino acids-SAAIG)	Ross 308	Day 17.5	Amnion	During incubation high temperature (39.6°C for 6 h daily) between 10 and 18 ED	in ovo SAA injection (methionine plus cysteine) in the embryonated eggs exposed to HS increased GSH-Px gene expression and antioxidant indices, and reduced HSP70 gene expression, corticosterone concentrations and lipid profile of newly hatched broiler chicks	Elnesr et al. (2019)

78:2562710

TABLE 1 (Continued)

Bioactive substance	Amount of dose used	Species	Injection time	Injection site	Time and duration of HS	Findings	References
Betaine	1 g/kg of betaine, 1 mL/egg	Ross 308	Day 17.5	Amniotic fluid	During rearing 4°C above optimum temperature from 7 to 28 days of age for 4 h per day	Dietary feeding of betaine resulted in an improvement in performance, immunity parameters, liver activity, blood cation–anion balance, and bone parameters and decreased the adverse effects of the high temperatures. In ovo administration of betaine had no significant effect on broilers performance parameters	Maddahian et al. (2021)
Vitamin C and VE	20 IU VE, 3 mg vitamin C and 20 IU VE + 3 mg vitamin C	Ross 308	Day 17	Amnion	During rearing 38 ± 1°C for 4 h every day at ages of 1–7 days	Exposing chicks to HS at an early age did not significantly affect body weight, feed intake and feed conversion ratio at 42 days of rearing. In ovo injection of VE and vitamin C is an effective mean of protection from lipid peroxidation in newly hatched chicks and from adverse effects of HS early in life	Altan et al. (2003)
Nano-Se and Nano-ZnO	40 μg Nano-Se and 500 μg Nano-ZnO	Cobb 500	Day 17	Amnion	During incubation 38.9°C from 19–21 ED	In ovo injection of Nano-Se and Nano-ZnO significantly increased activity of GSH-Px and SOD and total protein, but decreased the levels of corticosterone, cortisol, T4 and T3 at high EST. Injection of Nano-Se and Nano-ZnO had a significant role in alleviating the negative effects of high-temperature incubation and HS by increased antioxidant activity and reduced oxidative stress	Shokraneh et al. (2020)

Abbreviations: °C, degree Celsius; μg, microgram; μmol, micromolar; AA, ascorbic acid; BC, black cumin; BW, body weight; CHS, chronic heat stress; CWEWR, chick weight to egg weight ratio; D, day; ED, embryonic development; EST, eggshell temperature; GABA, gamma-aminobutyric acid, GOS, galactooligosaccharide; GSH-Px, glutathione peroxidase; H, hour; HS, heat stress; HSP, heat shock protein; mg, milligram; mL, millilitre; nano-Se, nano-selenium; nano-ZnO, nano-zinc oxide; NC, nano curcumin; pH, potential of hydrogen; PM, pectoral muscle; SAA, sulphur amino acid; SOD, superoxide dismutase; VE, vitamin E.

10 until Day 18 of the incubation as it improved the antioxidant status and posthatch performance of thermally challenged broiler chickens (Oke et al., 2021). Another study reported that the in ovo injection of Vitamin C and E on ED 17th and early exposure to HS (38°C for 4 h every day at ages of 1–7 days did not affect body weight gain feed intake and feed conversion ratio and was able to provide protection from lipid peroxidation in newly hatched chicks and from the detrimental effects of HS early in life (Altan et al., 2003). In a varied study, injection of Nano-Selenium and Nano-Zinc Oxide on 17 ED was reported to had a significant role in ameliorating the adverse effects of high-temperature incubation and HS by increased antioxidant activity and reduced oxidative stress (Shokraneh et al., 2020). In another study, they used different timepoint (ED 7) for in ovo injection and thereafter exposed the chicks to (35 ± 1°C for 180 min) and was found that in ovo injection of L-Leucine afforded thermotolerance in male broiler chicks under HS but not in female chicks (Han et al., 2018).

15 | CONCLUSION

The gradual increase in temperatures and global warming coinciding with the expansion of the poultry industry in the tropical and subtropical regions makes it important to provide novel intervention strategies to mitigate the effects of stress. Due to poultry birds poor heat tolerance, HS negatively affects their physiological and endocrinological functions, causes immune dysregulation and reduces production performance and health of birds consequently leading to great economic losses in the poultry sector. Over the past years, several intervention strategies have been used to tackle the deleterious effects of HS in poultry. However, only a few are effective and widely used in the poultry industry. Intervention strategies such as environmental modifications (adequate ventilation and proper cooling systems) and nutritional management (feed restriction, dual feeding, fat supplementation, adding bioactive substances to the diet) have been adopted to dissipate heat while maintaining acid–base imbalance. Other strategies are EHC and thermal manipulation, these help to provide thermotolerant to birds. The use of Na and F genes to combat HS has also been demonstrated to alleviate the adverse effects of HS. However, due to several factors such as bird's age, health status, sex, breed and geographical area cause limitations (efficiency, cost, time and laborious) to these strategies. Therefore, in recent years, the in ovo administration of bioactive substances to attenuate the negative effect of HS in poultry is gaining momentum.

The in ovo supplementation of bioactive substances has several advantages over the other strategies to mitigate HS in chickens. These include improving immune functions, increasing nutrient absorption and antioxidant activities while reducing rectal temperature and subsequently generating thermotolerance in chickens (Goel et al., 2023).

In ovo delivery of bioactive substances could also be more economical compared to the other conventional supplementation routes (i.e., in-feed and in-water). For example, 11 times less prebiotics via in ovo administration produce similar results compared with in-water delivery in broiler chickens (Bednarczyk et al., 2016). Similarly, 10 times less in ovo prebiotic supplementation (3.5 mg BI/embryo in ovo) compared to 40 mg BI/chick in-water showed similar results (Tavaniello et al., 2018). Furthermore, the administration of bioactive substances to the chick embryo could establish lifelong phenotypes, including superior performance, immunity and healthy gut microbiome in the bird (Siwek et al., 2018). The in ovo supplementation of bioactive substances also positively influences the development of both the embryo and its neonate while mitigating HS effects (Slawinska et al., 2016, 2020). With the numerous advantages and opportunities this method (in ovo injection of bioactive substances) provides us, it could be the most efficient and cost-effective approach to alleviate the adverse effects of HS in the poultry industry.

AUTHOR CONTRIBUTIONS

Modou Mangan and Maria Siwek conceptualized and drafted the manuscript. Modou Mangan wrote the draft manuscript and prepared the final version of the manuscript. Maria Siwek revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

This work was supported and funded by the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement [Number 955374] MonoGutHealth project.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data availability not applicable as this a review paper.

ORCID

Modou Mangan http://orcid.org/0000-0002-7601-3444

REFERENCES

Abdel-Moneim, A.-M. E., Shehata, A. M., Khidr, R. E., Paswan, V. K., Ibrahim, N. S., El-Ghoul, A. A., Aldhumri, S. A., Gabr, S. A., Mesalam, N. M., Elbaz, A. M., Elsayed, M. A., Wakwak, M. M., & Ebeid, T. A. (2021). Nutritional manipulation to combat heat stress in poultry—A comprehensive review. *Journal of Thermal Biology*, *98*, 102915.

Abdelqader, A., Abuajamieh, M., Hayajneh, F., & Al-Fataftah, A.-R. (2020). Probiotic bacteria maintain normal growth mechanisms of heat stressed broiler chickens. *Journal of Thermal Biology*, *92*, 102654.

Abdel-Wareth, A. A. A., Hammad, S., Khalaphallah, R., Salem, W. M., & Lohakare, J. (2019). Synbiotic as eco-friendly feed additive in diets of chickens under hot climatic conditions. *Poultry Science*, *98*, 4575–4583.

Ahmad, T., Khalid, T., Mushtaq, T., Mirza, M. A., Nadeem, A., Babar, M. E., & Ahmad, G. (2008). Effect of potassium chloride supplementation in drinking water on broiler performance under heat stress conditions. *Poultry Science*, *87*, 1276–1280.

Altan, Ö., Pabuçcuoğlu, A., Altan, A., Konyalioğlu, S., & Bayraktar, H. (2003). Effect of heat stress on oxidative stress, lipid peroxidation and some stress parameters in broilers. *British Poultry Science*, *44*, 545–550.

Al-Zghoul, M. B., & El-Bahr, S. M. (2019). Thermal manipulation of the broilers embryos: Expression of muscle markers genes and weights of body and internal organs during embryonic and post-hatch days. *BMC Veterinary Research*, *15*, 166.

Al-Zghoul, M. B., Sukker, H., & Ababneh, M. M. (2019). Effect of thermal manipulation of broilers embryos on the response to heat-induced oxidative stress. *Poultry Science*, *98*, 991–1001.

Arain, M. A., Mei, Z., Hassan, F. U., Saeed, M., Alagawany, M., Shar, A. H., & Rajput, I. R. (2018). Lycopene: A natural antioxidant for prevention of heat-induced oxidative stress in poultry. *World's Poultry Science Journal*, *74*, 89–100.

Arjona, A. A., Denbow, D. M., & Weaver, W. D. (1988). Effect of heat stress early in life on mortality of broilers exposed to high environmental temperatures just prior to marketing. *Poultry Science*, *67*, 226–231.

Ashraf, S., Zaneb, H., Yousaf, M. S., Ijaz, A., Sohail, M. U., Muti, S., Usman, M. M., Ijaz, S., & Rehman, H. (2013). Effect of dietary supplementation of prebiotics and probiotics on intestinal micro-architecture in broilers reared under cyclic heat stress. *Journal of Animal Physiology and Animal Nutrition*, *97*, 68–73.

Attia, Y. A., Abd El-Hamid, A. E.-H. E., Abedalla, A. A., Berika, M. A., Al-Harthi, M. A., Kucuk, O., Sahin, K., & Abou-Shehema, B. M. (2016). Laying performance, digestibility and plasma hormones in laying hens exposed to chronic heat stress as affected by betaine, vitamin C, and/or vitamin E supplementation. *SpringerPlus*, *5*, 1619.

Attia, Y. A., Al-Harthi, M. A., El-Shafey, A. S., Rehab, Y. A., & Kim, W. K. (2017). Enhancing tolerance of broiler chickens to heat stress by supplementation with vitamin E, vitamin C and/or probiotics. *Annals of Animal Science*, *17*, 1155–1169.

Attia, Y. A., Al-Harthi, M. A., & Sh. Elnaggar, A. (2018). Productive, physiological and immunological responses of two broiler strains fed different dietary regimens and exposed to heat stress. *Italian Journal of Animal Science*, *17*, 686–697.

Attia, Y. A., & Hassan, S. S. (2017). Broiler tolerance to heat stress at various dietary protein/energy levels. *European Poultry Science*, *81*, 1612–9199. http://www.european-poultryscience.com/artikel.dll/ROJ_VIEWJUMP?DOI=10.1399/eps.2017.171

Awad, W., Ghareeb, K., & Böhm, J. (2008). Intestinal structure and function of broiler chickens on diets supplemented with a synbiotic containing enterococcus faecium and oligosaccharides. *International Journal of Molecular Sciences*, *9*, 2205–2216.

Awad, E. A., Najaa, M., Zulaikha, Z. A., Zulkifli, I., & Soleimani, A. F. (2020). Effects of heat stress on growth performance, selected physiological and immunological parameters, caecal microflora, and meat quality in two broiler strains. *Asian-Australasian Journal of Animal Sciences*, *33*, 778–787.

Awad, E. A., Zulkifli, I., Ramiah, S. K., Khalil, E. S., & Abdallh, M. E. (2021). Prebiotics supplementation: An effective approach to mitigate the detrimental effects of heat stress in broiler chickens. *World's Poultry Science Journal*, *77*, 135–151.

Ayo, J. O., Obidi, J. A., & Rekwot, P. I. (2011). Effects of heat stress on the well-being, fertility, and hatchability of chickens in the Northern Guinea Savannah Zone of Nigeria: A review. *ISRN Veterinary Science*, *2011*, 1–10.

Babinszky, L., Halas, V., & Verstegen, M. W. A. (2011). In H. Kheradmand, *Impacts of climate change on animal production and quality of animal food products.in climate change—socioeconomic effects*. InTech.

Balnave, D., & Muheereza, S. (1997). Improving eggshell quality at high temperatures with dietary sodium bicarbonate. *Poultry Science*, *76*, 588–593.

Barrett, N. W., Rowland, K., Schmidt, C. J., Lamont, S. J., Rothschild, M. F., Ashwell, C. M., & Persia, M. E. (2019). Effects of acute and chronic heat stress on the performance, egg quality, body temperature, and blood gas parameters of laying hens. *Poultry Science*, *98*, 6684–6692.

De Basilio, V., Requena, F., Leon, A., Vilarino, M., & Picard, M. (2003). Early age thermal conditioning immediately reduces body temperature of broiler chicks in a tropical environment. *Poultry Science*, *82*, 1235–1241.

De Basilio, V., Vilariño, M., Yahav, S., & Picard, M. (2001). Early age thermal conditioning and a dual feeding program for male broilers challenged by heat stress. *Poultry Science*, *80*, 29–36.

Beck, C. N., McDaniel, C. D., Wamsley, K. G. S., & Kiess, A. S. (2019). The potential for inoculating Lactobacillus animalis and Enterococcus faecium alone or in combination using commercial in ovo technology without negatively impacting hatch and post-hatch performance. *Poultry Science*, *98*, 7050–7062.

Bednarczyk, M., Stadnicka, K., Kozłowska, I., Abiuso, C., Tavaniello, S., Dankowiakowska, A., Sławińska, A., & Maiorano, G. (2016). Influence of different prebiotics and mode of their administration on broiler chicken performance. *Animal*, *10*, 1271–1279.

Benton, C. E., Balnave, D., Brake, J., Boyd, L. J., & Brown, M. A. (1998). The use of dietary minerals during heat stress in broilers. *The Professional Animal Scientist*, *14*, 193–196.

Binsiya, T. K., Sejian, V., Bagath, M., Krishnan, G., Hyder, I., Manimaran, A., Lees, A. M., Gaughan, J. B., & Bhatta, R (2017). Significance of Hypothalamic-Pituitary-Adrenal Axis to adapt to Climate Change in Livestock.

Borges, S. A., Fischer Da Silva, A. V., Majorka, A., Hooge, D. M., & Cummings, K. R. (2004). Physiological responses of broiler chickens to heat stress and dietary electrolyte balance (sodium plus potassium minus chloride, milliequivalents per kilogram). *Poultry Science*, *83*, 1551–1558.

Brugaletta, G., Teyssier, J.-R., Rochell, S. J., Dridi, S., & Sirri, F. (2022). A review of heat stress in chickens. Part I: Insights into physiology and gut health. *Frontiers in Physiology*, *13*, 934381.

Cao, C., Chowdhury, V. S., Cline, M. A., & Gilbert, E. R. (2021). The microbiota-gut-brain axis during heat stress in chickens: A review. *Frontiers in Physiology*, *12*, 752265.

Carvalho, R. H., Soares, A. L., Grespan, M., Spurio, R. S., Coró, F. A. G., Oba, A., & Shimokomaki, M. (2015). The effects of the dark house system on growth, performance and meat quality of broiler chicken. *Animal Science Journal*, *86*, 189–193.

Cassuce, D. C., Tinôco, I. F. F., Baêta, F. C., Zolnier, S., Cecon, P. R., & Vieira, M. F. A. (2013). Thermal comfort temperature update for broiler chickens up to 21 days of age. *Engenharia Agrícola*, *33*, 28–36.

Çaylı, A., Akyüz, A., Üstün, S., & Yeter, B. (2021). Efficiency of two different types of evaporative cooling systems in broiler houses in Eastern Mediterranean climate conditions. *Thermal Science and Engineering Progress*, *22*, 100844.

Chung, M. K., Choi, J. H., Chung, Y. K., & Chee, K. M. (2005). Effects of dietary vitamins C and E on egg shell quality of broiler breeder hens exposed to heat stress. *Asian-Australasian Journal of Animal Sciences*, *18*, 545–551.

Chang, Q., Lu, Y., & Lan, R. (2020). Chitosan oligosaccharide as an effective feed additive to maintain growth performance, meat quality, muscle glycolytic metabolism, and oxidative status in yellow-feather broilers under heat stress. *Poultry Science*, *99*, 4824–4831.

Dalólio, F. S., Albino, L. F. T., Lima, H. J. D., Silva, J. N. D., & Moreira, J. (2015). Heat stress and vitamin E in diets for broilers as a mitigating measure. *Acta Scientiarum: Animal Sciences*, *37*, 419.

Das, R., Mishra, P., & Jha, R. (2021). In ovo feeding as a tool for improving performance and gut health of poultry: A review. *Frontiers in Veterinary Science*, *8*, 754246.

Deeb, N., & Cahaner, A. (2002). Genotype-by-environment interaction with broiler genotypes differing in growth rate. 3. Growth rate and water consumption of broiler progeny from weight-selected versus nonselected parents under normal and high ambient temperatures. *Poultry Science*, *81*, 293–301.

Deng, W., Dong, X. F., Tong, J. M., & Zhang, Q. (2012). The probiotic *Bacillus licheniformis* ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. *Poultry Science*, *91*, 575–582.

Ebeid, T. A., Suzuki, T., & Sugiyama, T. (2012). High ambient temperature influences eggshell quality and calbindin-D28k localization of eggshell gland and all intestinal segments of laying hens. *Poultry Science*, *91*, 2282–2287.

Elnesr, S. S., Elwan, H. A. M., Xu, Q. Q., Xie, C., Dong, X. Y., & Zou, X. T. (2019). Effects of in ovo injection of sulfur-containing amino acids on heat shock protein 70, corticosterone hormone, antioxidant indices, and lipid profile of newly hatched broiler chicks exposed to heat stress during incubation. *Poultry Science*, *98*, 2290–2298.

El-Senousey, H. K., Chen, B., Wang, J. Y., Atta, A. M., Mohamed, F. R., & Nie, Q. H. (2018). In ovo injection of ascorbic acid modulates antioxidant defense system and immune gene expression in newly hatched local Chinese yellow broiler chicks. *Poultry Science*, *97*, 425–429.

El-Tarabany, M. S. (2016). Effect of thermal stress on fertility and egg quality of Japanese quail. *Journal of Thermal Biology*, *61*, 38–43.

Fathi, M. M., Galal, A., Radwan, L. M., Abou-Emera, O. K., & Al-Homidan, I. H. (2022). Using major genes to mitigate the deleterious effects of heat stress in poultry: An updated review. *Poultry Science*, *101*, 102157.

Francis, C. A., Macleod, M. G., & Anderson, J. E. M. (1991). Alleviation of acute heat stress by food withdrawal or darkness. *British Poultry Science*, *32*, 219–225.

Galarza-Seeber, R., Latorre, J. D., Bielke, L. R., Kuttappan, V. A., Wolfenden, A. D., Hernandez-Velasco, X., Merino-Guzman, R., Vicente, J. L., Donoghue, A., Cross, D., Hargis, B. M., & Tellez, G. (2016). Leaky gut and mycotoxins: Aflatoxin B1 does not increase gut permeability in broiler chickens. *Frontiers in Veterinary Science*, *3*, 10. http://journal.frontiersin.org/Article/10.3389/fvets.2016.00010/abstract

Ghazalah, A. A., Abd - elsa, M. O., & Ali, A. M. (2008). Influence of dietary energy and poultry fat on the response of broiler chicks to heat therm. *International Journal of Poultry Science*, *7*, 355–359.

Glatz, P. (2013). Poultry housing and management in developing countries.

Goel, A. (2021). Heat stress management in poultry. *Journal of Animal Physiology and Animal Nutrition*, *105*, 1136–1145.

Goel, A., Bhanja, S. K., Mehra, M., Majumdar, S., & Mandal, A. (2017). In ovo silver nanoparticle supplementation for improving the post-hatch immunity status of broiler chickens. *Archives of Animal Nutrition*, *71*, 384–394.

Goel, A., Ncho, C. M., Gupta, V., & Choi, Y.-H. (2023). Embryonic modulation through thermal manipulation and in ovo feeding to develop heat tolerance in chickens. *Animal Nutrition*, *13*, 150–159.

Goel, A., Ncho, C. M., Kim, B.-J., Jeong, C.-M., Gupta, V., Jung, J.-Y., Ha, S.-Y., Yang, J.-K., & Choi, Y.-H. (2022). Dietary shredded steam-exploded pine particle supplementation as a strategy to mitigate chronic cyclic heat stress by modulating gut microbiota in broilers. *Scientific Reports*, *12*, 19704.

Gomes, A. V. S., Quinteiro-Filho, W. M., Ribeiro, A., Ferraz-de-Paula, V., Pinheiro, M. L., Baskeville, E., Akamine, A. T., Astolfi-Ferreira, C. S., Ferreira, A. J. P., & Palermo-Neto, J. (2014). Overcrowding stress decreases macrophage activity and increases *Salmonella* Enteritidis invasion in broiler chickens. *Avian Pathology*, *43*, 82–90.

Gupta, V., Ncho, C. M., Goel, A., Jeong, C.-M., & Choi, Y.-H. (2022). Effects of in ovo injection of α-ketoglutaric acid on hatchability, growth, plasma metabolites, and antioxidant status of broilers. *Antioxidants*, *11*, 2102.

Han, G., Yang, H., Bungo, T., Ikeda, H., Wang, Y., Nguyen, L. T. N., Eltahan, H. M., Furuse, M., & Chowdhury, V. S. (2018). In ovo L - leucine administration stimulates lipid metabolisms in heat-exposed male, but not female, chicks to afford thermotolerance. *Journal of Thermal Biology*, *71*, 74–82.

Hasan, S., Hossain, M. M., Alam, J., & Bhuiyan, M. E. R (2015). Benificial effects of probiotic on growth performance and hemato-biochemical parameters in broilers during heat stress. 10.

He, S. P., Arowolo, M. A., Medrano, R. F., Li, S., Yu, Q. F., Chen, J. Y., & He, J. H. (2018). Impact of heat stress and nutritional interventions on poultry production. *World's Poultry Science Journal*, *74*, 647–664.

He, S., Li, S., Arowolo, M. A., Yu, Q., Chen, F., Hu, R., & He, J. (2019). Effect of resveratrol on growth performance, rectal temperature and serum parameters of yellow-feather broilers under heat stress. *Animal Science Journal*, *90*, 401–411.

Heidary, M., Hassanabadi, A., & Mohebalian, H. (2020). Effects of in ovo injection of nanocurcumin and vitamin E on antioxidant status immune responses intestinal morphology and growth performance of broiler chickens exposed to heat stress. *Journal of Livestock Science and Technologies*, *8*, 17–27. https://doi.org/10.22103/jlst.2020.15352.1310

Hu, R., He, Y., Arowolo, M., Wu, S., & He, J. (2019). Polyphenols as potential attenuators of heat stress in poultry production. *Antioxidants*, *8*, 67.

Hulet, R., Gladys, G., Hill, D., Meijerhof, R., & El-Shiekh, T. (2007). Influence of egg shell embryonic incubation temperature and broiler breeder flock age on posthatch growth performance and carcass characteristics. *Poultry Science*, *86*, 408–412.

Iyasere, O. S., Bateson, M., Beard, A. P., & Guy, J. H. (2021). Provision of additional cup drinkers mildly alleviated moderate heat stress conditions in broiler chickens. *Journal of Applied Animal Welfare Science*, *24*, 188–199.

Johnston, P. A., Liu, H., O'Connell, T., Phelps, P., Bland, M., Tyczkowski, J., Kemper, A., Harding, T., Avakian, A., Haddad, E., Whitfill, C., Gildersleeve, R., & Ricks, C. A. (1997). Applications in in ovo technology. *Poultry Science*, *76*, 165–178.

Kang, D., & Shim, K. (2021). Early heat exposure effect on the heat shock proteins in broilers under acute heat stress. *Poultry Science*, *100*, 100964.

Kapetanov, M., Pajić, M., Ljubojević, D., & Pelić, M. (2016). Heat stress in poultry industry. *Archives of Veterinary Medicine*, *8*, 87–101.

Khalil, M. M., Abdollahi, M. R., Zaefarian, F., & Ravindran, V. (2021). Influence of feed form on the apparent metabolisable energy of feed ingredients for broiler chickens. *Animal Feed Science and Technology*, *271*, 114754.

Khan, R. U., Naz, S., Nikousefat, Z., Selvaggi, M., Laudadio, V., & Tufarelli, V. (2012). Effect of ascorbic acid in heat-stressed poultry. *World's Poultry Science Journal*, *68*, 477–490.

Khan, R. U., Naz, S., Nikousefat, Z., Tufarelli, V., Javdani, M., Rana, N., & Laudadio, V. (2011). Effect of vitamin E in heat-stressed poultry. *World's Poultry Science Journal*, *67*, 469–478.

Kim, H.-W., Cramer, T., Ogbeifun, O. O. E., Seo, J.-K., Yan, F., Cheng, H.-W., & Kim, Y. H. B. (2017). Breast meat quality and protein functionality of broilers with different probiotic levels and cyclic heat challenge exposure. *Meat and Muscle Biology*, *1*, 1. https://www.iastatedigitalpress.com/mmb/article/id/9446/

Kim, H., Kim, H., Seong, P., Arora, D., Shin, D., Park, W., & Park, J.-E. (2021). Transcriptomic response under heat stress in chickens revealed the regulation of genes and alteration of metabolism to maintain homeostasis. *Animals: An Open Access Journal from MDPI*, *11*, 2241.

Kpomasse, C. C., Oke, O. E., Houndonougbo, F. M., & Tona, K. (2021). Broiler production challenges in the tropics: A review. *Veterinary Medicine and Science*, *7*, 831–842.

Kucuk, O., Sahin, N., & Sahin, K. (2003). Supplemental zinc and vitamin A can alleviate negative effects of heat stress in broiler chickens. *Biological Trace Element Research*, *94*, 225–236.

Lara, L., & Rostagno, M. (2013). Impact of heat stress on poultry production. *Animals: An Open Access Journal from MDPI*, *3*, 356–369.

Li, X. J., Piao, X. S., Kim, S. W., Liu, P., Wang, L., Shen, Y. B., Jung, S. C., & Lee, H. S. (2007). Effects of chito-oligosaccharide supplementation on performance, nutrient digestibility, and serum composition in broiler chickens. *Poultry Science*, *86*, 1107–1114.

Lin, H., Jiao, H. C., Buyse, J., & Decuypere, E. (2006). Strategies for preventing heat stress in poultry. *World's Poultry Science Journal*, *62*, 71–86.

Lin, H., Wang, L. F., Song, J. L., Xie, Y. M., & Yang, Q. M. (2002). Effect of dietary supplemental levels of vitamin A on the egg production and immune responses of heat-stressed laying hens. *Poultry Science*, *81*, 458–465.

Liu, G., Zhu, H., Ma, T., Yan, Z., Zhang, Y., Geng, Y., Zhu, Y., & Shi, Y. (2020). Effect of chronic cyclic heat stress on the intestinal morphology, oxidative status and cecal bacterial communities in broilers. *Journal of Thermal Biology*, *91*, 102619.

Liu, L. L., He, J. H., Xie, H. B., Yang, Y. S., Li, J. C., & Zou, Y. (2014). Resveratrol induces antioxidant and heat shock protein mRNA expression in response to heat stress in black-boned chickens. *Poultry Science*, *93*, 54–62.

Lozano, C., De Basilio, V., Oliveros, I., Alvarez, R., Colina, I., Bastianelli, D., Yahav, S., & Picard, M. (2006). Is sequential feeding a suitable technique to compensate for the negative effects of a tropical climate in finishing broilers? *Animal Research*, *55*, 71–76.

Luo, J., Song, J., Liu, L., Xue, B., Tian, G., & Yang, Y. (2018). Effect of epigallocatechin gallate on growth performance and serum biochemical metabolites in heat-stressed broilers. *Poultry Science*, *97*, 599–606.

Løtvedt, P., Fallahshahroudi, A., Bektic, L., Altimiras, J., & Jensen, P. (2017). Chicken domestication changes expression of stress-related genes in brain, pituitary and adrenals. *Neurobiology of Stress*, *7*, 113–121.

Mack, L. A., Felver-Gant, J. N., Dennis, R. L., & Cheng, H. W. (2013). Genetic variations alter production and behavioral responses following heat stress in 2 strains of laying hens. *Poultry Science*, *92*, 285–294.

MacLeod, M. G., & Hocking, P. M. (1993). Thermoregulation at high ambient temperature in genetically fat and lean broiler hens fed *ad libitum* or on a controlled-feeding regime. *British Poultry Science*, *34*, 589–596.

Maddahian, A., Dadvar, P., Morovat, M., & Shamsaddini Bafti, M. (2021). The effect of in ovo feeding compared with dietary feeding of betaine on performance, immunity and liver activity of broiler chickens exposed to high temperatures. *Iranian Journal of Applied Animal Science*, *11*, 381–390.

Madkour, M., Salman, F. M., El-Wardany, I., Abdel-Fattah, S. A., Alagawany, M., Hashem, N. M., Abdelnour, S. A., El-Kholy, M. S., & Dhama, K. (2022). Mitigating the detrimental effects of heat stress in poultry through thermal conditioning and nutritional manipulation. *Journal of Thermal Biology*, *103*, 103169.

Majidi-Mosleh, A., Sadeghi, A., Mousavi, S., Chamani, M., & Zarei, A. (2017). Effects of in ovo infusion of probiotic strains on performance parameters. *Jejunal Bacterial Population and Mucin Gene Expression in Broiler Chicken Revista Brasileira de Ciência Avícola*, *19*, 97–102.

Mashaly, M. M., Hendricks, G. L., Kalama, M. A., Gehad, A. E., Abbas, A. O., & Patterson, P. H. (2004). Effect of heat stress on production parameters and immune responses of commercial laying hens. *Poultry Science*, *83*, 889–894.

McGruder, B. M., Zhai, W., Keralapurath, M. M., Bennett, L. W., Gerard, P. D., & Peebles, E. D. (2011). Effects of in ovo injection of electrolyte solutions on the pre- and posthatch physiological characteristics of broilers. *Poultry Science*, *90*, 1058–1066.

Mohamed, A. S. A., Lozovskiy, A. R., & Ali, A. M. A. (2019). Strategies to combat the deleterious impacts of heat stress through feed restrictions and dietary supplementation (vitamins, minerals) in broilers. *Journal of the Indonesian Tropical Animal Agriculture*, *44*, 155.

Mohammed, A. A., Jacobs, J. A., Murugesan, G. R., & Cheng, H. W. (2018). Effect of dietary synbiotic supplement on behavioral patterns and growth performance of broiler chickens reared under heat stress. *Poultry Science*, *97*, 1101–1108.

Mohammed, A. A., Jiang, S., Jacobs, J. A., & Cheng, H. W. (2019). Effect of a synbiotic supplement on cecal microbial ecology, antioxidant status, and immune response of broiler chickens reared under heat stress. *Poultry Science*, *98*, 4408–4415.

Mushtaq, M. M. H., Pasha, T. N., Mushtaq, T., & Parvin, R. (2013). Electrolytes, dietary electrolyte balance and salts in broilers: An updated review on growth performance, water intake and litter quality. *World's Poultry Science Journal*, *69*, 789–802.

Nawab, A., Ibtisham, F., Li, G., Kieser, B., Wu, J., Liu, W., Zhao, Y., Nawab, Y., Li, K., Xiao, M., & An, L. (2018). Heat stress in poultry production: Mitigation strategies to overcome the future challenges facing the global poultry industry. *Journal of Thermal Biology*, *78*, 131–139.

Nawaz, A. H., Amoah, K., Leng, Q. Y., Zheng, J. H., Zhang, W. L., & Zhang, L. (2021). Poultry response to heat stress: Its physiological, metabolic, and genetic implications on meat production and quality including strategies to improve broiler production in a warming world. *Frontiers in Veterinary Science*, *8*, 699081.

Ncho, C. M., Goel, A., Gupta, V., Jeong, C.-M., & Choi, Y.-H. (2022). Impact of embryonic manipulations on core body temperature dynamics and survival in broilers exposed to cyclic heat stress. *Scientific Reports*, *12*, 15110.

Ncho, C.-M., Goel, A., Jeong, C.-M., Gupta, V., & Choi, Y.-H. (2021). Effects of in ovo feeding of γ-aminobutyric acid on growth performances, plasma metabolites, and antioxidant status in broilers exposed to cyclic heat stress. *Sustainability*, *13*, 11032.

Ncho, C.-M., Goel, A., Jeong, C.-M., Youssouf, M., & Choi, Y.-H. (2021). In ovo injection of GABA can help body weight gain at hatch, increase chick weight to egg weight ratio, and improve broiler heat resistance. *Animals: An Open Access Journal from MDPI*, *11*, 1364.

Neves, D., Banhazi, T., & Nääs, I. (2014). Feeding behaviour of broiler chickens: A review on the biomechanical characteristics. *Revista Brasileira de Ciência Avícola*, *16*, 01–16.

Njoku, P. C. (1986). Effect of dietary ascorbic acid (vitamin C) supplementation on the performance of broiler chickens in a tropical environment. *Animal Feed Science and Technology*, *16*, 17–24.

Oke, O. E., Oyelola, O. B., Iyasere, O. S., Njoku, C. P., Oso, A. O., Oso, O. M., Fatoki, S. T., Bankole, K. O., Jimoh, I. O., Sybill, N. I., Awodipe, H. O., Adegbite, H. O., Rahman, S. A., & Daramola, J. O. (2021). In ovo injection of black cumin (Nigella sativa) extract on hatching and post hatch performance of thermally challenged broiler chickens during incubation. *Poultry Science*, *100*, 100831.

Oladokun, S., & Adewole, D. I. (2020). In ovo delivery of bioactive substances: An alternative to the use of antibiotic growth promoters in poultry production—a review. *Journal of Applied Poultry Research*, *29*, 744–763.

Oladokun, S., & Adewole, D. I. (2022). Biomarkers of heat stress and mechanism of heat stress response in Avian species: Current insights and future perspectives from poultry science. *Journal of Thermal Biology*, *110*, 103332.

Oladokun, S., Koehler, A., MacIsaac, J., Ibeagha-Awemu, E. M., & Adewole, D. I. (2021). *Bacillus subtilis* delivery route: effect on growth performance, intestinal morphology, cecal short-chain fatty acid concentration, and cecal microbiota in broiler chickens. *Poultry Science*, *100*, 100809.

Oloyo, A., & Ojerinde, A (2020). *Poultry housing and management in poultry—An ādvanced learning*. In A. Ali Kamboh (Ed.). IntechOpen.

Onagbesan, O. M., Uyanga, V. A., Oso, O., Tona, K., & Oke, O. E. (2023). Alleviating heat stress effects in poultry: updates on methods and mechanisms of actions. *Frontiers in Veterinary Science*, *10*, 1255520.

Pawar, S., Sajjanar, B., Lonkar, V., Kurade, N., Kadam, A., Nirmal, A., Brahmane, M., & Bal, S. (2016). Assessing and mitigating the impact of heat stress in poultry. *Advances in Animal and Veterinary Sciences*, *4*, 332–341.

Peebles, E. D. (2018). In ovo applications in poultry: A review. *Poultry Science*, *97*, 2322–2338.

Piestun, Y., Shinder, D., Ruzal, M., Halevy, O., Brake, J., & Yahav, S. (2008). Thermal manipulations during broiler embryogenesis: Effect on the acquisition of thermotolerance. *Poultry Science*, *87*, 1516–1525.

Pietrzak, E., Dunislawska, A., Siwek, M., Zampiga, M., Sirri, F., Meluzzi, A., Tavaniello, S., Maiorano, G., & Slawinska, A. (2020). Splenic gene expression signatures in slow-growing chickens stimulated in ovo with galactooligosaccharides and challenged with heat. *Animals: An Open Access Journal from MDPI*, *10*, 474.

Ratriyanto, A., & Mosenthin, R. (2018). Osmoregulatory function of betaine in alleviating heat stress in poultry. *Journal of Animal Physiology and Animal Nutrition*, *102*, 1634–1650.

Renaudeau, D., Collin, A., Yahav, S., De Basilio, V., Gourdine, J. L., & Collier, R. J. (2012). Adaptation to hot climate and strategies to alleviate heat stress in livestock production. *Animal*, *6*, 707–728.

Ricks, C., Mendu, N., & Phelps, P. (2003). The embryonated egg: A practical target for genetic based advances to improve poultry production. *Poultry Science*, *82*, 931–938.

Ringseis, R., & Eder, K. (2022). Heat stress in pigs and broilers: Role of gut dysbiosis in the impairment of the gut-liver axis and restoration of these effects by probiotics, prebiotics and synbiotics. *Journal of Animal Science and Biotechnology*, *13*, 126.

Roberfroid, M. (2007). Prebiotics: The concept revisited. *The Journal of Nutrition*, *137*, 830S–837S.

Rostagno, M. H. (2020). Effects of heat stress on the gut health of poultry. *Journal of Animal Science*, *98*, skaa090.

Saeed, M., Abbas, G., Alagawany, M., Kamboh, A. A., Abd El-Hack, M. E., Khafaga, A. F., & Chao, S. (2019). Heat stress management in poultry farms: A comprehensive overview. *Journal of Thermal Biology*, *84*, 414–425.

Saeed, M., Babazadeh, D., Naveed, M., Alagawany, M., Abd El-Hack, M. E., Arain, M. A., Tiwari, R., Sachan, S., Karthik, K., Dhama, K., Elnesr, S. S., & Chao, S. (2019). In ovo delivery of various biological supplements, vaccines and drugs in poultry: current knowledge. *Journal of the Science of Food and Agriculture*, *99*, 3727–3739.

Sahin, K., & Kucuk, O. (2001). Effects of vitamin E and selenium on performance, digestibility of nutrients, and carcass characteristics of Japanese quails reared under heat stress (34°C). *Journal of Animal Physiology and Animal Nutrition*, *85*, 342–348.

Sahin, K., & Kucuk, O. (2003). Zinc supplementation alleviates heat stress in laying Japanese quail. *The Journal of Nutrition*, *133*, 2808–2811.

Sahin, K., Orhan, C., Tuzcu, M., Ali, S., Sahin, N., & Hayirli, A. (2010). Epigallocatechin-3-gallate prevents lipid peroxidation and enhances antioxidant defense system via modulating hepatic nuclear transcription factors in heat-stressed quails. *Poultry Science*, *89*, 2251–2258.

Sahin, K., Orhan, C., Tuzcu, M., Sahin, N., Hayirli, A., Bilgili, S., & Kucuk, O. (2016). Lycopene activates antioxidant enzymes and nuclear transcription factor systems in heat-stressed broilers. *Poultry Science*, *95*, 1088–1095.

Sahin, N., Onderci, M., Sahin, K., & Kucuk, O. (2008). Supplementation with organic or inorganic selenium in heat-distressed quail. *Biological Trace Element Research*, *122*, 229–237.

Saleh, K. M. M., Tarkhan, A. H., & Al-Zghoul, M. B. (2020). Embryonic thermal manipulation affects the antioxidant response to post-hatch thermal exposure in broiler chickens. *Animals: An Open Access Journal from MDPI*, *10*, 126.

Sgavioli, S., De Almeida, V. R., Matos Júnior, J. B., Zanirato, G. L., Borges, L. L., & Boleli, I. C. (2019). In ovo injection of ascorbic acid and higher incubation temperature modulate blood parameters in response to heat exposure in broilers. *British Poultry Science*, *60*, 279–287.

Shakeri, M., Cottrell, J. J., Wilkinson, S., Le, H. H., Suleria, H. A. R., Warner, R. D., & Dunshea, F. R. (2020). Dietary betaine reduces the negative effects of cyclic heat exposure on growth performance, blood gas status and meat quality in broiler chickens. *Agriculture (London)*, *10*, 176.

Shakeri, M., Cottrell, J. J., Wilkinson, S., Zhao, W., Le, H. H., McQuade, R., Furness, J. B., & Dunshea, F. R. (2019). Dietary betaine improves intestinal barrier function and ameliorates the impact of heat stress in multiple vital organs as measured by Evans blue dye in broiler chickens. *Animals: An Open Access Journal from MDPI*, *10*, 38.

Shakeri, M., & Le, H. H. (2022). Deleterious effects of heat stress on poultry production: Unveiling the benefits of betaine and polyphenols. *Poultry*, *1*, 147–156.

Shakeri, M., Zulkifli, I., Soleimani, A. F., O'Reilly, E. L., Eckersall, P. D., Anna, A. A., Kumari, S., & Abdullah, F. F. J. (2014). Response to dietary supplementation of l-glutamine and l-glutamate in broiler chickens reared at different stocking densities under hot, humid tropical conditions. *Poultry Science*, *93*, 2700–2708.

Sharifi, A. R., Horst, P., & Simianer, H. (2010). The effect of frizzle gene and dwarf gene on reproductive performance of broiler breeder dams under high and normal ambient temperatures. *Poultry Science*, *89*, 2356–2369.

Sharma, J. M., & Burmester, B. R. (1982). Resistance of Marek's disease at hatching in chickens vaccinated as embryos with the Turkey herpesvirus. *Avian Diseases*, *26*, 134.

Shokraneh, M., Sadeghi, A. A., Mousavi, S. N., Esmaeilkhanian, S., & Chamani, M. (2020). Effects of in ovo injection of nano-selenium and nano-zinc oxide and high eggshell temperature during late incubation on antioxidant activity, thyroid and glucocorticoid hormones and some blood metabolites in broiler hatchlings. *Acta Scientiarum. Animal Sciences*, *42*, e46029.

Silva, V. K., Silva, J. D. T., Gravena, R. A., Marques, R. H., Hada, F. H., & Moraes, V. M. B. (2010). Yeast extract and prebiotic in pre-initial phase diet for broiler chickens raised under different temperatures. *Revista Brasileira de Zootecnia*, *39*, 165–174.

Siwek, M., Slawinska, A., Stadnicka, K., Bogucka, J., Dunislawska, A., & Bednarczyk, M. (2018). Prebiotics and synbiotics—in ovo delivery for improved lifespan condition in chicken. *BMC Veterinary Research*, *14*, 402.

Slawinska, A., Mendes, S., Dunislawska, A., Siwek, M., Zampiga, M., Sirri, F., Meluzzi, A., Tavaniello, S., & Maiorano, G. (2019). Avian model to mitigate gut-derived immune response and oxidative stress during heat. *Biosystems*, *178*, 10–15.

Slawinska, A., Plowiec, A., Siwek, M., Jaroszewski, M., & Bednarczyk, M. (2016). Long-term transcriptomic effects of prebiotics and synbiotics delivered in ovo in broiler chickens. *PLoS ONE*, *11*, e0168899.

Slawinska, A., Zampiga, M., Sirri, F., Meluzzi, A., Bertocchi, M., Tavaniello, S., & Maiorano, G. (2020). Impact of galactooligosaccharides delivered in ovo on mitigating negative effects of heat stress on performance and welfare of broilers. *Poultry Science*, *99*, 407–415.

Smith, M. O., & Teeter, R. G. (1987). Potassium balance of the 5 to 8-week-old broiler exposed to constant heat or cycling high temperature stress and the effects of supplemental potassium chloride on body weight gain and feed efficiency. *Poultry Science*, *66*, 487–492.

Sohail, M. U., Hume, M. E., Byrd, J. A., Nisbet, D. J., Ijaz, A., Sohail, A., Shabbir, M. Z., & Rehman, H. (2012). Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress. *Poultry Science*, *91*, 2235–2240.

Sohail, M. U., Ijaz, A., Yousaf, M. S., Ashraf, K., Zaneb, H., Aleem, M., & Rehman, H. (2010). Alleviation of cyclic heat stress in broilers by dietary supplementation of mannan-oligosaccharide and Lactobacillus-based probiotic: Dynamics of cortisol, thyroid hormones, cholesterol, C-reactive protein, and humoral immunity. *Poultry Science*, *89*, 1934–1938.

Song, D., Li, A., Wang, Y., Song, G., Cheng, J., Wang, L., Liu, K., Min, Y., & Wang, W. (2022). Effects of synbiotic on growth, digestibility, immune and antioxidant performance in broilers. *Animal*, *16*, 100497.

Song, D. J., & King, A. J. (2015). Effects of heat stress on broiler meat quality. *World's Poultry Science Journal*, *71*, 701–709.

Song, J., Jiao, L. F., Xiao, K., Luan, Z. S., Hu, C. H., Shi, B., & Zhan, X. A. (2013). Cello-oligosaccharide ameliorates heat stress-induced impairment of intestinal microflora, morphology and barrier integrity in broilers. *Animal Feed Science and Technology*, *185*, 175–181.

Song, J., Xiao, K., Ke, Y. L., Jiao, L. F., Hu, C. H., Diao, Q. Y., Shi, B., & Zou, X. T. (2014). Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. *Poultry Science*, *93*, 581–588.

Souza, L. F. A., Espinha, L. P., Almeida, E. A., Lunedo, R., Furlan, R. L., & Macari, M. (2016). How heat stress (continuous or cyclical) interferes with nutrient digestibility, energy and nitrogen balances and performance in broilers. *Livestock Science*, *192*, 39–43.

St-Pierre, N. R., Cobanov, B., & Schnitkey, G. (2003). Economic losses from heat stress by US livestock industries. *Journal of Dairy Science*, *86*, E52–E77.

Sugiharto, S. (2020). Alleviation of heat stress in broiler chicken using turmeric (Curcuma longa)—a short review. *Journal of Animal Behaviour and Biometeorology*, *8*, 215–222.

Syafwan, S., Kwakkel, R. P., & Verstegen, M. W. A. (2011). Heat stress and feeding strategies in meat-type chickens. *World's Poultry Science Journal*, *67*, 653–674.

Tavaniello, S., Maiorano, G., Stadnicka, K., Mucci, R., Bogucka, J., & Bednarczyk, M. (2018). Prebiotics offered to broiler chicken exert positive effect on meat quality traits irrespective of delivery route. *Poultry Science*, *97*, 2979–2987.

Tavaniello, S., Slawinska, A., Prioriello, D., Petrecca, V., Bertocchi, M., Zampiga, M., Salvatori, G., & Maiorano, G. (2020). Effect of galactooligosaccharides delivered in ovo on meat quality traits of broiler chickens exposed to heat stress. *Poultry Science*, *99*, 612–619.

Teyssier, J.-R., Brugaletta, G., Sirri, F., Dridi, S., & Rochell, S. J. (2022). A review of heat stress in chickens. Part II: Insights into protein and energy utilization and feeding. *Frontiers in Physiology*, *13*, 943612.

Tixier-Boichard, M., Huybrechts, L. M., Kühn, E., Decuypere, E., Charrier, J., & Mongin, P (1989). Physiological studies on the sex-linked dwarfism of the fowl: A review on the search for the gene's primary effect.

Tóth, R., Tokodyné Szabadi, N., Lázár, B., Buda, K., Végi, B., Barna, J., Patakiné Várkonyi, E., Liptói, K., Pain, B., & Gócza, E. (2021). Effect of post-hatch heat-treatment in heat-stressed transylvanian naked neck chicken. *Animals: An Open Access Journal from MDPI*, *11*, 1575.

Uzum, M. H., & Toplu, H. D. O. (2013). Effects of stocking density and feed restriction on performance, carcass, meat quality characteristics and some stress parameters in broilers under heat stress. M. H.

Villaluenga, C. M., Wardeńska, M., Pilarski, R., Bednarczyk, M., & Gulewicz, K. (2004). Utilization of the chicken embryo model for assessment of biological activity of different oligosaccharides. *Folia Biologica*, *52*, 135–142.

Wang, W. C., Yan, F. F., Hu, J. Y., Amen, O. A., & Cheng, H. W. (2018). Supplementation of *Bacillus subtilis*-based probiotic reduces heat stress-related behaviors and inflammatory response in broiler chickens1. *Journal of Animal Science*, *96*, 1654–1666.

Wasti, S., Sah, N., & Mishra, B. (2020). Impact of heat stress on poultry health and performances, and potential mitigation strategies. *Animals: An Open Access Journal from MDPI*, *10*, 1266.

Wasti, S., Sah, N., Singh, A. K., Lee, C. N., Jha, R., & Mishra, B. (2021). Dietary supplementation of dried plum: A novel strategy to mitigate heat stress in broiler chickens. *Journal of Animal Science and Biotechnology*, *12*, 58.

Wiernusz, C. J., & Teeter, R. G. (1996). Acclimation effects on fed and fasted broiler thermobalance during thermoneutral and high ambient temperature exposure. *British Poultry Science*, *37*, 677–687.

Williams, C. J., & Zedek, A. S. (2010). Comparative field evaluations of in ovo applied technology. *Poultry Science*, *89*, 189–193.

Wolfenson, D., Bachrach, D., Maman, M., Graber, Y., & Rozenboim, I. (2001). Evaporative cooling of ventral regions of the skin in heat-stressed laying hens. *Poultry Science*, *80*, 958–964.

Wu, X.-Y., Wang, F.-Y., Chen, H.-X., Dong, H.-L., Zhao, Z.-Q., & Si, L.-F. (2023). Chronic heat stress induces lung injury in broiler chickens by disrupting the pulmonary blood-air barrier and activating TLRs/NF-κB signaling pathway. *Poultry Science*, *102*, 103066.

Yahav, S., & McMurtry, J. P. (2001). Thermotolerance acquisition in broiler chickens by temperature conditioning early in life—The effect of timing and ambient temperature. *Poultry Science*, *80*, 1662–1666.

Yalçin, S., Özkan, S., Çabuk, M., & Siegel, P. B. (2003). Criteria for evaluating husbandry practices to alleviate heat stress in broilers. *Journal of Applied Poultry Research*, *12*, 382–388.

Yalcin, S., Testik, A., Ozkan, S., Settar, P., Celen, F., & Cahaner, A. (1997). Performance of naked neck and normal broilers in hot, warm, and temperate climates. *Poultry Science*, *76*, 930–937.

Zaboli, G., Huang, X., Feng, X., & Ahn, D. U. (2019). How can heat stress affect chicken meat quality?—a review. *Poultry Science*, *98*, 1551–1556.

Zaboli, G.-R., Rahimi, S., Shariatmadari, F., Torshizi, M. A. K., Baghbanzadeh, A., & Mehri, M. (2017). Thermal manipulation during pre and post-hatch on thermotolerance of male broiler chickens exposed to chronic heat stress. *Poultry Science*, *96*, 478–485.

Zampiga, M., Laghi, L., Zhu, C., Cartoni Mancinelli, A., Mattioli, S., & Sirri, F. (2021). Breast muscle and plasma metabolomics profile of broiler chickens exposed to chronic heat stress conditions. *Animal*, *15*, 100275.

Zhang, J. F., Bai, K. W., Su, W. P., Wang, A. A., Zhang, L. L., Huang, K. H., & Wang, T. (2018). Curcumin attenuates heat-stress-induced oxidant damage by simultaneous activation of GSH-related antioxidant enzymes and Nrf2-mediated phase II detoxifying enzyme systems in broiler chickens. *Poultry Science*, *97*, 1209–1219.

Zhang, J., Wang, J., Wang, Q., Cui, H., Ding, J., Wang, Z., Thiam, M., Li, Q., & Zhao, G. (2023). Immunogenetic basis of chicken's heterophil to lymphocyte ratio revealed by genome-wide indel variants analysis. *Journal of Integrative Agriculture*, *22*, 2810–2823.

Zhang, P., Yan, T., Wang, X., Kuang, S., Xiao, Y., Lu, W., & Bi, D. (2017). Probiotic mixture ameliorates heat stress of laying hens by enhancing intestinal barrier function and improving gut microbiota. *Italian Journal of Animal Science*, *16*, 292–300.

Zhou, W. T., Fujita, M., Ito, T., & Yamamoto, S. (1997). Effects of early heat exposure on thermoregulatory responses and blood viscosity of broilers prior to marketing. *British Poultry Science*, *38*, 301–306.

How to cite this article: Mangan, M., & Siwek, M. (2024). Strategies to combat heat stress in poultry production—A review. *Journal of Animal Physiology and Animal Nutrition*, *108*, 576–595. https://doi.org/10.1111/jpn.13916

Journal of Animal Physiology and Animal Nutrition

WILEY

Journal of Animal Physiology and Animal Nutrition

ORIGINAL ARTICLE OPEN ACCESS

Poultry

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

M. Mangan[1] | P. Reszka[2] | K. Połtowicz[3] | M. Siwek[1]

[1]Department of Animal Biotechnology and Genetics, Bydgoszcz University of Science and Technology (PBS), Bydgoszcz, Poland | [2]Department of Animal Physiology, Physiotherapy and Nutrition, Bydgoszcz University of Science and Technology (PBS), Bydgoszcz, Poland | [3]Department of Poultry Breeding, National Research Institute of Animal Production, Balice, Poland

Correspondence: M. Mangan (modou.mangan@pbs.edu.pl)

Received: 10 July 2024 | **Revised:** 28 November 2024 | **Accepted:** 29 November 2024

Funding: This study was supported by H2020 Marie Sklodowska-Curie Actions and subsidy from the Polish Ministry of Science and Higher Education research project: BN-WHiBZ-0/2022.

Keywords: chick quality | gut health | in ovo technology | post-hatch performance | prebiotic | probiotic

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.

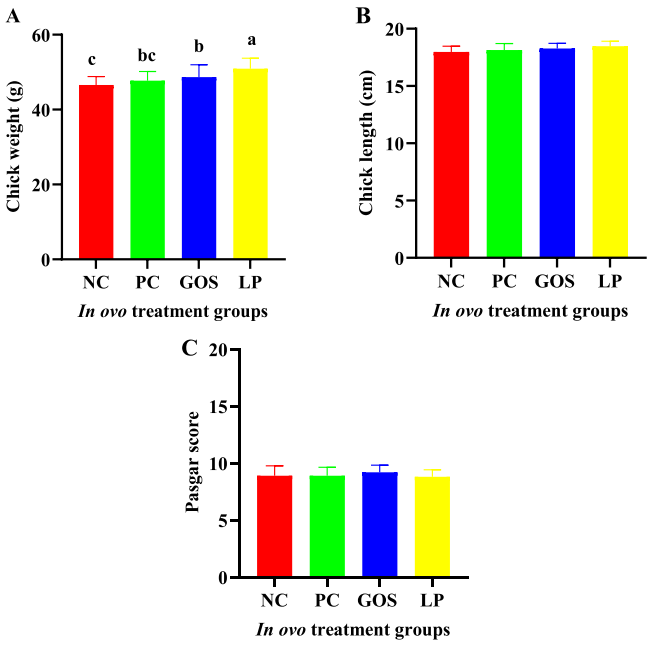

FIGURE 1 | The assessment of chick quality parameters: (A) hatchling weight (g), (B) length (cm) and (C) Pasgar score of the four in ovo treatment groups. Error bars: ±SD. Tukey HSD test ($p < 0.05$) was used to check for significant differences with different letters a, b, c. GOS, galactooligosaccharides; 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.

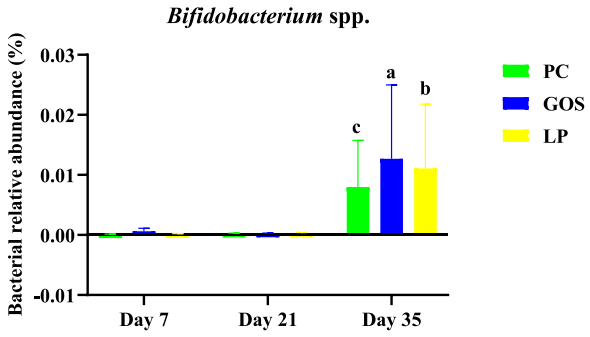

FIGURE 2 | The bacterial relative abundance of *Bifidobacterium* spp. in the faeces of in ovo treated chickens on Days 7, 21 and 35. Error bars: ±SE. a, b letters having different superscripts differ significantly ($p < 0.05$). GOS, galactooligosaccharide; LP, *Lactiplantibacillus plantarum*; PC, positive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

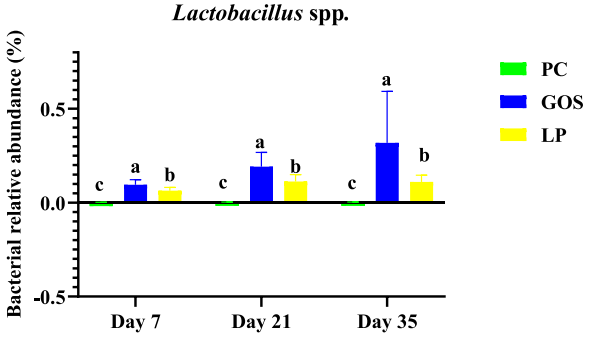

FIGURE 3 | The bacterial relative abundance of *Lactobacillus* spp. in the faeces of in ovo-treated chickens on Days 7, 21 and 35. Error bars: ±SE. a, b letters having different superscripts differ significantly ($p < 0.05$). GOS, galactooligosaccharide; LP, *Lactiplantibacillus plantarum*; PC, positive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

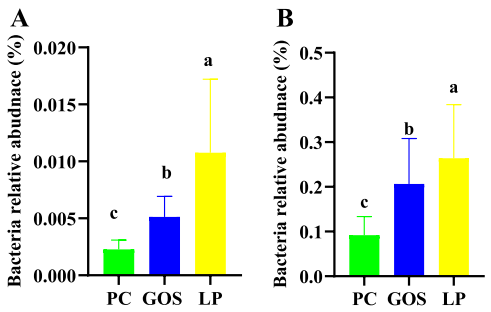

FIGURE 4 | The relative abundance of bacteria in the caecal content of in ovo-treated chickens: (A) *Lactobacillus* spp. and (B) *Bifidobacterium* spp. Error bars: ±SE. a, b letters having different superscripts differ significantly ($p < 0.05$). GOS, galactooligosaccharide; LP, *Lactiplantibacillus 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.

Traits	Treatment groups			SD	*p* value
	PC	GOS	LP		
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.

Acknowledgements

This study was financially supported by the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement number [955374] MonoGutHealth project. This study was also funded by a subsidy from the Polish Ministry of Science and Higher Education research project: BN-WHiBZ-0/2022.

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.

References

Adhikari, P. A., and W. K. Kim. 2017. "Overview of Prebiotics and Probiotics: Focus on Performance, Gut Health and Immunity—A Review." *Annals of Animal Science* 17: 949–966.

Ahmed, M. M. N., Z. S. H. Ismail, I. Elwardany, J. Lohakare, and A. A. A. Abdel-Wareth. 2023. "In Ovo Feeding Techniques of Green Nanoparticles of Silver and Probiotics: Evaluation of Performance, Physiological, and Microbiological Responses of Hatched One-Day-Old Broiler Chicks." *Animals* 13: 3725.

Akosile, O. A., F. O. Kehinde, A. I. Oni, and O. E. Oke. 2023. "Potential Implication of In Ovo Feeding of Phytogenics in Poultry Production." *Translational Animal Science* 7: txad094.

Alizadeh, M., J. Bavananthasivam, B. Shojadoost, et al. 2021. "In Ovo and Oral Administration of Probiotic Lactobacilli Modulate Cell- and Antibody-Mediated Immune Responses in Newly Hatched Chicks." *Frontiers in Immunology* 12: 664387.

Angwech, H., S., Tavaniello, A., Ongwech, A. N., Kaaya, and Maiorano, G. 2019. "Efficacy of in Ovo Delivered Prebiotics on Growth Performance, Meat Quality and Gut Health of Kuroiler Chickens in the Face of a Natural Coccidiosis Challenge." *Animals* 9, no. 11: 876.

Awad, W. A., K. Ghareeb, S. Abdel-Raheem, and J. Böhm. 2009. "Effects of Dietary Inclusion of Probiotic and Synbiotic on Growth Performance, Organ Weights, and Intestinal Histomorphology of Broiler Chickens." *Poultry Science* 88: 49–56.

Bednarczyk, M., K. Stadnicka, I. Kozłowska, et al. 2016. "Influence of Different Prebiotics and Mode of Their Administration on Broiler Chicken Performance." *Animal* 10: 1271–1279.

Bilalissi, A., O. N'nanle, and D. Nideou, et al. 2019. "The Appropriate Time to Improve Day-Old Chick Production and Post-Hatch Growth through *Moringa oleifera* Leaf Extract Inoculation Into the Hatching Egg." *European Poultry Science (EPS)* 83.

Bogucka, J., A. Dankowiakowska, G. Elminowska-Wenda, A. Sobolewska, A. Szczerba, and M. Bednarczyk. 2016. "Effects of Prebiotics and Synbiotics Delivered in Ovo on Broiler Small Intestine Histomorphology During the First Days After Hatching." *Folia Biologica (Kraków)* 64, no. 3: 131–143.

Chen, P., H. Lv, W. Liu, et al. 2023. "Effects of *Lactobacillus plantarum* HW1 on Growth Performance, Intestinal Immune Response, Barrier Function, and Cecal Microflora of Broilers With Necrotic Enteritis." *Animals* 13: 3810.

Cheng, Y., Y. Chen, X. Li, et al. 2017. "Effects of Synbiotic Supplementation on Growth Performance, Carcass Characteristics, Meat Quality and Muscular Antioxidant Capacity and Mineral Contents in Broilers." *Journal of the Science of Food and Agriculture* 97: 3699–3705.

Cramer, T. A., H. W. Kim, Y. Chao, W. Wang, H. W. Cheng, and Y. H. B. Kim. 2018. "Effects of Probiotic (Bacillus Subtilis) Supplementation on Meat Quality Characteristics of Breast Muscle From Broilers Exposed to Chronic Heat Stress." *Poultry Science* 97, no. 9: 3358–3368.

Ciszewski, A., Ł. S. Jarosz, A. Bielecka, et al. 2023. "Effect of In Ovo Administration of a Multi-Strain Probiotic and Zinc Glycine Chelate on Antioxidant Capacity and Selected Immune Parameters in Newly Hatched Chicks." *Antioxidants* 12: 1905.

Dankowiakowska, A., J. Bogucka, A. Sobolewska, S. Tavaniello, G. Maiorano, and M. Bednarczyk. 2019. "Effects of In Ovo Injection of Prebiotics and Synbiotics on the Productive Performance and Microstructural Features of the Superficial Pectoral Muscle in Broiler Chickens." *Poultry Science* 98: 5157–5165.

Deng, W., X. F. Dong, J. M. Tong, and Q. Zhang. 2012. "The Probiotic *Bacillus licheniformis* Ameliorates Heat Stress-Induced Impairment of Egg Production, Gut Morphology, and Intestinal Mucosal Immunity in Laying Hens." *Poultry Science* 91: 575–582.

Duan, A., A. Ju, Y. Zhang, et al. 2021. "The Effects of In Ovo Injection of Synbiotics on the Early Growth Performance and Intestinal Health of Chicks." *Frontiers in Veterinary Science* 8: 658301.

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

Dunisławska, A., E. Pietrzak, A. Bełdowska, A. Sławińska, and M. Siwek. 2023. "Response in Liver Gene Expression and DNA Methylationto Changes in the Intestinal Microbial Profileafter In Ovo Stimulation of Chickens." *Journal of Animal and Feed Sciences* 32: 152–163.

Dunislawska, A., A. Slawinska, and K. Stadnicka, et al. 2017. "Synbiotics for Broiler Chickens—In Vitro Design and Evaluation of the Influence on Host and Selected Microbiota Populations Following In Ovo Delivery." *PLoS One* 12: e0168587.

Gao, M., Y. Ren, S. Lu, R. Reddyvari, K. Venkitanarayanan, and M. A. Amalaradjou. 2024. "In Ovo Probiotic Supplementation Supports Hatchability and Improves Hatchling Quality in Broilers." *Poultry Science* 103: 103624.

Guo, W., J. Zhou, Y. Liu, et al. 2023. "Embryonic Injection of *Lactobacillus plantarum* PA01 Alters the Microbial Diversity in the Gastrointestinal Tract of the Broilers before and after Hatching." *Poultry Science* 102: 102764.

Hou, T., and E. Tako. 2018. "The In Ovo Feeding Administration (*Gallus gallus*)—An Emerging In Vivo Approach to Assess Bioactive Compounds with Potential Nutritional Benefits." *Nutrients* 10: 418.

Jha, R., R. Das, S. Oak, and P. Mishra. 2020. "Probiotics (Direct-Fed Microbials) in Poultry Nutrition and Their Effects on Nutrient Utilization, Growth and Laying Performance, and Gut Health: A Systematic Review." *Animals* 10: 1863.

Jung, S. J., R. Houde, B. Baurhoo, X. Zhao, and B. H. Lee. 2008. "Effects of Galacto-Oligosaccharides and a *Bifidobacteria lactis*-Based Probiotic Strain on the Growth Performance and Fecal Microflora of Broiler Chickens." *Poultry Science* 87: 1694–1699.

Khochamit, N., S. Siripornadulsil, P. Sukon, and W. Siripornadulsil. 2020. "*Bacillus subtilis* and Lactic Acid Bacteria Improve the Growth Performance and Blood Parameters and Reduce *Salmonella* Infection in Broilers." *Veterinary World* 13: 2663–2672.

Kim, J., H. Kim, S. Jeon, J. Jo, Y. Kim, and H. Kim. 2020. "Synergistic Antibacterial Effects of Probiotic Lactic Acid Bacteria With *Curcuma longa* Rhizome Extract as Synbiotic against Cutibacterium Acnes." *Applied Sciences* 10: 8955.

Lee, D., H. J. Lee, D. Y. Jung, H. J. Kim, A. Jang, and C. Jo. 2022. "Effect of an Animal-Friendly Raising Environment on the Quality, Storage Stability, and Metabolomic Profiles of Chicken Thigh Meat." *Food Research International* 155: 111046.

Liu, L., L. Li, and C. Li, et al. 2023. "Effects of *Lactiplantibacillus plantarum* LPJZ-658 Supplementation on the Production, Meat Quality, Intestinal Morphology, and Cecal Microbiota of Broilers Chickens." *Microorganisms* 11: 1549.

Maiorano, G., A. Sobolewska, D. Cianciullo, et al. 2012. "Influence of In Ovo Prebiotic and Synbiotic Administration on Meat Quality of Broiler Chickens." *Poultry Science* 91: 2963–2969.

Majidi-Mosleh, A., A. Sadeghi, S. Mousavi, M. Chamani, and A. Zarei. 2017. "Effects of In Ovo Infusion of Probiotic Strains on Performance Parameters, Jejunal Bacterial Population and Mucin Gene Expression in Broiler Chicken." *Revista Brasileira de Ciência Avícola* 19: 97–102.

Mookiah, S., C. C. Sieo, K. Ramasamy, N. Abdullah, and Y. W. Ho. 2014. "Effects of Dietary Prebiotics, Probiotic and Synbiotics on Performance, Caecal Bacterial Populations and Caecal Fermentation Concentrations of Broiler Chickens: Effects of Dietary Prebiotics, Probiotic and Synbiotics on Performance." *Journal of the Science of Food and Agriculture* 94: 341–348.

Mukhtar, N., S. H. Khan, and M. S. Anjum. 2013. "Hatchling Length Is a Potential Chick Quality Parameter in Meat Type Chickens." *World's Poultry Science Journal* 69: 889–896.

Munyaka, P. M., H. Echeverry, A. Yitbarek, et al. 2012. "Local and Systemic Innate Immunity in Broiler Chickens Supplemented With Yeast-Derived Carbohydrates." *Poultry Science* 91: 2164–2172.

O'Dea, E. E., G. M. Fasenko, G. E. Allison, D. R. Korver, G. W. Tannock, and L. L. Guan. 2006. "Investigating the Effects of Commercial Probiotics on Broiler Chick Quality and Production Efficiency." *Poultry Science* 85: 1855–1863.

Oladokun, S., and D. I. Adewole. 2020. "In Ovo Delivery of Bioactive Substances: An Alternative to the Use of Antibiotic Growth Promoters in Poultry Production—A Review." *Journal of Applied Poultry Research* 29: 744–763.

Oladokun, S., and D. I. Adewole. 2022. "Biomarkers of Heat Stress and Mechanism of Heat Stress Response in Avian Species: Current Insights and Future Perspectives From Poultry Science." *Journal of Thermal Biology* 110: 103332.

Oladokun, S., S. Dridi, and D. Adewole. 2023. "An Evaluation of the Thermoregulatory Potential of In Ovo Delivered Bioactive Substances (Probiotic, Folic Acid, and Essential Oil) in Broiler Chickens." *Poultry Science* 102: 102602.

Oladokun, S., A. Koehler, J. MacIsaac, E. M. Ibeagha-Awemu, and D. I. Adewole. 2021. "*Bacillus subtilis* Delivery Route: Effect on Growth Performance, Intestinal Morphology, Cecal Short-Chain Fatty Acid Concentration, and Cecal Microbiota in Broiler Chickens." *Poultry Science* 100: 100809.

Penders, J., C. Vink, C. Driessen, N. London, C. Thijs, and E. E. Stobberingh. 2005. "Quantification of Bifidobacterium spp., Escherichia Coli and Clostridium Difficile in Faecal Samples of Breast-Fed and Formula-Fed Infants by Real-Time PCR." *FEMS Microbiology Letters* 243, no. 1: 141–147.

Połtowicz, K., J. Nowak, and D. Wojtysiak. 2015. "Effect of Feed Restriction on Performance, Carcass Composition and Physicochemical Properties of the m. Pectoralis Superficialis of Broiler Chickens." *Annals of Animal Science* 15, no. 4: 1019–1029.

Pruszynska-Oszmalek, E., P. A. Kolodziejski, K. Stadnicka, et al. 2015. "In Ovo Injection of Prebiotics and Synbiotics Affects the Digestive Potency of the Pancreas in Growing Chickens." *Poultry Science* 94: 1909–1916.

Sakamoto, K., H. Hirose, A. Onizuka, M. Hayashi, N. Futamura, Y. Kawamura, and T. Ezaki. 2000. "Quantitative Study of Changes in Intestinal Morphology and Mucus Gel on Total Parenteral Nutrition in Rats." *Journal of Surgical Research* 94, no. 2: 99–106.

Shehata, A. M., V. K. Paswan, Y. A. Attia, et al. 2021. "Managing Gut Microbiota through In Ovo Nutrition Influences Early-Life Programming in Broiler Chickens." *Animals* 11: 3491.

Siwek, M., A. Slawinska, K. Stadnicka, J. Bogucka, A. Dunislawska, and M. Bednarczyk. 2018. "Prebiotics and Synbiotics—In Ovo Delivery for Improved Lifespan Condition in Chicken." *BMC Veterinary Research* 14: 402.

Slawinska, A., A. Dunislawska, and A. Plowiec, et al. 2019. "Modulation of Microbial Communities and Mucosal Gene Expression in Chicken Intestines After Galactooligosaccharides Delivery In Ovo." *PLoS One* 14: e0212318.

Slawinska, A., S. Mendes, A. Dunislawska, et al. 2019. "Avian Model to Mitigate Gut-Derived Immune Response and Oxidative Stress During Heat." *Biosystems* 178: 10–15.

Slawinska, A., A. Plowiec, M. Siwek, M. Jaroszewski, and M. Bednarczyk. 2016. "Long-Term Transcriptomic Effects of Prebiotics and Synbiotics Delivered In Ovo in Broiler Chickens." *PLoS One* 11: e0168899.

Slawinska, A., M. Zampiga, F. Sirri, et al. 2020. "Impact of Galactooligosaccharides Delivered In Ovo on Mitigating Negative Effects of Heat Stress on Performance and Welfare of Broilers." *Poultry Science* 99: 407–415.

Smialek, M., E. Kaczorek, E. Szczucińska, et al. 2018. "Evaluation of *Lactobacillus* Spp. and Yeast Based Probiotic (Lavipan) Supplementation for the Reduction of Salmonella Enteritidis After Infection of Broiler Chickens." *Polish Journal of Veterinary Sciences*: 5–10.

Smuliikowska and Rutkowski. 2018. Recommended Allowances and Nutritive Value of Feedstuffs–Poultry Feeding Standards [in Polish].

Sobolewska, A., G. Elminowska-Wenda, J. Bogucka, et al. 2017. "The Influence of In Ovo Injection With the Prebiotic Dinovo® on the Development of Histomorphological Parameters of the Duodenum, Body Mass and Productivity in Large-Scale Poultry Production Conditions." *Journal of Animal Science and Biotechnology* 8: 45.

Sozcu, A., and A. Ipek. 2015. "Quality Assessment Chicks From Different Hatcher Temperatures With Different Scoring Methods and Prediction of Broiler Growth Performance." *Journal of Applied Animal Research* 43, no. 4: 409–416.

Tannock, G. W., A. Tilsala-Timisjarvi, S. N. G. J. Rodtong, J. Ng, K. Munro, and T. Alatossava. 1999. "Identification of Lactobacillus Isolates From the Gastrointestinal Tract, Silage, and Yoghurt by 16S-23S rRNA Gene Intergenic Spacer Region Sequence Comparisons." *Applied and Environmental Microbiology* 65, no. 9: 4264–4267.

Tavaniello, S., D. De Marzo, M. Bednarczyk, et al. 2023. "Influence of a Commercial Synbiotic Administered In Ovo and In-Water on Broiler Chicken Performance and Meat Quality." *Foods* 12: 2470.

Tavaniello, S., R. Mucci, K. Stadnicka, O. Acaye, M. Bednarczyk, and G. Maiorano. 2019. "Effect of in Ovo Administration of Different Synbiotics on Carcass and Meat Quality Traits in Broiler Chickens." *Poultry Science* 98, no. 1: 464–472.

Tavaniello, S., A. Slawinska, F. Sirri, et al. 2022. "Performance and Meat Quality Traits of Slow-Growing Chickens Stimulated In Ovo With Galactooligosaccharides and Exposed to Heat Stress." *Poultry Science* 101: 101972.

Tavaniello, S., A. Slawinska, D. Prioriello, et al. 2020. "Effect of Galactooligosaccharides Delivered in Ovo on Meat Quality Traits of Broiler Chickens Exposed to Heat Stress." *Poultry Science* 99, no. 1: 612–619. https://doi.org/10.3382/ps/pez556.

Wiersema, M. L., L. R. Koester, S. Schmitz-Esser, and D. A. Koltes. 2021. "Comparison of Intestinal Permeability, Morphology, and Ileal Microbial Communities of Commercial Hens Housed in Conventional Cages and Cage-Free Housing Systems." *Poultry Science* 100: 1178–1191.

Wishna-Kadawarage, R. N., K. Połtowicz, A. Dankowiakowska, R. M. Hickey, and M. Siwek. 2024. "Prophybiotics for In-Ovo Stimulation; Validation of Effects on Gut Health and Production of Broiler Chickens." *Poultry Science* 103: 103512.

Yang, C., M. Qiu, Z. Zhang, et al. 2022. "Galacto-Oligosaccharides and Xylo-Oligosaccharides Affect Meat Flavor by Altering the Cecal Microbiome, Metabolome, and Transcriptome of Chickens." *Poultry Science* 101: 102122.

Ye, Y., Z. Li, P. Wang, et al. 2021. "Effects of Probiotic Supplements on Growth Performance and Intestinal Microbiota of Partridge Shank Broiler Chicks." *PeerJ* 9: e12538.

Journal of Applied Genetics
https://doi.org/10.1007/s13353-024-00931-7

ANIMAL GENETICS • ORIGINAL PAPER

Modulatory effects of *in ovo* delivery of galactooligosaccharide and *Lactiplantibacillus plantarum* on antioxidant capacity, gene expression, and selected plasma metabolite parameters of broiler chickens

Modou Mangan[1] · Katarzyna Połtowicz[2] · Cornelia C. Metges[3] · Maria Siwek[1]

Received: 28 August 2024 / Revised: 22 November 2024 / Accepted: 3 December 2024

Abstract
A stable gut microbiota promotes a healthy gut and enhances immune function, antioxidant status, and metabolic activities in chickens. The present research work aimed to investigate the modulatory impacts of *in ovo* delivery of prebiotic and probiotic on oxidative stress, the intestinal transcriptome, and various plasma metabolites in chickens. Fertilized Ross 308 eggs were administered *in ovo* either with galactooligosaccharide (GOS) (3.5 mg/egg or *Lactiplantibacillus plantarum* (LP) 1×10^6/egg on the 12th day of egg incubation. Three hundred viable Ross 308 broiler hatching eggs in total were randomly assigned to four groups, namely, the negative control not injected group, the group receiving physiological saline injections as the positive control, GOS, and LP. The analysis of genes associated with immune functions, antioxidants, barrier functions, and free fatty acid receptors were determined via qPCR. The analysis of the selected plasma blood metabolites was performed automatically with Pentra C 400. The antioxidant capacity of the chickens' liver, breast muscle, and spleen was enhanced by the *in ovo* injection of GOS and LP. The immune-related gene expression levels were upregulated after *in ovo* stimulation with either GOS or LP which improved the gut health of broiler chickens. In addition, several genes related to gut barrier functions were upregulated, thus ensuring epithelial integrity. As for blood plasma metabolites, no adverse effects were observed. In summary, we report that *in ovo* stimulation with either GOS or LP stimulates the immune system and improves the antioxidant status and gut health of chickens with no negative impact on plasma blood metabolite indices.

Keywords Antioxidant · Broiler · Gene expression · Gut health · *In ovo*

Introduction

Broiler chickens are among the most important sources of animal protein for humans, it is more affordable and is widely accepted in different regions and religions across the globe (Mottet and Tempio 2017). This has led to the intensification and commercialization of the broiler industry worldwide, with a rapid increase in production. However, this practice is associated with several consequences, such as metabolic disorders, pathogen infection, oxidative stress, morbidity, and mortality (Yang et al. 2024). In light of these adverse effects, leading to production losses in the broiler industry, the search for sustainable alternative strategies to maximize and ensure the continuous and efficient production of healthy and high-quality broiler chickens has gained much attention. In recent years, different probiotics and prebiotics have been developed and used via different

Communicated by: Tomasz Strabel

✉ Modou Mangan
modou.mangan@pbs.edu.pl

[1] Department of Animal Biotechnology and Genetics, Bydgoszcz University of Science and Technology (PBS), Mazowiecka 28, 85-084 Bydgoszcz, Poland

[2] Department of Poultry Breeding, National Research Institute of Animal Production, Krakow-Ska 1, 32-083 Balice, Poland

[3] Research Institute for Farm Animal Biology (FBN), Nutritional Physiology, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

Published online: 12 December 2024
101:30707482

strategies to promote poultry health and performance (Slawinska et al. 2020b; Wishna-Kadawarage et al. 2024). These bioactive substances are supplemented in the diet or water offered to the chickens or *in ovo* methods (Bednarczyk et al. 2016). However, the latter has received much attention due to early gut colonization and improvements in the immune system and health during embryonic development (Alagawany et al. 2018; Slawinska et al. 2020b; Das et al. 2021; Kpodo and Proszkowiec-Weglarz 2023).

Prebiotics, such as galactooligosaccharides (GOS), are food components that cannot be digested by the body's own enzymes but have positive impacts on the host by directing the growth and activity of beneficial gut bacteria associated with improved gut health nutrient and absorption (Roberfroid 2007; Bertocchi et al. 2019; Slawinska et al. 2020b). Live microorganism known as probiotics improves the host's health by enhancing the balance of the gut microbiota and improving overall health and welfare (Dunislawska et al. 2019). Among the *Lactobacillus species*, *Lactiplantibacillus plantarum* has been used as a probiotic in chicken feed and administered *in ovo* during embryonic development and has been reported to confer beneficial effects. *Lactiplantibacillus plantarum* is characterized by its ability to produce lactic acid and its resilience in the gastrointestinal tract (Jha et al. 2020; Fathima et al. 2022) and its antioxidant capacity in chickens (Yang et al. 2019). The use of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) in vitro assay demonstrated that several probiotics such as *L. plantarum*, can scavenge free radicals and thereby counteract oxidative stress and subsequently alleviate multiple stressors, such as heat stress, pathogen infection, and metabolic disorders, later in the life of broiler chickens (Mu et al. 2018; Mounir et al. 2022; Yang et al. 2024).

In poultry, heat stress causes an increase in reactive oxygen species (ROS), leading to oxidative stress in chickens, ultimately causing an imbalance between oxidants and antioxidants (Wilson et al. 2014; Sumanu et al. 2023). while increasing the malondialdehyde levels (Zeng et al. 2014). It is reported that the supplementation of LP and GOS boosts the level of antioxidant expression in chickens (Slawinska et al. 2020b; Sumanu et al. 2023).

Although the *in ovo* injection of GOS or LP in chickens has already been tested (Slawinska et al. 2020b; Yang et al. 2024), results in the literature are inconsistent and the understanding of the associated biological and molecular processes is limited. Therefore the first aim of the present study was to evaluate the radical scavenging ability of the *Lactiplantibacillus plantarum* via in vitro studies. Secondly, we investigated the effects of GOS and LP administered *in ovo* on plasma biochemical indices and transcriptomic analysis of genes related to gut health, immune functions, and antioxidant activities in broiler chickens.

Materials and methods

Probiotic strains and culture conditions

The probiotics used in this research work (Table 1) were acquired from the JHJ Company (Nowa Wies, Gizalki, Poland). Before use, the probiotic cultures were kept at – 80 °C in 50% glycerol. All strains were cultured and maintained in MRS broth at 37 °C. A total of 6.82 g of MRS agar (de Man, Rogosa and Sharpe, Merck KGaA, Darmstadt) was dissolved in 100 mL of distilled water and stirred (IKA® RCT basic IKAMAG™ Safety Control Magnetic Stirrer) to dissolve the agar completely. Next, it was autoclaved at 121 °C for 15 min and vortexed for 10 s. Stock cultures of probiotic strains were established on agar plates, and the plates underwent a 24-h incubation period at 37 °C. A bacterial suspension was prepared for each strain in 10 mL of DeMan, Rogosa, and Sharpe broth (MRS) (Merck KGaA, Darmstadt). Subsequently, a 96-well microplate was filled with 250 µL of MRS broth and 10 µL of each bacterial suspension. The microplate was then incubated for 48 h at 37 °C under aerobic conditions. Three replicates of each sample were included for each of the three repetitions for each probiotic. MRS broth without inoculum was used as a control. Bacterial growth measurements (OD600) were performed every 12 h using a Multiskan™ FC Microplate Photometer and SkanIt software version 7.0 (Thermo Fisher Scientific, Waltham, MA). Microtiter plates were shaken for 10 s before the microplate readings were taken to ensure homogeneity in the samples.

Table 1 Concentrations of probiotics used for the 2,2-diphenyl-1-picrylhydrazyl (DPPH) DPPH test

Probiotic concentrations	
L. casei	*Lacticaseibacillus casei* 1.4×10^6
	Lacticaseibacillus casei 7.0×10^5
	Lacticaseibacillus casei 3.5×10^5
L. plantarum	*Lactiplantibacillus plantarum* 4.4×10^6
	Lactiplantibacillus plantarum 2.1×10^6
	Lactiplantibacillus plantarum 1.0×10^6
L. reuteri	*Limosilactobacillus reuteri* 7.9×10^6
	Limosilactobacillus reuteri 3.9×10^6
	Limosilactobacillus reuteri 1.9×10^6
L. rhamnosus	*Lacticaseibacillus rhamnosus* 1.1×10^8
	Lacticaseibacillus rhamnosus 5.5×10^7
	Lacticaseibacillus rhamnosus 2.7×10^7

In vitro determination of the antioxidant activities of the selected probiotics

The list of *Lactobacillus* strains used in this study is provided in Table 1. To pre-select probiotics for *in ovo* injection, we used the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma−Aldrich, St. Louis, MO) assay to measure free radical scavenging activities according to (Kao and Chen 2006).

Briefly, 0.1 mM of DPPH was dissolved in 100 mL of ethanol. The mixture was vigorously shaken and left to react for 30 min at room temperature in the dark. It was always used fresh on the day of analysis. Prior to the DPPH assay, the *Lactobacillus* samples were serially diluted, and 10 µl of sample (with appropriate dilution), 190 µl of sample, and the control (200 µl of DPPH ethanolic solution) were added to a 96-well microtiter plate. The blank group contained MRS broth media and ethanol. The optical absorbance at 520 nm was measured in triplicate using a MultiskanTM FC Microplate Photometer. Using the following equation below, the percentage of free radical scavenging activity was determined.

$$(\%)\text{scavenging activity} = [(\text{Ac} - \text{As})/\text{Ac}] \times 100$$

where Ac is the absorbance of the control and As is the absorbance of the sample.

The results are expressed as the EC50 (µg/mL), which is the lowest antioxidant concentration needed to reduce 50% of the initial DPPH reaction from the moment the extract reached stability. Based on the growth curve and the DPPH assay results, the bioactive compound with the best growth and highest antioxidant activity was selected for *in ovo* application to validate its effects on Ross 308 broiler chickens. The prebiotic GOS was selected for *in ovo* application studies based on results of previous studies by our group showing its ability to mitigate heat stress in Ross 308 broilers (Slawinska et al. 2020b).

Egg incubation and in ovo protocol

A total of three hundred (300) fertile Ross 308 broiler eggs were incubated under standard incubation conditions (Midi series I, Fest Incubators, Gostyń, Poland). On day 7 of embryonic development, eggs were taken out of the incubator and sterilized using 70% ethanol, then candled, and the infertile and dead embryos were discarded. The remaining fertile eggs were randomly allotted into four groups: negative control (NC), positive control (PC), GOS, and LP. Next, a 20G needle was used to make a hole in the air chamber of the eggs. Subsequently, *in ovo* injection was manually performed on the 12th day of egg incubation in all the groups except the NC group. A 0.2 mL sterile 0.9% physiological saline solution was injected into the PC group eggs while the GOS group eggs were injected with 3.5 mg of GOS/egg suspended in 0.2 mL of physiological saline and the LP group was injected with 10^6 CFU of LP bacteria/egg suspended in 0.2 mL of physiological saline solution. After injection, each egg was sealed using organic glue (Elmer's school glue, Elmer's Products Inc., USA), and immediately returned to the incubator.

Birds and housing

The experiment was conducted in compliance with the Ethics Committee for Experiments with Animals guidelines and the Polish Act on the Protection of Animals Used for Scientific or Educational Purposes regulations of January 15, 2015 (which was implemented by the European Parliament and Council of September 22, 2010, Directive 2010/63/EU on the protection of animals used for scientific purposes). All birds in each experimental group consisting of 32 birds/pens were housed in separate pens with similar optimized environmental conditions during the experiment. Water and feed were made available to the chickens at all times. The birds were fed the following three types of age-dependent diets throughout the experimental period: starter (1–21 days), grower (22–28 days), and finisher (29–35 days), consisting of 12.45, 13.01, and 13.01 MJ/kg of metabolizable energy and 22.3%, 20.2%, and 20.2% crude protein, respectively. The dietary mixtures were in accordance with broiler chicken dietary requirements (Smulikowska and Rutkowski, 2018). The initial environmental temperature in the pens was 32–33 °C on day one of life, and the temperature steadily reduced reaching approximately 21 °C at the end of the trial period.

Sample collection

At the end of the rearing period, 8 birds per group ($n = 24$) with a final average body weight of 2.43–2.53 kg were randomly chosen. The birds were slaughtered by decapitation after being deprived of feed for 10 h and left to bleed for approximately 90 s. Following the slaughtering of each bird, two milliliters of blood were collected in K-EDTA tubes and centrifuged at 3000 × g for 15 min to extract plasma. Next, the plasma samples were immediately placed on dry ice and transported to the laboratory. Upon arrival, all the samples were kept at − 80 °C until analysis. In addition, cecal mucosa, liver, spleen, and breast muscle were collected and preserved in RNA stabilization reagent (fix RNA: E0280, EURx, Gdańsk, Poland) and transported at room temperature, and the fixed RNA was poured off and the tubes with the samples were kept at − 80 °C until use.

RNA extraction, RT−PCR, and qPCR gene expression analysis

Tissues were homogenized with a TissueRuptor homogenizer (990,890, Qiagen, Wrocław, Poland) and immersed in a tube containing 1 mL of RNA extracol solution (E3700, EURx, Gdańsk, Poland) for the RNA isolation procedure. Next, each sample was centrifuged using 0.2 mL of chloroform (112,344,305, Chempur, Piekary Śląskie, Poland). A commercial kit (Universal RNA purification kit (E3598, EURx, Gdańsk, Poland)) was used to carry out the subsequent steps of the RNA isolation process. A NanoDrop 2000 spectrophotometer (Thermo Scientific, Warsaw, Poland) was used to measure the quantity and quality of the RNA, while a 2% agarose gel was used to assess RNA integrity. RNA samples were stored at − 80 °C until use. Using the smART First Strand cDNA Synthesis Kit (0804, EURx, Poland), the RT-PCR process was performed following the manufacturer's protocol. Next, the cDNA obtained was diluted to 100 ng/µl. Afterward, RT−qPCR was carried out using a total volume of 10 µL. The reaction mixture included Maxima SYBR Green qPCR Master Mix (0401, EURx, Gdańsk, Poland), 1 µM of each primer, and 2 µl of diluted cDNA. Thermal cycling was conducted using a LightCycler II 480 (Roche Diagnostics, Basel, Switzerland). Each RT−qPCR was carried out in two technical replicates in 96-well plates (4TI-0955, AZENTA, Genomed, Warszawa, Poland). The qPCR protocol for the gene expression analysis consisted of initial denaturation for 15 min (95 °C), followed by 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 30 s).

The expression levels of the target genes were determined via geometric means of two housekeeping genes (*Actb* and *G6pdh*). The target genes analyzed for each tissue and the reference genes are listed in Table 2. The relative gene expression was calculated using the ΔΔCt method. The ΔCt of the control group was subtracted from the ΔCt of each of the treatment groups. The fold change (FC) of the target gene in the treatment group against the control group was calculated as $2^{-\Delta\Delta Ct}$.

Blood plasma metabolite analysis

Blood plasma from eight 35 d old birds per each experimental group was randomly chosen to analyze metabolite concentrations and enzyme activities. An automatic enzyme analyzer (Pentra C 400, Axon Lab AG, Germany) was used to determine aspartate aminotransferase (AST): Kit No. A11A01629; alanine aminotransferase (ALT): A11A01627; high-density lipoprotein (HDL): A11A01636; low-density lipoprotein (LDL): A11A01638; total cholesterol: Kit No. A11A01634; triglyceride (TG): Kit No. A11A01640 (Horiba ABX), non-esterified fatty acid (NEFA): Kit No. 434–91,795 (Wako Chemicals GmbH, Neuss, Germany)); uric acid: Kit No. A11A01670; glucose: Kit No. A11A01667; lactose dehydrogenase (LDH): Kit No. A11A01871; and gamma-glutamyl transferase (GGT): Kit No. A11A01630 (Axon Lab AG, Reichenbach, Germany). These selected plasma metabolite parameters were analyzed at the Institute of Nutritional Physiology at the Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany.

Statistical analysis

All the data were checked for distribution normality, presence of outliers, and homogeneity of variance using the Shapiro–Wilk test and Levene test, respectively. The DPPH in vitro results and plasma metabolites were analyzed using GraphPad Prism version 10.1.2. The one-way ANOVA was used for the DPPH in vitro data. To analyze the plasma metabolites, we used principal component analysis (PCA). Tukey's HSD test was used to determine the differences between means ($P < 0.05$). The ΔCt values of every treatment group were compared with those of the control group for the gene expression analysis using GraphPad Prism and Student's *t*-test to identify significant differences among the treatments ($P < 0.05$), and plotting of graphs was done with Microsoft Excel.

Results and discussion

DPPH antioxidant assay

The antioxidant potential and heat stress alleviation effects of prebiotic galactooligosaccharides were previously reported by our research group (Pietrzak et al. 2020). In this study, we tested the antioxidant capacity of several *Lactobacillus* species (Table 1) and found that *Lactiplantibacillus plantarum* 1.0×10^6, exhibited the highest radical scavenging ability (68.89%) ($P < 0.05$) compared to the other probiotic bacteria (Fig. 1). Therefore we suggest that *Lactiplantibacillus plantarum* 1.0×10^6 was able to influence the activities of *Sod*, *Cat*, *Nrf2*, and *Gpx1* and reduced heat stress in poultry (Mangan and Siwek 2023). In an in vitro study, it is reported that *Lactobacillus* species such as *L. plantarum* have antibacterial, antipathogen, and antifungal features (Li et al. 2012). In addition, *L. curvatus* and *L. plantarum* C88 demonstrated high antioxidant activities (59.67%) and (53.05%) respectively (Li et al. 2012; Zhang et al. 2017). (Mu et al. 2018) reported high antioxidant activities of *L. casei* Y3, Y4 and Y16 and *L. plantarum* Y41, Y42, and Y44 with the DPPH assay ($P < 0.05$).

Table 2 List of target genes used for qPCR gene expression analysis

Tissues	Gene	Primer Sequences (5′−3′)	References
Cecal mucosa	Claudin1 (*Cldn1*)	F: TCTTCATCATTGCAGGTCTGTC R: AACGGGTGTGAAAGGGTCAT	(Slawinska et al. 2019)
	Mucin 6 (*Muc6*)	F: TTCAACATTCAGTTCCGCCG R: TTGATGACACCGACACTCCT	(Slawinska et al. 2019)
	Avian beta defensin 1 (*Avbd1*)	F: AAACCATTGTCAGCCCTGTG R: TTCCTAGAGCCTGGGAGGAT	(Slawinska et al. 2019)
	Free fatty acid receptor 2 (*Ffar2*)	F: GCTCGACCCCTTCATCTTCT R: ACACATTGTGCCCCGAATTG	(Slawinska et al. 2019)
	Tight junction-associated protein 1 (*Tjap1*)	F: AGGAAGCGATGAATCCCTGTT R: TCACTCAGATGCCAGATCCAA	(Slawinska et al. 2019)
	Interleukin 1 beta (*Il1b*)	F: GGAGGTTTTTGAGCCCGTC TCGAAGATGTCGAAGGACTG	(Dunislawska et al. 2017)
	Interleukin 10 (*Il10*)	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG	(Rothwell et al. 2004)
	Cathelicidin 2 (*Cathl2*)	F: AGGAGAATGGGGTCATCAGG R: GGATCTTTCTCAGGAAGCGG	(Slawinska et al. 2019)
Liver	Glutathione peroxidase- 1 (*Gpx1*)	F: TTGTAAACATCAGGGGCAAA R: ATGGGCCAAGATCTTTCTGTAA	(Akbarian et al. 2014)
	Heme oxygenase 1 (*Ho1*)	F: CTCAAGGGCATTCATTCG R: ACCCTGTCTATGCTCCTGTT	(Wu et al. 2019)
	Nuclear factor erythroid 2-related factor 2 (*Nrf*2)	F: ATCACCTCTTCTGCACCGAA R: GCTTTCTCCCGCTCTTTCTG	(Wu et al. 2019)
	Interleukin 1 beta (*Il1b*)	F: GGAGGTTTTTGAGCCCGTC TCGAAGATGTCGAAGGACTG	(Dunislawska et al. 2017)
	Occludin	F: TCATCCTGCTCTGCCTCATCT R: CATCCGCCACGTTCTTCAC	(Wu et al. 2019)
	Free fatty acid receptor 4 (*Ffar4*)	F: AGTGTCACTGGTGAGGAGATT R:ACAGCAACAGCATAGGTCAC	(Slawinska et al. 2019)
Breast muscle	Superoxide dismutase 1 (*Sod1*)	F: AGGGGGTCATCCACTTCC R: CCCATTTGTGTTGTCTCCAA	(El-Deep et al. 2014)
	Catalase (*Cat*)	F: GGGGAGCTGTTTACTGCAAG R: CTTCCATTGGCTATGGCATT	(El-Deep et al. 2014)
	Nuclear factor erythroid 2- related factor 2 (*Nrf2*)	F: ATCACCTCTTCTGCACCGAA R: GCTTTCTCCCGCTCTTTCTG	(Wu et al. 2019)
	Manganese superoxide dismutase (*Mnsod*)	F:TTCCTGACCTGCCTTACGACTAT R: CCAGCGCCTCTTTGTATTTCT	(Li et al. 2011)
	Zonula Occludens 1 (*Zo1*)	F:CTTCAGGTGTTTCTCTTCCTCCTC R:CTGTGG TTTCATGGCTGG ATC	(Chang et al. 2020)
Spleen	Cathelicidin 2 (*Cathl2*)	F: AGGAGAATGGGGTCATCAGG R: GGATCTTTCTCAGGAAGCGG	(Slawinska et al. 2019)
	Interleukin 4 (*Il4*)	F: GCTCTCAGTGCCGCTGATG R: GGAAACCTCTCCCTGGATGTC	(Sławinska et al. 2014a)
	Interleukin 8 (*Il8*)	F: CCACTGCTCCCTGGGTACAG R:TCAGAATTGAGCTGAGCC TTG	(Sławinska et al. 2014a)
	Interleukin 12p40 (*Il12p40*)	F: TTGCCGAAGAGCACCAGCCG R: CGGTGTGCTCCAGGTCTTGGG	(Brisbin et al. 2010)
	Reference genes		
	Actin, beta (*Actb*)	F: CACAGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	(Sevane et al. 2014)
	Glucose-6-phosphate dehydrogenase (*G6pdh*)	F: CGGGAACCAAATGCACTTCGT R: GGCTGCCGTAGAGGTATGGGA	(Sevane et al. 2014)

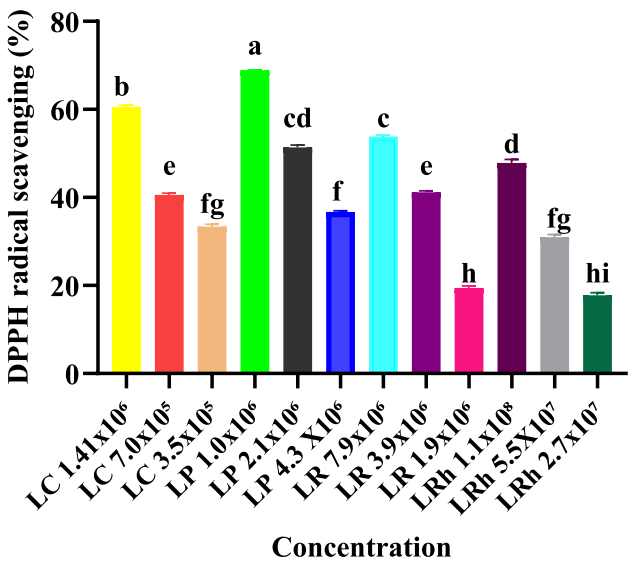

Fig. 1 Free radical scavenging activities of the *Lactobacillus* species 2,2-diphenyl-1-picrylhydrazyl (DPPH). The results are expressed as means ± SEMs ($n = 3$). Different lowercase letters (a–i) indicate significantly different means ($P < 0.05$). LP, *Lactiplantibacillus plantarum*; LC, *Lacticaseibacillus casei*; LR, *Limosilactobacillus*; LRh, *Lacticaseibacillus* rhamnosus

Gene expression analysis

Relative expression of different genes in cecal mucosal tissue

In our study, we deduced that the *in ovo* administration of GOS led to significant upregulation ($P < 0.05$) of *Muc6*, *Avbd1*, *Ffar2*, *Il1b*, and *Cathl*2 (Fig. 2B, C, D, E and F). On the other hand, *Cldn1*, *Avbd1*, *Il1b,* and *Cathl2* were upregulated ($P < 0.05$) by the *in ovo* administration of LP (Fig. 2A, B, C, E and F). The *Muc6* gene was significantly upregulated ($P < 0.05$) in both of our treatment groups when compared to the positive control group. The *Muc6* gene is part of the mucus layer and is responsible for mucin secretion and plays an integral part in gut barrier functions (Forder et al. 2012); therefore, GOS and LP delivered *in ovo* were able to ensure the production of mucin in the cecal mucosa, thus conferring protection to chickens against pathogen infection. During the innate immune response, the *Avbd1* gene regulates the secretion of avian beta defensin1 which plays a crucial role in the inhibition of pathogens (Zhang and Sunkara 2014; Lyu et al. 2020). Even though high expression levels of *Avbd1* are prominent during infection, SCFAs like butyrate and acetate influence and promote defensin synthesis in epithelial cells without causing any inflammation or dysbiosis (Zhang and Sunkara 2014; Chen et al. 2020; Wishna-Kadawarage et al. 2024). According to our result, no form of inflammation was found in the cecal mucosa that might affect the chicken's gut health. Therefore, the upregulation of the *Avbd1* gene may be caused by increased SCFA production through the modulation of the gut microbiota (Wishna-Kadawarage et al. 2024). In addition, our results revealed that *in ovo* delivery of GOS leads to the upregulation of *Ffar2*; therefore, it could be suggested that this gene plays a vital role in metabolic activities and immune cell recruitment in chicken cecal mucosa and subsequently modulates the gut microbiota (Slawinska et al. 2019). The *Ffar2* and *Ffar4* are nutrient-sensing genes that significantly influence the production of immune cells via SCFA production (Burns and Moniri 2010; Den Besten et al. 2013; Corrêa-Oliveira et al. 2016; Alvarez-Curto and Milligan 2016; Kolodziejski et al. 2018; Schlatterer et al. 2021). The *Cathl2* gene also supports gut barrier functions and regulates the inflammatory immune response (Volf et al. 2017; Slawinska et al. 2019). Our results confirmed that GOS and LP protect the gut barrier and reduce the risk of pathogen infection by increasing *Cathl2* expression (Fig. 2F). Interestingly, we found significant upregulation of *Il1b* in both GOS and LP ($P < 0.05$). *Il1b* plays a pivotal role in both proinflammatory cytokine production and protection against infection and therefore improves chicken gut health (Slawinska et al. 2019). According to (Khosravi and Mazmanian 2013; Slawinska et al. 2019), the gut of animals colonized by beneficial bacteria ensures a healthy gut which is correlated with high production of *IL1b*. *Cldn1* is a component of tight junctions that participates in preventing epithelial wall/cell permeability (Kawabe et al. 2001). In our study, LP enhanced *Cldn1* expression and therefore ensured epithelial cell integrity (Fig. 2A). Surprisingly, *in ovo* stimulation of either GOS or LP had no significant effect on *Tjap1* or *Il10*, suggesting that their functions were not compromised (Fathima et al. 2022).

Relative expression of different genes in splenic tissue

With regard to the spleen, we observed a significantly increased ($P < 0.05$) expression levels of *Sod*, *Il12p40*, *Il4,* and *Il8* (Fig. 3A, B, C and D) while the expression of *Il2* and *Cathl2* genes were not significantly affected.

Il12p40 encodes the *p40* subunit, which is a key component of both *Il12* and *Il23*. In our study, the upregulation of *Il12p40* in GOS *in ovo*-treated chickens may be explained by the ability of GOS to produce high amounts of *Il12* and *Il23* by activating certain splenic antigen-presenting cells, namely dendritic cells and macrophages (Slawinska et al. 2020a). In the literature, *Lactobacillus salivarius* with GOS and *Lactiplantibacillus plantarum* with RFO delivered *in ovo* were shown to upregulate the immune-related genes, including *Il12p40* (Sławinska et al. 2014b; Dunislawska et al. 2017). A different study by (Dunislawska et al. 2019) reported that *in ovo* injection of a synbiotic consisting of *Lactobacillus salivarius* and GOS increased the gene expression levels in the spleens of chickens while *Lactiplantibacillus plantarum*

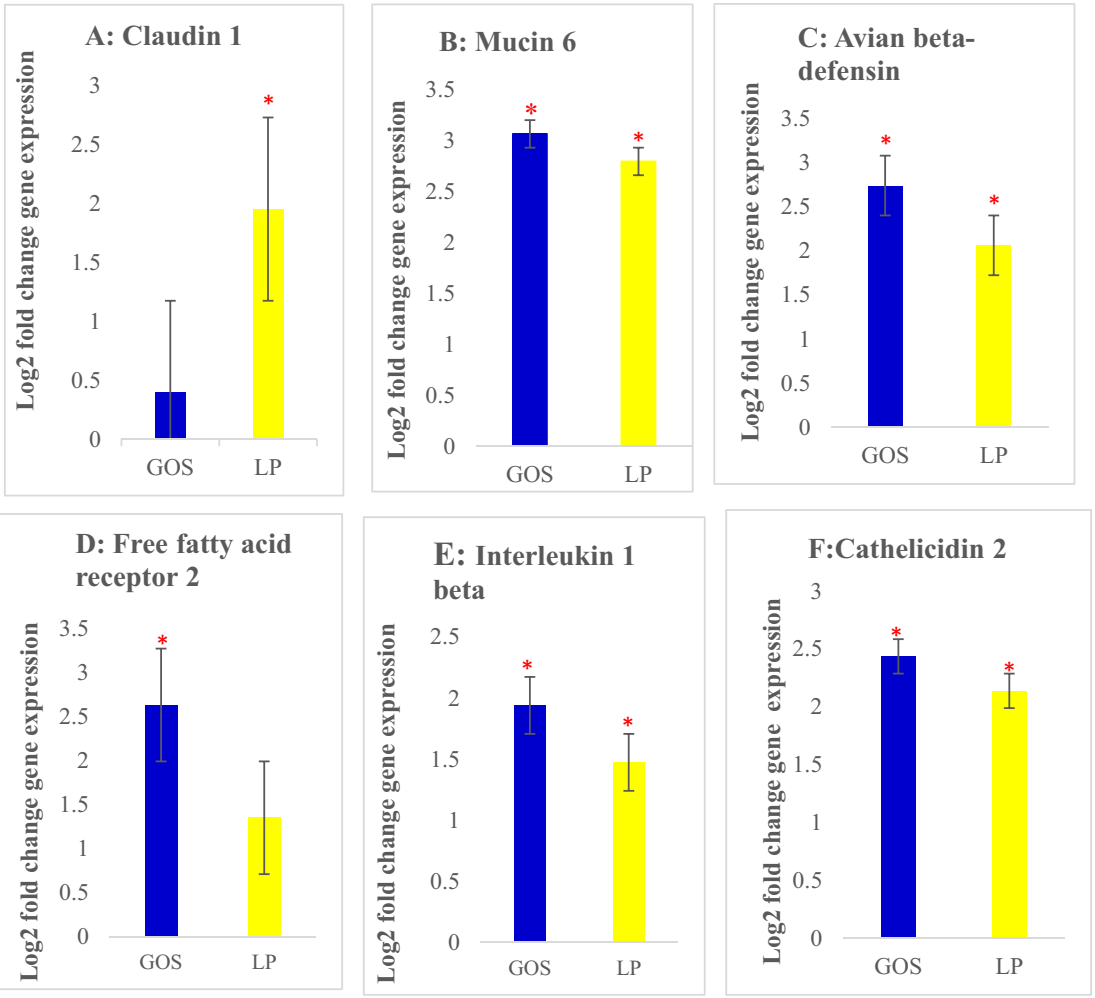

Fig. 2 Gene expression levels in the cecal mucosa of chickens treated *in ovo* with either galactooligosaccharide (GOS) or *Lactiplantibacillus plantarum* (LP). **A** *Cldn1*, **B** *Muc6*, **C** *Avbd1*, **D** *Ffar2*, **E** *Il1b*, and **F** *Cathl2*. Error bars: ± SE. Red asterisks (*) indicate significant changes ($P < 0.05$)

with RFO had no effects on gene expression. The *Il4* gene was highly expressed ($P < 0.05$) in both of our treatment groups and this may be explained by the ability of GOS and LP to modulate the gut microbiome through IgA-mediated mechanisms and regulation of the peripheral immune system in the spleen (Sławinska et al. 2014a, b). Although the expression of *Il8* is associated with infection, the *Il8* gene is often involved in routine immune regulation and homeostasis and is important in recruiting immune cells such as heterophils to the spleen (Jarosinski et al. 2005; Yu et al. 2020; Pietrzak et al. 2020; Elnagar et al. 2021). The *Sod1* is the first line of defense against oxidative stress; thus it stabilizes the oxidant/antioxidant equilibrium by catalyzing the dismutation of superoxide radicals to hydrogen peroxide. We observed significant differences in the expression levels ($P < 0.05$) of *Sod1* in both of our treatment groups. Similarly, (Pietrzak et al. 2020; Ncho et al. 2021) reported an upregulation of *Sod* in heat-stressed birds. Therefore, we suggest that GOS and LP possess antioxidant potential and can alleviate oxidative stress in chickens.

Relative expression of different genes in breast muscle

In our study, we demonstrated the *in ovo* delivery of GOS and LP increased the expression levels of *Gpx1*, *Cat*, *Sod*1, *Mnsod*, and *Nrf2* with no significant effect on Ho1 in chicken breast muscle. Oxidative stress destabilizes antioxidant levels in chickens, thereby causing an increase in *Mda* levels (Georgieva et al. 2006). Due to global warming and increasing temperatures worldwide strategies to alleviate oxidative stress and heat stress have gained much attention in the poultry industry (Mangan and Siwek 2023). Probiotic bacteria such as *Lactobacillus spp*. can activate the *Nrf2* pathway and other antioxidants such as catalase (*Cat*). Hydrogen peroxide is broken down into water and oxygen by the catalase antioxidant enzyme thereby preventing the accumulation

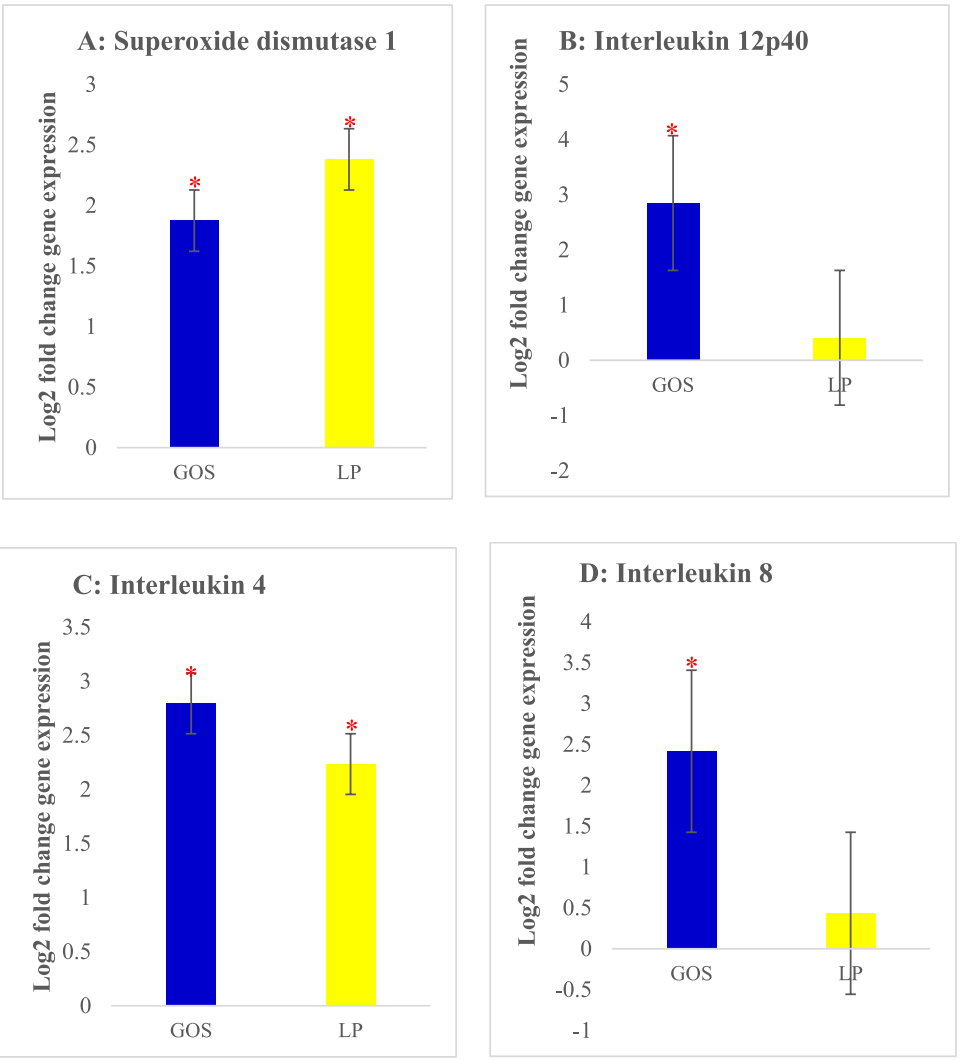

Fig. 3 Gene expression levels in the splenic tissue of chickens treated *in ovo* with either galactooligosaccharide (GOS) or *Lactiplantibacillus plantarum* (LP). **A** *Sod1*, **B** *Il12p40*, **C** *Il4*, and **D** *Il8*. Error bars: ± SE. Red asterisks (*) indicate significant changes ($P < 0.05$)

of ROS (Surai et al. 2019; Karaca et al. 2022). The *in ovo* stimulation of GOS upregulated both *Cat* and *Nrf2*, (Surai et al. 2019). In our study, we found that GOS and LP significantly upregulated the antioxidants tested in chicken breast muscle suggesting that the oxidant/antioxidant balance of the chickens in this group was well-balanced while *Nrf2* was not affected (Fig 4). Similarly, several studies reported that *in ovo* delivery of prebiotics and probiotics upregulated the expression pattern of *Sod* and *Mnsod*, thereby reducing oxidative stress (Bai et al. 2017; Cheng et al. 2017; Cao et al. 2019; Pietrzak et al. 2020).

Relative expression of different genes in liver tissue

Our results showed that *in ovo* administration of GOS increased the expression levels ($P < 0.05$) of *Gpx1* and *Nrf2* (Fig. 5A and B) in the livers of *in ovo*-treated chickens. Surprisingly, GOS affects *Nrf2*, occludin, *Ho1*, or *Ffar4* expression levels. On the other hand, LP led to the upregulation ($P < 0.05$) of *Gpx1*, *Nrf2*, *Il1b*, and occludin genes, while no significant effects were found on *Ho1* and *Ffar4* genes. The upregulation of the *Gpx1* gene by GOS and LP plays a vital role in the detoxification of hydrogen peroxide and lipid peroxides in poultry, thereby preventing oxidative stress. The positive effects exerted by GOS and LP may be explained by the modulatory ability of antioxidant enzymes and transcription factors involved in the *Nrf2* and *Gpx1* pathways (Surai et al. 2019; Gao et al. 2023).

Il1b and occludin were not highly expressed in GOS *in ovo*-treated chickens. However, *Il1b* and occludin were significantly upregulated ($P < 0.05$) upon *in ovo* supplementation with LP. Immune cells release the proinflammatory cytokine *Il1b* in response to infections or tissue damage. The high expression of *Il1b* suggests that *L. plantarum* triggered an inflammatory response in the liver, potentially due to its recognition as a foreign microorganism by the host's immune system. However, this inflammatory response may be beneficial, as probiotics such as *L. plantarum* are known

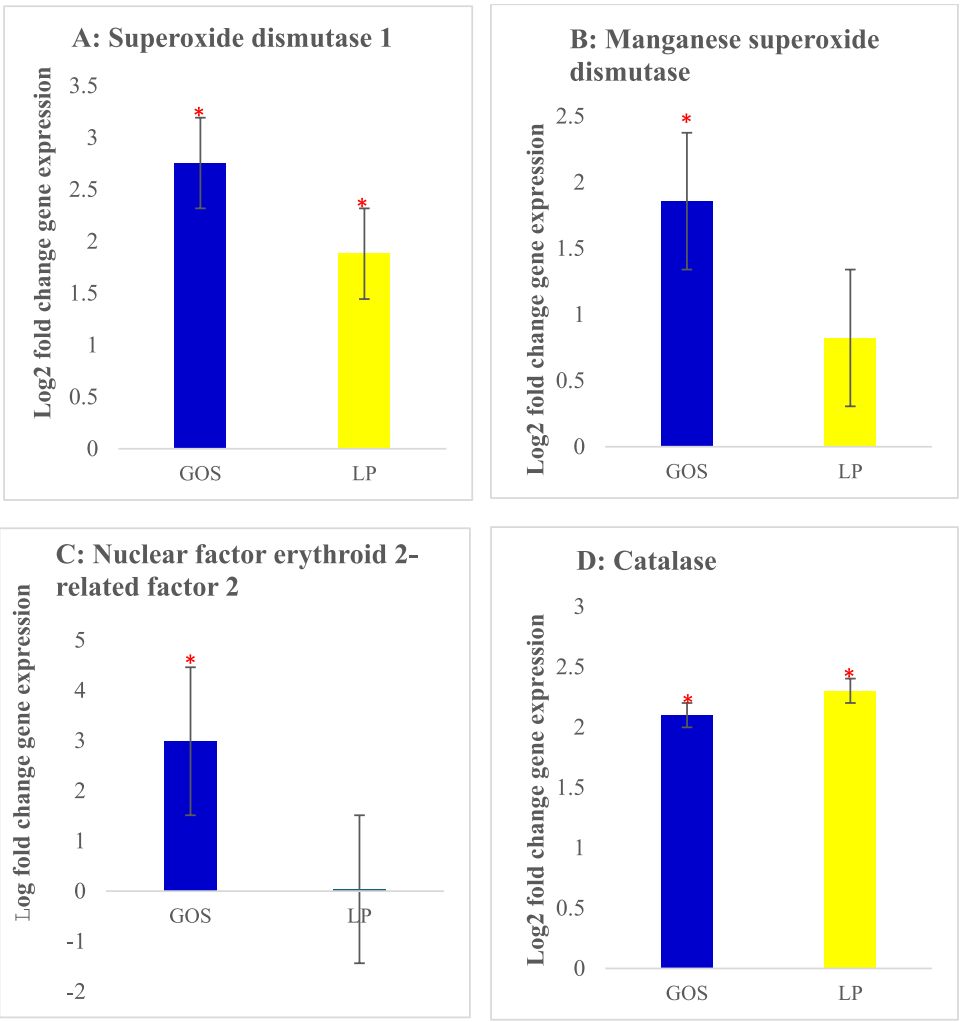

Fig. 4 Gene expression levels in the breast muscle of chickens treated *in ovo* with either galactooligosaccharide (GOS) or *Lactiplantibacillus plantarum* (LP) **A** *Sod1*, **B** *Mnsod*, **C** *Nrf2*, and **D** *Cat*. Error bars: ± SE. Red asterisks (*) indicate significant changes ($P < 0.05$)

to induce a mild inflammatory response that can prime the immune system and enhance its ability to fight off harmful pathogens (Wang et al. 2018; Gao et al. 2022).

Plasma blood metabolite analysis

The *in ovo* administration of either GOS or LP had no significant effects on the measured plasma metabolite parameters (Table 3). Surprisingly, our study, showed that the LP group had higher LDL than the PC and GOS groups. The high LDL concentration may be explained by the ability of LP to trigger compensatory mechanisms in lipid metabolism thus temporarily increasing lipid production. LDL cholesterol is mainly synthesized in the liver and plays an essential role in the transportation of lipids to peripheral tissues, however, when lipid metabolism is altered due to metabolic stress or other stressors, the liver may increase the production of LDL to ensure lipid homeostasis and therefore avert the accumulation of excess lipids in other tissues (Trapani et al. 2012). Additionally, our result showed an increased LDL which correlates to a numerically increased AST in the LP group compared to the GOS and PC groups (Table 3). In chickens, an increased level of AST could indicate mild liver stress. The slightly higher AST in the LP group may potentially be associated with increased lipid metabolism and LDL cholesterol (Lee et al. 2022). However, according to our Principal Component Analysis (PCA) result, the increased levels of LDL and AST in the LP group did not cause any negative effects on the chickens. The (PCA) was performed to study the effects of *in ovo* stimulation of GOS and LP on chicken plasma metabolites and enzymes. According to our results, no clear separation was observed between samples from the three groups (samples dot plot; Fig. 6A). The variable arrow plot (Fig. 6B) did not show a clear separation between the experimental groups for the various parameters. However, all the studied parameters tend to conglomerate together and were positively correlated, except for cholesterol, HDL, GGT and glucose. Based on the results, there were no substantial differences in metabolites between the groups. When chickens are exposed to stressful conditions such as

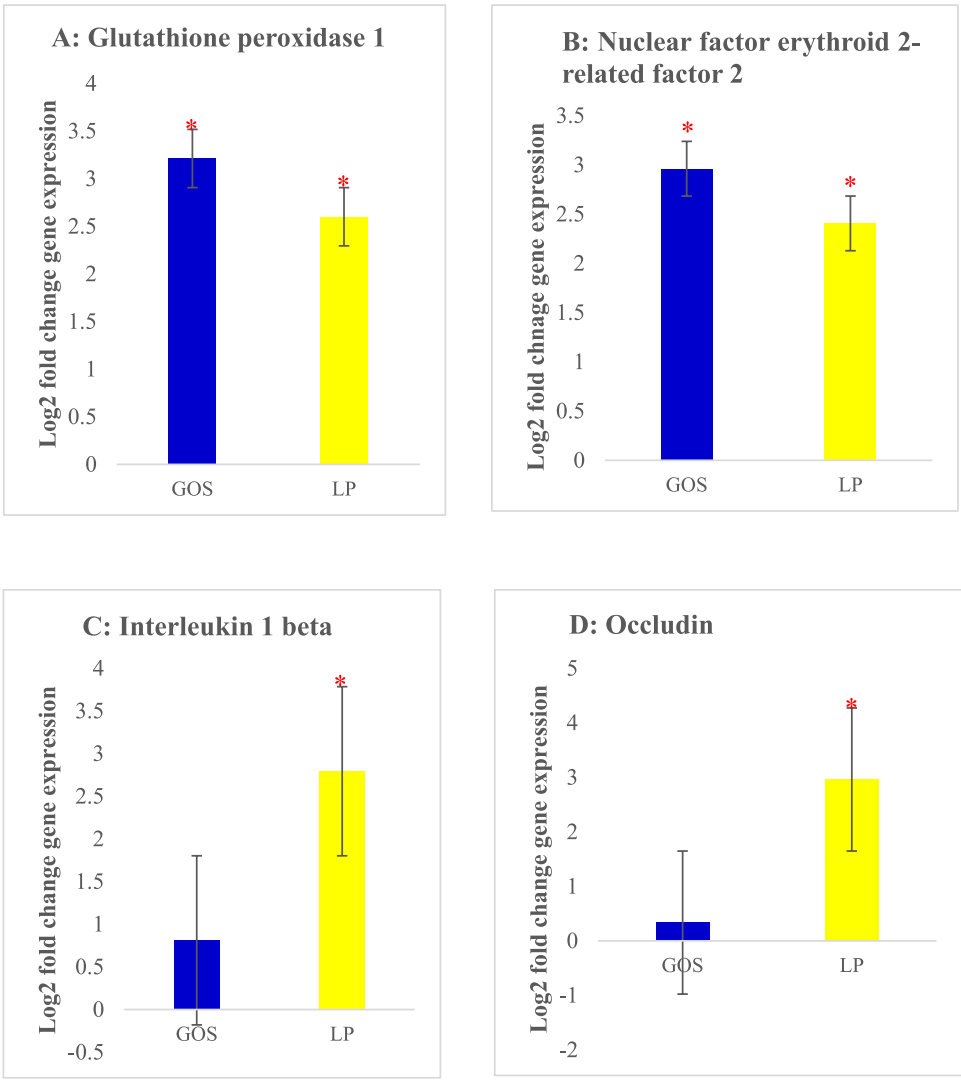

Fig. 5 Gene expression levels in the liver of chickens treated *in ovo* with either GOS, galactooligosaccharide group; LP, *Lactiplantibacillus plantarum* group **A** *Gpx1*, **B** *Nrf2*, **C** *Il1b*, and **D** Occludin. Error bars: ± SE. The red asterisk (*) indicates significant changes ($P < 0.05$)

Table 3 Effect of *in ovo* administration of GOS and LP on chicken plasma metabolites

Parameters	Treatments			*P*-value
	Control	GOS	LP	
ALT (U/L)	10.38 ± 3.25	10.13 ± 1.73	10.75 ± 1.67	0.865
AST (U/L)	601.64 ± 248.55	452.46 ± 146.77	677.41 ± 278.62	0.167
HDL (mmol/L)	2.11 ± 0.19	2.18 ± 0.32	2.06 ± 0.36	0.708
LDL (mmol/L)	0.48 ± 0.13	0.41 ± 0.08	0.62 ± 0.20	0.022
Cholesterol (mmol/L	3.31 ± 0.25	3.34 ± 0.33	3.40 ± 0.45	0.884
Glucose (mmol/L	15.75 ± 4.03	18.00 ± 4.50	14.75 ± 4.20	0.307
GGT (U/L)	11.98 ± 1.48	12.77 ± 0.63	12.18 ± 0.81	0.313
LDH (U/L)	2300.13 ± 1583.05	1473.61 ± 948.33	2614.04 ± 2026.84	0.176
NEFA (µmol/L)	882.13 ± 362.18	676.00 ± 147.77	532.75 ± 124.26	0.055
TG (mmol/L)	0.46 ± 0.08	0.47 ± 0.18	0.59 ± 0.16	0.187
UA (µmol/L)	304.38 ± 101.41	241.13 ± 98.73	203.13 ± 99.78	0.148

oxidative stress, infections, and lipolysis, several metabolic changes may occur and consequently increase NEFA levels (Abramowicz et al. 2019). Our PCA revealed no significant effects on NEFA levels despite the ANOVA results showing that the control group had a numerically greater NEFA concentration than the GOS and LP groups. The unchanged

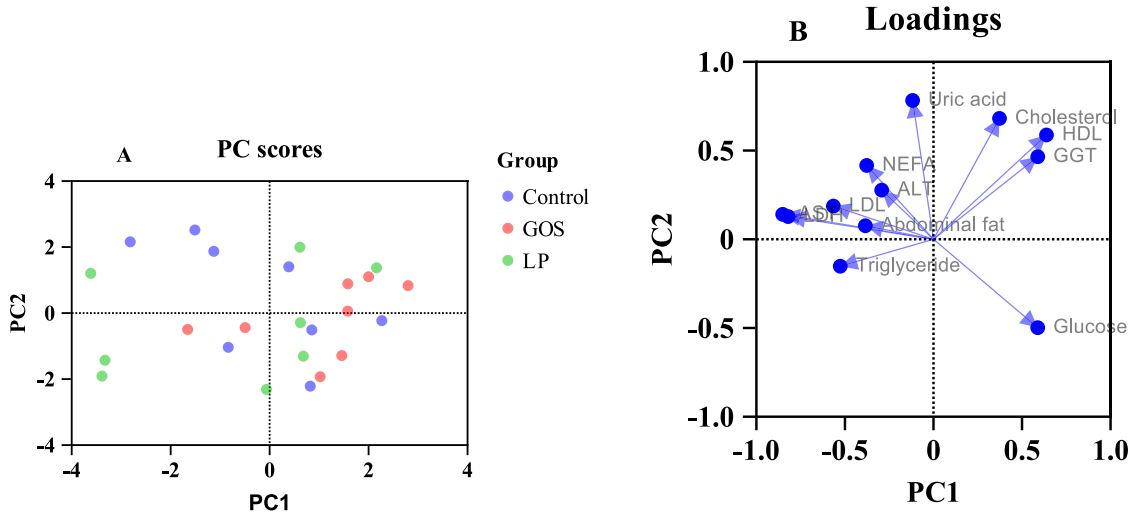

Fig. 6 **A** Principal component analysis (PCA) plot of PCA score **B** and variables/plasma metabolite parameters. An individual refers to a sampled bird per treatment, while a variable is a biological parameter analyzed. The individuals have been colored according to treatment C, control; GOS, galactooligoaccharide; or LP, *Lactiplantibacillus plantarum*

NEFA levels observed in our study could be regarded as a positive effect, according to (Verago et al. 2001), lower/unchanged NEFA levels may indicate the ability of chickens to adapt to their environment or other stressors. The other parameters remain unaffected indicating that the *in ovo* stimulation of GOS or LP did not cause any negative effect on the health of the chickens (Table 3) and (Fig. 6A) which was confirmed by the transcriptomic analysis and our production data results (body weight, FCR, and feed intake M. Mangan et al., personal communication).

Conclusion

The present study provided evidence suggesting that the *in ovo* administration of either GOS or LP may positively influence gut health and immune functions in broiler chickens as indicated by changes in mRNA expression of relevant genes. Additionally, GOS and LP delivered *in ovo* prevented oxidative stress as indicated by the upregulation of certain antioxidants, such as *Cat*, *Sod1*, *Mnsod*, and *Nrf2*. Furthermore, the *in ovo* administration of either GOS or LP did not cause any negative effects on the selected plasma metabolites, indicating that the chickens were healthy and in good condition. Based on these findings, we showed that the *in ovo* administration of GOS and LP on the 12th day of egg incubation may provide long-lasting beneficial effects on chicken gut health, immunity, and antioxidant status. Overall, our results suggest that the *in ovo* administration of GOS (3.5 mg/egg) significantly influenced the gut health, immune functions, antioxidant activity and performance of chickens more than the *in ovo* injection of 1×10^6 *Lactiplantibacillus plantarum*. We suggest that further research be conducted to understand the mechanisms involved in the antioxidant, molecular, and biological processes in specific chicken tissues and their effects on chicken health. Additionally, while our results showed beneficial effects of *in ovo* administration of GOS and LP as demonstrated by the mRNA expression levels of the selected genes, we suggest that further studies on enzymatic activities and protein expression levels be performed in other to understand their effects on chicken health and performance.

Abbreviations AC: Absorbance of control; *Actb*: Actin, beta; ALT: Alanine transaminase; ANOVA: Analysis of variance; AS: Absorbance of sample; AST: Aspartate aminotransferase; *Avbd1*: Avian beta defensin 1; *Cat*: Catalase; *Cathl2*: Cathelicidin 2; CFU: Colony forming units; cDNA: Complementary DNA; *Cldn1*: Claudin 1; DNA: Deoxyribonucleic acid; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; EU: European Union; ED: Embryonic development; EC50: Half maximal effective concentration; *Ffar2*: Free fatty acid receptor 2; *Ffar4*: Free fatty acid receptor 4; *G6pdh*: Glucose-6-phosphate dehydrogenase; GGT: Gamma-glutamyl transferase; GOS: Galactooligosaccharide; *Gpx*1: Glutathione peroxidase 1; HDL: High-density lipoprotein; *Ho1*: Heme oxygenase 1; *Il1b*: Interleukin 1 beta; *Il4*: Interleukin 4; *Il8*: Interleukin 8; *Il10*: Interleukin 10; *Il12p40*: Interleukin 12p40; LAB: Lactic acid bacteria; LC: *Lacticaseibacillus casei*; LDH: Lactose dehydrogenase; LDL: Low-density lipoprotein; LP: *Lactiplantibacillus plantarum*; LR: *Limosilactobacillus reuteri*; LRh: *Lacticaseibacillus rhamnosus*; mg: Milligram; MJ/Kg: Mega Jules per kilogram; mL: Milliliter; mM: Millimolar; *Mnsod*: Manganese superoxide dismutase; MRS agar: De Man-Rogosa-Sharpe agar; *Muc6*: Mucin 6; NC: Negative control; NEFA: Non-esterified fatty acid; nm: Nanometer; *Nrf2*: Nuclear factor erythroid 2-related factor 2; OD600: Optical density at 600 nm; PC: Positive control; PCA: Principal component analysis; PCR: Polymerase chain reaction; qPCR: Quantitative polymerase chain reaction; RNA: Ribonucleic acid; rpm: Revolutions per minute; SCFA: Short-chain fatty acids; SD: Standard deviation; SE: Standard error; SEM: Standard error of means; *Sod1*: Superoxide

dismutase 1; TG: Triglyceride; *Tjap1*: Tight junction-associated protein 1; UA: Uric acid; *Zo1*: Zonula occludens 1

Acknowledgements The authors would like to thank JHJ sp z o.o., Poland, for kindly providing the probiotics and Clasado Biosciences Ltd, Reading, UK for providing the prebiotic (galactooligosaccharide) used in this study. We are also grateful to the European Union's Horizon 2020 research and innovation program for the financial support of this work.

Author contribution All authors contributed to the design and conceptualization of the study. MM and MS performed the *in ovo* study, data collection, data analysis, visualization and writing of the draft manuscript was done by MM. KP supervised the in vivo experiment while CM supervised the blood biochemical analysis. MS, KP, and CM revised and edited the final manuscript. All authors read and approved the final version of the manuscript for submission.

Funding This research was funded by the European Union's Horizon 2020 research and innovation program under grant agreement number [955374]. This article has been financially supported by the Minister of Science under the program "Regional Initiative of Excellence" (RID/SP/0017/2024/01).

Data availability All the data used in this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

References

Abramowicz K, Krauze M, Ognik K (2019) The effect of a probiotic preparation containing *Bacillus subtilis* PB6 in the diet of chickens on redox and biochemical parameters in their blood. Annals of Animal Science 19:433–451. https://doi.org/10.2478/aoas-2018-0059

Akbarian A, Michiels J, Golian A et al (2014) Gene expression of heat shock protein 70 and antioxidant enzymes, oxidative status, and meat oxidative stability of cyclically heat-challenged finishing broilers fedOriganum compactum andCurcuma xanthorrhiza essential oils. Poult Sci 93:1930–1941. https://doi.org/10.3382/ps.2014-03896

Alagawany M, Abd El-Hack ME, Farag MR et al (2018) The use of probiotics as eco-friendly alternatives for antibiotics in poultry nutrition. Environ Sci Pollut Res 25:10611–10618. https://doi.org/10.1007/s11356-018-1687-x

Alvarez-Curto E, Milligan G (2016) Metabolism meets immunity: the role of free fatty acid receptors in the immune system. Biochem Pharmacol 114:3–13. https://doi.org/10.1016/j.bcp.2016.03.017

Bai K, Huang Q, Zhang J et al (2017) Supplemental effects of probiotic Bacillus subtilis fmbJ on growth performance, antioxidant capacity, and meat quality of broiler chickens. Poult Sci 96:74–82. https://doi.org/10.3382/ps/pew246

Bednarczyk M, Stadnicka K, Kozłowska I et al (2016) Influence of different prebiotics and mode of their administration on broiler chicken performance. Animal 10:1271–1279. https://doi.org/10.1017/S1751731116000173

Bertocchi M, Zampiga M, Luise D et al (2019) In ovo Injection of a Galacto-Oligosaccharide prebiotic in broiler chickens submitted to heat-stress: impact on transcriptomic profile and plasma immune parameters. Animals 9:1067. https://doi.org/10.3390/ani9121067

Brisbin JT, Gong J, Parvizi P, Sharif S (2010) Effects of Lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. Clin Vaccine Immunol 17:1337–1343. https://doi.org/10.1128/CVI.00143-10

Burns RN, Moniri NH (2010) Agonism with the omega-3 fatty acids α-linolenic acid and docosahexaenoic acid mediates phosphorylation of both the short and long isoforms of the human GPR120 receptor. Biochem Biophys Res Commun 396:1030–1035. https://doi.org/10.1016/j.bbrc.2010.05.057

Cao L, Wu XH, Bai YL et al (2019) Anti-inflammatory and antioxidant activities of probiotic powder containing Lactobacillus plantarum 1.2567 in necrotic enteritis model of broiler chickens. Livest Sci 223:157–163. https://doi.org/10.1016/j.livsci.2019.03.009

Chang CH, Teng PY, Lee TT, Yu B (2020) Effects of multi-strain probiotic supplementation on intestinal microbiota, tight junctions, and inflammation in young broiler chickens challenged with Salmonella enterica subsp. enterica. Asian-Australas J Anim Sci 33:1797–1808. https://doi.org/10.5713/ajas.19.0427

Chen J, Zhai Z, Long H et al (2020) Inducible expression of defensins and cathelicidins by nutrients and associated regulatory mechanisms. Peptides 123:170177. https://doi.org/10.1016/j.peptides.2019.170177

Cheng Y, Chen Y, Li X et al (2017) Effects of synbiotic supplementation on growth performance, carcass characteristics, meat quality and muscular antioxidant capacity and mineral contents in broilers. J Sci Food Agric 97:3699–3705. https://doi.org/10.1002/jsfa.8230

Corrêa-Oliveira R, Fachi JL, Vieira A et al (2016) Regulation of immune cell function by short-chain fatty acids. Clinical & Translational Immunology 5:e73. https://doi.org/10.1038/cti.2016.17

Das R, Mishra P, Jha R (2021) In ovo feeding as a tool for improving performance and gut health of poultry: a review. Front Vet Sci 8:754246. https://doi.org/10.3389/fvets.2021.754246

Den Besten G, Van Eunen K, Groen AK et al (2013) The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res 54:2325–2340. https://doi.org/10.1194/jlr.R036012

Directive 2010/63/EU Of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Text with EEA relevance

Dunislawska A, Slawinska A, Stadnicka K et al (2017) Synbiotics for broiler chickens—in vitro design and evaluation of the influence on host and selected microbiota populations following in ovo delivery. PLoS ONE 12:e0168587. https://doi.org/10.1371/journal.pone.0168587

Dunislawska A, Slawinska A, Bednarczyk M, Siwek M (2019) Transcriptome modulation by in ovo delivered Lactobacillus synbiotics in a range of chicken tissues. Gene 698:27–33. https://doi.org/10.1016/j.gene.2019.02.068

Elnagar R, Elkenany R, Younis G (2021) Interleukin gene expression in broiler chickens infected by different Escherichia coli serotypes. Vet World 2727–2734. https://doi.org/10.14202/vetworld.2021.2727-2734

El-Deep, Ijiri D, Z Eid Y et al (2014) Effects of dietary supplementation with Aspergillus awamori on growth performance and antioxidative status of broiler chickens exposed to high ambient temperature. J Poult Sci 51:281–288. https://doi.org/10.2141/jpsa.0130154

Fathima S, Shanmugasundaram R, Adams D, Selvaraj RK (2022) Gastrointestinal microbiota and their manipulation for improved growth and performance in chickens. Foods 11:1401. https://doi.org/10.3390/foods11101401

Forder REA, Nattrass GS, Geier MS et al (2012) Quantitative analyses of genes associated with mucin synthesis of broiler chickens with induced necrotic enteritis. Poult Sci 91:1335–1341. https://doi.org/10.3382/ps.2011-02062

Gao J, Wang R, Liu J et al (2022) Effects of novel microecologics combined with traditional Chinese medicine and probiotics on growth performance and health of broilers. Poult Sci 101:101412. https://doi.org/10.1016/j.psj.2021.101412

Gao S, Wang K, Xiong K et al (2023) Unraveling the Nrf2-ARE signaling pathway in the DF-1 chicken fibroblast cell line: insights into T-2 toxin-induced oxidative stress regulation. Toxins 15:627. https://doi.org/10.3390/toxins15110627

Georgieva NV, Koinarski V, Gadjeva V (2006) Antioxidant status during the course of *Eimeria tenella* infection in broiler chickens. Vet J 172:488–492. https://doi.org/10.1016/j.tvjl.2005.07.016

Jarosinski KW, Njaa BL, O'connell PH, Schat KA (2005) Pro-inflammatory responses in chicken spleen and brain tissues after infection with very virulent plus Marek's disease virus. Viral Immunol 18:148–161. https://doi.org/10.1089/vim.2005.18.148

Jha R, Das R, Oak S, Mishra P (2020) Probiotics (direct-fed microbials) in poultry nutrition and their effects on nutrient utilization, growth and laying performance, and gut health: a systematic review. Animals 10:1863. https://doi.org/10.3390/ani10101863

Kao T-H, Chen B-H (2006) Functional components in soybean cake and their effects on antioxidant activity. J Agric Food Chem 54:7544–7555. https://doi.org/10.1021/jf061586x

Karaca B, Yilmaz M, Gursoy UK (2022) Targeting Nrf2 with probiotics and postbiotics in the treatment of periodontitis. Biomolecules 12:729. https://doi.org/10.3390/biom12050729

Kawabe H, Nakanishi H, Asada M et al (2001) Pilt, a novel peripheral membrane protein at tight junctions in epithelial cells. J Biol Chem 276:48350–48355. https://doi.org/10.1074/jbc.M107335200

Khosravi A, Mazmanian SK (2013) Disruption of the gut microbiome as a risk factor for microbial infections. Curr Opin Microbiol 16:221–227. https://doi.org/10.1016/j.mib.2013.03.009

Kolodziejski PA, Sassek M, Chalupka D et al (2018) GLP1 and GIP are involved in the action of synbiotics in broiler chickens. J Animal Sci Biotechnol 9:13. https://doi.org/10.1186/s40104-017-0227-8

Kpodo KR, Proszkowiec-Weglarz M (2023) Physiological effects of in ovo delivery of bioactive substances in broiler chickens. Front Vet Sci 10:1124007. https://doi.org/10.3389/fvets.2023.1124007

Lee B, Kim JY, Choi YM (2022) Effects of serum aspartate aminotransferase activity on levels of serum Ca2+, Ca2+ channel genes, apoptotic factors, and meat quality characteristics of chicken pectoralis major muscle. Poult Sci 101:102214. https://doi.org/10.1016/j.psj.2022.102214

Li S, Lu L, Hao S et al (2011) Dietary manganese modulates expression of the manganese-containing superoxide dismutase gene in chickens. J Nutr 141:189–194. https://doi.org/10.3945/jn.110.126680

Li S, Zhao Y, Zhang L et al (2012) Antioxidant activity of Lactobacillus plantarum strains isolated from traditional Chinese fermented foods. Food Chem 135:1914–1919. https://doi.org/10.1016/j.foodchem.2012.06.048

Lyu W, Zhang L, Gong Y et al (2020) Developmental and tissue patterns of the basal expression of chicken avian β-defensins. Biomed Res Int 2020:1–12. https://doi.org/10.1155/2020/2567861

Mangan M, Siwek M (2023) Strategies to combat heat stress in poultry production—A review. J Anim Physiol Anim Nutr 108:576–595. https://doi.org/10.1111/jpn.13916

Mottet A, Tempio G (2017) Global poultry production: current state and future outlook and challenges. World's Poultry Science Journal 73:245–256. https://doi.org/10.1017/S0043933917000071

Mounir M, Ibijbijen A, Farih K et al (2022) Synbiotics and their antioxidant properties, mechanisms, and benefits on human and animal health: a narrative review. Biomolecules 12:1443. https://doi.org/10.3390/biom12101443

Mu G, Gao Y, Tuo Y et al (2018) Assessing and comparing antioxidant activities of lactobacilli strains by using different chemical and cellular antioxidant methods. J Dairy Sci 101:10792–10806. https://doi.org/10.3168/jds.2018-14989

Ncho C-M, Goel A, Jeong C-M et al (2021) In ovo injection of GABA can help body weight gain at hatch, increase chick weight to egg weight ratio, and improve broiler heat resistance. Animals 11:1364. https://doi.org/10.3390/ani11051364

Pietrzak E, Dunislawska A, Siwek M et al (2020) Splenic gene expression signatures in slow-growing chickens stimulated in ovo with galactooligosaccharides and challenged with heat. Animals 10:474. https://doi.org/10.3390/ani10030474

Roberfroid M (2007) Prebiotics: the concept revisited. J Nutr 137:830S-837S. https://doi.org/10.1093/jn/137.3.830S

Rothwell L, Young JR, Zoorob R et al (2004) Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. J Immunol 173:2675–2682. https://doi.org/10.4049/jimmunol.173.4.2675

Schlatterer K, Peschel A, Kretschmer D (2021) Short-chain fatty acid and FFAR2 activation – a new option for treating infections? Front Cell Infect Microbiol 11:785833. https://doi.org/10.3389/fcimb.2021.785833

Sevane N, Bialade F, Velasco S et al (2014) Dietary inulin supplementation modifies significantly the liver transcriptomic profile of broiler chickens. PLoS ONE 9:e98942. https://doi.org/10.1371/journal.pone.0098942

Slawinska A, Dunislawska A, Plowiec A et al (2019) Modulation of microbial communities and mucosal gene expression in chicken intestines after galactooligosaccharides delivery In Ovo. PLoS ONE 14:e0212318. https://doi.org/10.1371/journal.pone.0212318

Slawinska A, Zampiga M, Sirri F et al (2020b) Impact of galactooligosaccharides delivered in ovo on mitigating negative effects of heat stress on performance and welfare of broilers. Poult Sci 99:407–415. https://doi.org/10.3382/ps/pez512

Sławinska A, Siwek MZ, Bednarczyk MF (2014a) Effects of synbiotics injected in ovo on regulation of immune-related gene expression in adult chickens. ajvr 75:997–1003. https://doi.org/10.2460/ajvr.75.11.997

Sławinska A, Siwek MZ, Bednarczyk MF (2014b) Effects of synbiotics injected in ovo on regulation of immune-related gene expression in adult chickens. ajvr 75:997–1003. https://doi.org/10.2460/ajvr.75.11.997

Slawinska A, Dunislawska A, Kowalczyk A, et al (2020a) Mucosal and systemic immune responses modulated by in ovo-delivered bioactive compounds in distinct chicken genotypes

Sumanu VO, Naidoo V, Oosthuizen M, Chamunorwa JP (2023) A technical report on the potential effects of heat stress on antioxidant enzymes activities, performance and small intestinal morphology in broiler chickens administered probiotic and ascorbic acid during the hot summer season. Animals 13:3407. https://doi.org/10.3390/ani13213407

Surai P, Kochish, Fisinin V, Kidd MT (2019) Antioxidant defence systems and oxidative stress in poultry biology: an update. Antioxidants 8:235. https://doi.org/10.3390/antiox8070235

Trapani L, Segatto M, Pallottini V (2012) Regulation and deregulation of cholesterol homeostasis: the liver as a metabolic "power station." World J Hepatol 4:184. https://doi.org/10.4254/wjh.v4.i6.184

Verago JL, Grassi-Kassisse DM, Spadari-Bratfisch RC (2001) Metabolic markers following beta-adrenoceptor agonist infusion in footshock-stressed rats. Braz J Med Biol Res 34:1197–1207. https://doi.org/10.1590/s0100-879x2001000900014

Volf J, Polansky O, Sekelova Z et al (2017) Gene expression in the chicken caecum is dependent on microbiota composition. Vet Res 48:85. https://doi.org/10.1186/s13567-017-0493-7

Wang L, Li L, Lv Y et al (2018) Lactobacillus plantarum restores intestinal permeability disrupted by salmonella infection in newly-hatched chicks. Sci Rep 8:2229. https://doi.org/10.1038/s41598-018-20752-z

Wilson RA, Sangha MK, Banga SS et al (2014) Heat stress tolerance in relation to oxidative stress and antioxidants in Brassica juncea. J Environ Biol 35:383–387

Wishna-Kadawarage RN, Połtowicz K, Dankowiakowska A et al (2024) Prophybiotics for in-ovo stimulation; validation of effects on gut health and production of broiler chickens. Poult Sci 103:103512. https://doi.org/10.1016/j.psj.2024.103512

Wu Y, Wang B, Zeng Z et al (2019) Effects of probiotics Lactobacillus plantarum 16 and Paenibacillus polymyxa 10 on intestinal barrier function, antioxidative capacity, apoptosis, immune response, and biochemical parameters in broilers. Poult Sci 98:5028–5039. https://doi.org/10.3382/ps/pez226

Yang S-J, Lee J-E, Lim S-M et al (2019) Antioxidant and immune-enhancing effects of probiotic Lactobacillus plantarum 200655 isolated from kimchi. Food Sci Biotechnol 28:491–499. https://doi.org/10.1007/s10068-018-0473-3

Yang C, Wang S, Li Q et al (2024) Effects of probiotic Lactiplantibacillus plantarum HJLP-1 on growth performance, selected antioxidant capacity, immune function indices in the serum, and cecal microbiota in broiler chicken. Animals 14:668. https://doi.org/10.3390/ani14050668

Yu H, Zou W, Wang X et al (2020) Research note: correlation analysis of interleukin-6, interleukin-8, and C-C motif chemokine ligand 2 gene expression in chicken spleen and cecal tissues after Eimeria tenella infection in vivo. Poult Sci 99:1326–1331. https://doi.org/10.1016/j.psj.2019.10.071

Zeng T, Li J, Wang D et al (2014) Effects of heat stress on antioxidant defense system, inflammatory injury, and heat shock proteins of Muscovy and Pekin ducks: evidence for differential thermal sensitivities. Cell Stress Chaperones 19:895–901. https://doi.org/10.1007/s12192-014-0514-7

Zhang G, Sunkara L (2014) Avian antimicrobial host defense peptides: from biology to therapeutic applications. Pharmaceuticals 7:220–247. https://doi.org/10.3390/ph7030220

Zhang Y, Hu P, Lou L et al (2017) Antioxidant activities of lactic acid bacteria for quality improvement of fermented sausage. J Food Sci 82:2960–2967. https://doi.org/10.1111/1750-3841.13975

Statement of the author of the doctoral dissertation

Mgr. Modou Mangan

Department of Animal Biotechnology and Genetics
Faculty of Animal Breeding and Biology
Bydgoszcz University of Science and Technology
Mazowiecka 28, 85-084, Bydgoszcz, Poland

STATEMENT

I declare that my authorial contribution to the scientific articles listed below, constituting the series of publications of the doctoral dissertation, was as follows*:

1. **Modou Mangan** and Maria Siwek, Strategies to combat heat stress in poultry production-A review, Journal of Animal Physiology and Animal Nutrition (Wiley online library) 2024 December;108(3): 576-595. doi: 10.1111/jpn.13916. MNiSW points $_{2024}$:100, Impact Factor $_{2024}$: 2.2

Tasks performed by the PhD student as part of the article:
a) Planning and designing of the review article content
b) Performed thorough literature search
c) Writing and revision of the first draft manuscript

2. **Modou Mangan,** Patrycja Reszka, Katarzyna Połtowicz and Maria Siwek, Effects of *Lactiplantibacillus plantarum* and galactoligosaccharide administered *in ovo* on hatchability, chick quality, performance, cecal histomorphology and meat quality traits of broiler chickens, Journal of Animal Physiology and Animal Nutrition (Wiley online library) 2024 December. doi: 10.1111/jpn.14082. MNiSW $_{2024}$: 100, Impact Factor $_{2024}$: 2.2

Tasks performed by the PhD student as part of the article:
a) Planning and designing of the experiment

* *In the case of two- or multi-authored works, statements are required from the candidate for the doctoral degree and co-authors, indicating their substantive contribution to the creation of each work (e.g. creator of the research hypothesis, originator of the research, carrying out specific research - e.g. carrying out specific experiments, developing and collecting surveys, etc., analyzing the results, preparing an article manuscript and others). Determining the contribution of a given author, including a candidate for a doctoral degree, should be precise enough to enable an accurate assessment of his or her participation and role in the creation of each work.*

b) Conducted and managed the *in ovo* experiment

c) Sample collection

d) Laboratory experiment, data collection and data analysis

e) Writing and revision of the first draft manuscript

3. **Modou Mangan,** Katarzyna Połtowicz, Cornelia C. Metges and Maria Siwek, Modulatory effects of *in ovo* delivery of galactooligosaccharide and *Lactiplantibacillus plantarum* on antioxidant capacity, gene expression and selected plasma metabolite parameters of broiler chickens, Journal of Applied Genetics (Springer) 2024 December. doi:10.1007/s13353-024-00931-7. MNiSW $_{2024}$: 140, Impact Factor $_{2024}$: 2.0

Tasks performed by the PhD student as part of the article:

a) Planning and designing of the experiment

b) Conducted and managed the *in ovo* experiment

c) Sample collection

d) Laboratory experiment, data collection and data analysis

e) Writing and revision of the first draft manuscript

18.02.2025 .....

Place, date

.....

Signature of the author of the doctoral dissertation

.....

Signature of the second supervisor

.....

Signature of the first supervisor

Co-author's Declaration

Professor Maria Siwek - Gapińska
Department of Animal Biotechnology and Genetics
Faculty of Animal Breeding and Biology
Bydgoszcz University of Science and Technology
Mazowiecka 28, 85-084, Bydgoszcz, Poland

DECLARATION

I declare that my authorial contribution to the scientific article(s) mentioned below was as follows*:

1. Modou Mangan and **Maria Siwek**, Strategies to combat heat stress in poultry production-A review, Journal of Animal Physiology and Animal Nutrition (Wiley online library) 2024 December;108(3): 576-595. doi: 10.1111/jpn.13916. MNiSW points $_{2024}$:100, Impact Factor $_{2024}$: 2.2
Tasks completed as part of the article:
a) Funding acquisition
b) Planning and designing of the content of the review article
c) Supervision
d) Reviewing and editing of the manuscript

2. Modou Mangan, Patrycja Reszka, Katarzyna Połtowicz and **Maria Siwek**, Effects of *Lactiplantibacillus plantarum* and galactooligosaccharide administered *in ovo* on hatchability, chick quality, performance, cecal histomorphology and meat quality traits of broiler chickens, Journal of Animal Physiology and Animal Nutrition (Wiley online library) 2024 December. doi: 10.1111/jpn.14082. MNiSW $_{2024}$: 100, Impact Factor $_{2024}$: 2.2
Tasks completed as part of the article:
a) Funding acquisition
b) Planning and designing of the experiment
c) Supervision
d) Reviewing and editing of the manuscript

3. Modou Mangan, Katarzyna Połtowicz, Cornelia C. Metges and **Maria Siwek**, Modulatory effects of *in ovo* delivery of galactooligosaccharide and *Lactiplantibacillus plantarum* on antioxidant capacity, gene expression and selected plasma metabolite parameters of broiler chickens, Journal of Applied Genetics (Springer) 2024 December. doi:10.1007/s13353-024-00931-7. MNiSW $_{2024}$: 140, Impact Factor $_{2024}$: 2.0

Task completed as part of the article:

a) Funding acquisition

b) Planning and designing of the experiment

c) Supervision

d) Reviewing and editing of the manuscript

At the same time, I consent to the submission of the above-mentioned work(s) by **Mgr. Modou Mangan** as part of a doctoral dissertation based on a set of published and thematically related scientific articles.

Bydgoszcz 18.02 2025 ..... Maria Siwek .....

Place, date Co-author's signature

Co-author's Declaration

Professor Dr. Cornelia C. Metges
Research Institute for Farm Animal Biology (FBN)
Wilhelm-Stahl-Allee 2
18196 Dummerstorf
Germany

DECLARATION

I declare that my author's contribution to the journal article/articles mentioned below was as follows*:

1. Modou Mangan, Katarzyna Połtowicz, **Cornelia C. Metges** and Maria Siwek, Modulatory effects of *in ovo* delivery of galactooligosaccharide and *Lactiplantibacillus plantarum* on antioxidant capacity, gene expression and selected plasma metabolite parameters of broiler chickens, Journal of Applied Genetics (Springer) 2024 December. doi:10.1007/s13353-024-00931-7. MNiSW $_{2024}$: 140, Impact Factor $_{2024}$: 2.0

Tasks completed as part of the article:
a) Funding acquisition
b) Planning and design of experiment
c) Supervision
d) Reviewing and editing of the manuscript

At the same time, I hereby agree to the submission of the above-mentioned paper(s) by **Mgr. Modou Mangan** as part of the doctoral dissertation based on a collection of published and thematically related scientific papers.

Dummerstorf, 17.2.2025

....

Place, date Co-author's signature

* *In the case of two- or multi-author papers, declarations of a candidate for the doctoral degree and co-authors are required, indicating their substantive contribution to the creation of each paper (e.g. the creator of the research hypothesis, the originator of the research, performance of specific research – e.g. carrying out particular experiments, developing and collecting questionnaires, etc., analysis of the results, preparation of the article manuscript and others). Identification of the contribution of a given author, including a candidate for the doctoral degree, should be precise enough to allow for an accurate assessment of his/her participation and role in the creation of each paper.*

Co-author's Declaration

Dr hab. Katarzyna Połtowicz, prof. IZ

Department of Poultry Breeding

National Research Institute of Animal Production

Krakowska 1,

Balice 32-083

Poland

DECLARATION

I declare that my author's contribution to the journal article/articles mentioned below was as follows*:

1. Modou Mangan, Patrycja Reszka, **Katarzyna Połtowicz** and Maria Siwek, Effects of *Lactiplantibacillus plantarum* and galactooligosaccharide administered *in ovo* on hatchability, chick quality, performance, cecal histomorphology and meat quality traits of broiler chickens, Journal of Animal Physiology and Animal Nutrition (Wiley online library) 2024 December. doi: 10.1111/jpn.14082. MNiSW $_{2024}$: 100, Impact Factor $_{2024}$: 2.2

Tasks completed as part of the article:

a) Planning and design of experiment

b) Supervision of the animal trial, carcass traits and meat quality analysis

c) Reviewing and editing of the manuscript

2. Modou Mangan, **Katarzyna Połtowicz**, Cornelia C. Metges and Maria Siwek, Modulatory effects of *in ovo* delivery of galactooligosaccharide and *Lactiplantibacillus plantarum* on antioxidant capacity, gene expression and selected plasma metabolite parameters of broiler chickens, Journal of Applied Genetics (Springer) 2024 December. doi:10.1007/s13353-024-00931-7. MNiSW $_{2024}$: 140, Impact Factor $_{2024}$: 2.0

Tasks completed as part of the article:

a) Planning and design of experiment

b) Supervision of the in vivo experiment (animal trial)

c) Reviewing and editing of the manuscript

---

* *In the case of two- or multi-author papers, declarations of a candidate for the doctoral degree and co-authors are required, indicating their substantive contribution to the creation of each paper (e.g. the creator of the research hypothesis, the originator of the research, performance of specific research – e.g. carrying out particular experiments, developing and collecting questionnaires, etc., analysis of the results, preparation of the article manuscript and others). Identification of the contribution of a given author, including a candidate for the doctoral degree, should be precise enough to allow for an accurate assessment of his/her participation and role in the creation of each paper.*

At the same time, I hereby agree to the submission of the above-mentioned paper(s) by **Mgr. Modou Mangan** as part of the doctoral dissertation based on a collection of published and thematically related scientific papers.

Bellice, 14.02.2025 .... Place, date

Uuetenyne Poltolni .... Co-author's signature

Co-author's Declaration

Dr inż. Patrycja Reszka
Department of Animal Physiology and Physiotherapy
Faculty of Animal Breeding and Biology
Bydgoszcz University of Science and Technology
Mazowiecka 28, 85.084
Poland

DECLARATION

I declare that my author's contribution to the journal article/articles mentioned below was as follows*:

1. Modou Mangan, **Patrycja Reszka**, Katarzyna Połtowicz and Maria Siwek, Effects of *Lactiplantibacillus plantarum* and galactooligosaccharide administered *in ovo* on hatchability, chick quality, performance, cecal histomorphology and meat quality traits of broiler chickens, Journal of Animal Physiology and Animal Nutrition (Wiley online library) 2024 December. doi: 10.1111/jpn.14082. MNiSW $_{2024}$: 100, Impact Factor $_{2024}$: 2.2

Tasks completed as part of the article:

a) Planning and design of experiment

b) Supervision of the histology analysis

c) Reviewing and editing of the manuscript

At the same time, I hereby agree to the submission of the above-mentioned paper(s) by **Mgr. Modou Mangan** as part of the doctoral dissertation based on a collection of published and thematically related scientific papers.

Bydgoszcz, 14.02.25 — Place, date

Patrycja Reszka — Co-author's signature

* *In the case of two- or multi-author papers, declarations of a candidate for the doctoral degree and co-authors are required, indicating their substantive contribution to the creation of each paper (e.g. the creator of the research hypothesis, the originator of the research, performance of specific research – e.g. carrying out particular experiments, developing and collecting questionnaires, etc., analysis of the results, preparation of the article manuscript and others). Identification of the contribution of a given author, including a candidate for the doctoral degree, should be precise enough to allow for an accurate assessment of his/her participation and role in the creation of each paper.*