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

SCIENTIFIC COUNCIL OF THE DISCIPLINE ANIMAL SCIENCES AND FISHERIES

DISSERTATION

Ramesha Nirmali Wishna Kadawarage, MSc.

IN-OVO STIMULATION AS A TOOL TO IMPROVE GUT HEALTH OF BROILER CHICKENS

Stymulacja in-ovo jako narzędzie do poprawy zdrowia jelit kurcząt brojlerów.

FIELD: Agricultural Sciences
DISCIPLINE: Animal Sciences and Fisheries

FIRST SUPERVISOR.

PROF. DR HAB. INŻ. MARIA SIWEK-GAPIŃSKA

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

SECOND SUPERVISOR

DR. RITA M. HICKEY

TEAGASC FOOD RESEARCH CENTRE, MOOREPARK, FERMOY, IRELAND

Bydgoszcz, 2024

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

Source of financing

This research was carried out under the funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 955374. This researcher was funded and trained by Monoguthealth MSCA Innovative Training Network funded by the same grant agreement.

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

Acknowledgments

I would like to thank my son for supporting me throughout my academic journey abroad. He was my strength in every stressful situation. Secondly, I thank my husband for being my supporting in continuing my academic journey. I also thank my mother and sister for always believing in me and giving me strength when necessary. I thank both my supervisors Prof. Maria Siwek and Dr. Rita Hickey for guiding me throughout this PhD journey which was not a bed full of roses. Their kind understanding, assistance and guidance helped me to successfully finish my PhD on time. I further acknowledge all the co-authors of my PhD publications for their excellent contribution. Moreover, I thank all the people who were involved in Monoguthealth project for always making great questions and suggestions for my research in project meetings, and everyone who supported me in my academic secondment at Teagasc Food Research Centre, Ireland and industrial secondments, Vetdiagnostica and Drobex Agro in Poland. Furthermore, I thank European Union for funding my PhD project allowing me do engage in excellent science. I thank Prof. Aleksandra Dunislawska, the head of our department for always backing me up in difficult situations related to experiments and consumables. I also thank JHJ Sp. Zoo, Poland for providing probiotics, Kaesler Nutrition Gmbh, Germany for providing Turmeric and Green tea extracts, Prof. Martin Jensen, Aarhus University, Denmark for providing garlic powder and the advices for garlic extract preparation, and Prof. Ricarda, Aarhus University, Denmark for providing *Campylobacter jejuni* strain, for my research work. I further thank my previous supervisors Prof. Manjula Magamage and Dr. Tatiana Zerjal for guiding me well to achieve this success in my academic career. Last but not least I thank all the academic and non-academic staff of my university/faculty who helped me to feel welcome at a foreign country to successfully complete a very demanding research project.

CONTENTS

1. Introduction

Gut health is a topic that has gained increasing interest over the past decade. Despite this, there is no consensus definition of a healthy gut, to date. Staudacher and Loughman (2021) recently compiled a description for a healthy gut from literature as "the absence of gastrointestinal symptoms and disease, as well as an absence of other unfavorable local conditions including increased intestinal permeability, mucosal inflammation, or deficiency (or even excess) of short-chain fatty acids". However, the health of the gut is known to be linked to the physiology and health of other important organs such as liver (Pabst et al., 2023), brain (Appleton, 2018), gonads (Ashonibare et al., 2024), lungs (Enaud et al., 2020), skin (De Pessemier et al., 2021) and eyes (Campagnoli et al., 2023). Therefore, gut health is not merely an absence of gastrointestinal issues but a complex physiological status with utmost importance for an organism.

With the increasing world population and changes in food habits, a 15% increment in global poultry meat consumption has been foreseen by 2032 where poultry meat consumption is expected to account for 41% of the total meat consumption worldwide (OECD, 2023). Therefore, an expansion in broiler production is expected going forward. On the other hand, rising global concerns such as food security, food safety and antibiotic resistance, urge the broiler industry to produce safe and healthy meat for human consumption in a sustainable way (Wickramasuriya et al., 2022). In this context, ensuring optimal gut health in broilers is imperative as the production and welfare of birds and food safety of the broiler meat products are greatly influenced by their gut health (Oviedo-Rondón, 2019).

However, broilers have some unique challenges associated with gut health when compared to mammalian livestock species. Broiler chicks hatch in a relatively sterile external environment as the eggs, incubators and hatchers are sterilized before the incubation in commercial hatcheries. In addition, they lack maternal contact at birth unlike mammals (Kogut, 2019; Dunislawska et al., 2021). Moreover, due to longer hatching windows, they experience delays in access to feed and water which are the first sources of exogenous inoculum to build up the gut microbiome (Proszkowiec-Weglarz et al., 2022). For these reasons, the colonization of the gut with beneficial commensal bacteria is delayed in newly hatched chicks which adversely affects the development and maturation of the intestinal tract and immune system (Shehata et al., 2021). These factors ultimately predispose the chickens to colonization by

environmental foodborne pathogens such as *Campylobacter* and *Salmonella*. According to the latest reports, these are the two most prominent zoonotic pathogens reported within the European Union and broiler meat has been identified as one of the major sources of human cases of these zoonotic infections (Authority and European Centre for Disease Prevention and Control, 2022). Therefore, maintaining a healthy gut microbiome is imperative in broiler production to control foodborne gastrointestinal pathogens and maintain efficient energy utilization for efficient production.

As a core responsibility of the antibiotic stewardship, many scientists have investigated a variety of strategies to maintain optimal gut health in broiler chickens and mitigate the above mentioned challenges. These methods involve, feed quality control, inclusion of enzymes in feed, providing feed or water supplemented with prebiotic, probiotic, synbiotic, phytobiotic, organic acids or essential oils (as reviewed by Zhu et al., 2021). Apart from these *in-vivo* strategies, research has also been conducted to elucidate the potential of *in-ovo* intervention strategies on the post hatch gut health and production performance. Interestingly, Kogut (2019) claims that *in-ovo* delivery of bioactive substances may be more efficient than the other *in-vivo* methods (in feed/water, microbiome transplants etc.), as there should be less influence from confounding environmental factors which might reduce the efficacy of bioactive delivery.

In-ovo delivery of bioactive substances are mainly divided in to two methods based on the day of injection (Kpodo and Proszkowiec-Weglarz, 2023a). Administration of bioactive substances on 12^{th} day of incubation is referred to as *in-ovo* stimulation whereas on 17^{th} -18^{th} days administration is referred to as *in-ovo* feeding. The basis of *in-ovo* stimulation is to inject bioactive substances into the air cell allowing the passage of these substances to the embryos via the chorioallantoic membrane which is highly vascularized at the time (Siwek et al., 2018). There are accumulating evidence that the hatching eggs contain indigenous microbiome (Kizerwetter-Świda and Binek, 2008; Gantois et al., 2009; Siwek et al., 2018) and the main purpose of *in-ovo* stimulation is to stimulate the growth of indigenous commensal bacteria in the gut of the developing embryos. Consequently, a boost in the innate immune system and gastrointestinal tract development can be achieved, despite the regular stressors encountered in commercial hatchery operations such as long hatch windows and delays in access to feed and water (Siwek et al., 2018). In contrast, *in-ovo* feeding mostly involves a nutritional aspect where the delivery of nutrients (eg: carbohydrates, vitamins, amino acids or trace elements) into the amniotic fluid is performed with the intention of mitigating perinatal nutritional deficiencies

and aiding the nutritional transition from the yolk to post hatch feeding (Oladokun and Adewole, 2020a). However, this approach might also have a direct or indirect impact on the gut health.

The perinatal period (21 days of incubation) of commercial fast growing broilers (of slaughter age 42 days) is approximately one third of the total life span and is crucial for the development of gastrointestinal tract, immune system and microbiome of broilers (Siwek et al., 2018). Moreover, recent evidence demonstrates that the indigenous microbiome of chicken eggs, particularly in the yolk sac and amniotic fluid, changes during the course of embryonic development displaying functional associations to early, mid and late stages of embryonic development (Akinyemi et al., 2020). This suggests a role of native *in-ovo* bacteria in the embryonic development of the broiler chickens. Stimulating the growth of these native beneficial bacteria during embryonic development stage (*in-ovo* stimulation) therefore, can be considered as an excellent strategy to improve gut health in broiler chickens. Moreover, Dunislawska et al. (2021) concluded that *in-ovo* stimulation resulted in epigenetic regulation of gene expression in immune related organs such as the liver and spleen of broiler chickens indicating possible lifelong benefits of microbiome modulation as a result of *in-ovo* stimulation. Furthermore, the size of the embryo when *in-ovo* stimulation is performed is smaller when compared to that when *in-ovo* feeding is performed. Therefore, *in-ovo* stimulation has less impact on hatchability and chick quality when compared to *in-ovo* feeding as the risk of the injection needle causing damage to the developing embryos is less during *in-ovo* stimulation due to the smaller size of the embryo at the time of injection (Siwek et al., 2018).

To date, a multitude of bioactive substances have been studied with respect to *in-ovo* stimulation on day 12 of embryonic development. These mainly include, prebiotics, probiotics and synbiotics and each has demonstrated bioactive specific effects in number of different studies (Oladokun and Adewole, 2020). Kpodo and Proszkowiec-Weglarz (2023) recently concluded that *in-ovo* stimulation with different bioactive substances confers beneficial effects on digestion, metabolism, muscle development, production efficiency, immune related gene expression, heat stress response and mitigation of pathogenic infections. Furthermore, Akosile et al., (2023) also reviewed the beneficial effects of *in-ovo* delivery of plant based bioactive substances (phytobiotics) on hatchability, chick quality, antioxidant activity and gut development via mechanisms such as modulating gut microbiome and gene expression of the host.

Therefore, this PhD project was conducted to optimize an effective *in-ovo* stimulation protocol to improve the gut health of broiler chickens. In this project, *in-vitro* screening of a variety of prebiotics, probiotics and plant extracts was performed to select the most promising bioactive substances to be used in this protocol. Finally, an investigation of *in-vivo* effects on the gut health and production parameters was performed to validate the efficacy of the protocol as a tool to improve the gut health 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

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

1. **Ramesha N. Wishna-Kadawarage**, Martin Jensen, Szymon Powałowski, Rita M. Hickey and Maria Siwek, *In-vitro* screening of compatible synbiotics and (introducing) “prophybiotics” as a tool to improve gut health, International Microbiology (Springer), 2024, 27, 645–657, https://doi.org/10.1007/s10123-023-00417-2, MNiSW points $_{2024}$: 40, Impact Factor $_{2023}$: 2.3

2. **Ramesha N. Wishna-Kadawarage**, Rita M. Hickey and Maria Siwek, *In-vitro* selection of lactic acid bacteria to combat *Salmonella enterica* and *Campylobacter jejuni* in broiler chickens, World Journal of Microbiology and Biotechnology (Springer), 2024, 40, 133, https://doi.org/10.1007/s11274-024-03946-8, MNiSW points $_{2024}$: 70, Impact Factor $_{2023}$: 4.0

3. **Ramesha N. Wishna-Kadawarage**, Katarzyna Połtowicz, Agata Dankowiakowska, Rita M. Hickey and Maria Siwek, Prophybiotics for *in-ovo* stimulation; validation of effects on gut health and production of broiler chickens, Poultry Science (Elsevier), 2024, 103, 4, https://doi.org/10.1016/j.psj.2024.103512, MNiSW points $_{2024}$: 140, Impact Factor $_{2023}$: 3.8

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

3.1 List of abbreviations, symbols and units

ACTB	-	Actin, beta
AVBD1	-	Avian beta-defensin 1
CATHL2	-	Cathelicidin 2
CFU	-	Colony forming units
CLDN1	-	Claudin 1
CUR	-	Turmeric
FCR	-	Feed conversion ratio
FFAR2	-	Free fatty acid receptor 2
G	-	Garlic
G6PDH	-	Glucose-6-Phosphate Dehyfrogenase
GT	-	Green tea
IL10	-	Interleukin 10
IL1-β	-	Interleukin 1 beta
IL2	-	Interleukin 2
IL6	-	Interleukin 6
IL8	-	Interleukin 8
INU	-	Inulin
LAB	-	Lactic acid bacteria
LC	-	*Lacticaseibacillus casei*
LM	-	*Leuconostoc mesenteroides*
LP	-	*Lactiplantibacillus plantarum*
LR	-	*Limosilactobacillus reuteri*
LRh	-	*Lacticaseibacillus rhamnosus*
MJ/Kg	-	Mega Jules per Kilo gram
MUC6	-	Mucin 6
NC	-	Negative control

OD600	-	Optical density at 600nm
PAS	-	Periodic acid-Schiff
PB	-	Probiotic
PBS	-	Phosphate buffered saline
PC	-	Positive control
PP	-	Pediococcus pentosaceus
PPB	-	Prophybiotic
RAF	-	Rafinose
rpm	-	revolutions per minute
SAC	-	Saccharicterpenin
SCFA	-	Short chain fatty acids
SD	-	Standard deviation
SE	-	Standard error
w/v	-	weight/volume
WDA	-	Well diffusion assay

3.2 Hypothesis, objectives and scope of research

Hypothesis:

In-ovo stimulation of broiler chickens with carefully selected bioactives, will reprogram the gut microbiome and boost the immune system to activate direct and indirect pathogen exclusion pathways in the gut. These beneficial effects will last until the end of the production period even without continuous supplementation of special feed additives throughout the rearing period (Figure 1).

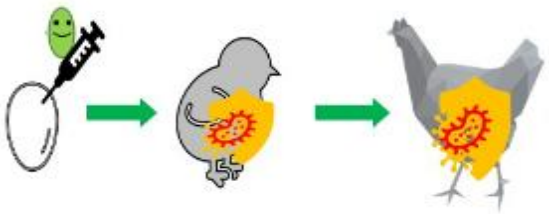

Figure 1: Graphical illustration of the research hypothesis

Main objective:

To develop a perinatal protocol for gut microbiome programming with proven anti-pathogenic effects, to improve the gut health of broiler chickens.

Sub objectives:

1. To identify the **most efficacious bioactives** in terms of **anti-pathogenic properties** (anti-*Campylobacter* and anti-*Salmonella*)
2. To identify the **optimal dose** of the selected bioactives to be administered into the chicken embryo.
3. To test the **efficacy** of *in-ovo* injection of the bioactives, in terms of **gut health and production**

Scope of research:

Although there are many advantages associated with the *in-ovo* stimulation method, the potential benefits of the treatment depend largely on the bioactive substances and the doses used in the protocol. Therefore, careful optimization of an *in-ovo* stimulation protocol is imperative to bring about the desired benefits while avoiding potential harmful effects. This PhD project was therefore, conducted to develop and validate an *in-ovo* stimulation protocol to improve the gut health of broiler chickens.

After performing a thorough literature search, six lactic acid bacteria all of which were probiotic strains, three prebiotics and three antimicrobial phytobiotics were pre-screened for the *in-ovo* protocol. The combined use of probiotics and prebiotics (as a synbiotic) has been investigated extensively in the *in-ovo* approach and can be divided into two types, complementary and synergistic synbiotics. Complementary synbiotics act individually on the host and do not necessarily need to be compatible whereas, synergistic synbiotics are required to be compatible in order to enhance the growth of the probiotic conferring better results for the host (Wishna-Kadawarage et al., 2024b). Therefore, it is important to investigate the compatibility of the selected prebiotics and probiotics to be used in an *in-ovo* protocol. Conversely, certain phytobiotics possess anti-pathogenic as well as prebiotic properties, making those interesting candidates to use in combination with a probiotic in an *in-ovo* approach. However, to the best of my knowledge, such a combination has not been studied to date. Therefore, I coined the term "**prophybiotics**" (**Pro**biotic + **Phy**tobiotic) to describe this unique

combination. Indeed, the compatibility of the probiotic and the phytobiotic is crucial for such a combined use.

The **first experiment** of this PhD project was therefore, designed and performed to identify compatible bioactive pairs (either synbiotic or prophybiotic) to be used for this application. There were two specific objectives of the experiment, to identify the preference of each probiotic in using a particular prebiotic as the sole energy source and to identify the phytobiotics and their doses at which the growth of each probiotic is not inhibited. Accordingly, the first publication of the PhD thesis (Wishna-Kadawarage et al., 2024b) reported the results of this experiment where *in-vitro* growth curve analysis of each of the six pre-screened probiotic strains was performed in the presence of each prebiotic (as the sole energy source) and phytobiotic (in different doses). The maximum growth, maximum growth rate and lag time were used to determine the effect of each supplementation on probiotic growth. This publication highlighted that the prebiotics are selective in stimulating the growth of a particular probiotic strain while antimicrobial phytobiotics seemed to be non-inhibitory to a wide range of probiotics at certain doses. Based on this outcome and the inherent bioactivity of the phytobiotics over the prebiotics, it was decided to proceed with prophybiotic combinations for the *in-ovo* stimulation protocol.

The **second experiment** was then, conducted to identify the probiotic with the most potent anti-pathogenic effects for use in the *in-ovo* stimulation protocol. The second publication of the PhD thesis (Wishna-Kadawarage et al., 2024a) reports the results of this experiment where, a variety of anti-microbial assays (spot overlay, well diffusion, co-culture and co-aggregation assays) against three strains of *Salmonella enterica* and well diffusion assays against a *Campylobacter jejuni* strain (the most common foodborne pathogens transmitting to humans from broilers) were performed.

Based on the two *in-vitro* experiments described above, the probiotic strain and the phytobiotic component and its dose were determined for the *in-ovo* protocol. The dose of the probiotic was obtained based on a literature search (Duan et al., 2021; Huang et al., 2024). The **third experiment** was then, conducted to validate the *in-vivo* effects of the selected bioactive substances (in the selected doses) when administered *in-ovo* (on day 12 of embryonic development) on the gut health and production parameters of ROSS308 broiler chickens (Wishna-Kadawarage et al., 2024c). The relative abundance of selected bacterial communities

in the feces of early life (1 week old) and late life (5 weeks old) chickens and the content of the ceca, was determined via a qPCR method. Moreover, histomorphological changes (using the PAS staining method) in the ceca and the expression of immune related genes in the cecal mucosa (via qPCR), were determined to investigate the health status of the gut. Stimulating the immune system can be a double edged sword. It can be beneficial in terms of mitigating pathogenic infections whereas, excessive stimulation may divert energy from production to maintaining the immune system. In this regard, the production parameters such as body weight, feed conversion ratio, carcass and meat quality characteristics were also determined to identify possible immune-metabolism trade-offs associated with the application.

Therefore, the research work conducted and reported by the three publications listed in this PhD thesis are well aligned and coherent with the main goal and the sub objectives of the PhD project (Figure 2). The first two publications report the results of the optimization of the *in-ovo* stimulation protocol. The data from these 2 publications support the conclusions of the first two sub-objectives of the PhD project (Determination of the most potent bioactive and optimal dose). The third publication reports the results of validation of the optimized *in-ovo* protocol. The data from this publication support the conclusions of the third sub-objective of the PhD project (to determine the efficacy in terms of gut health and production).

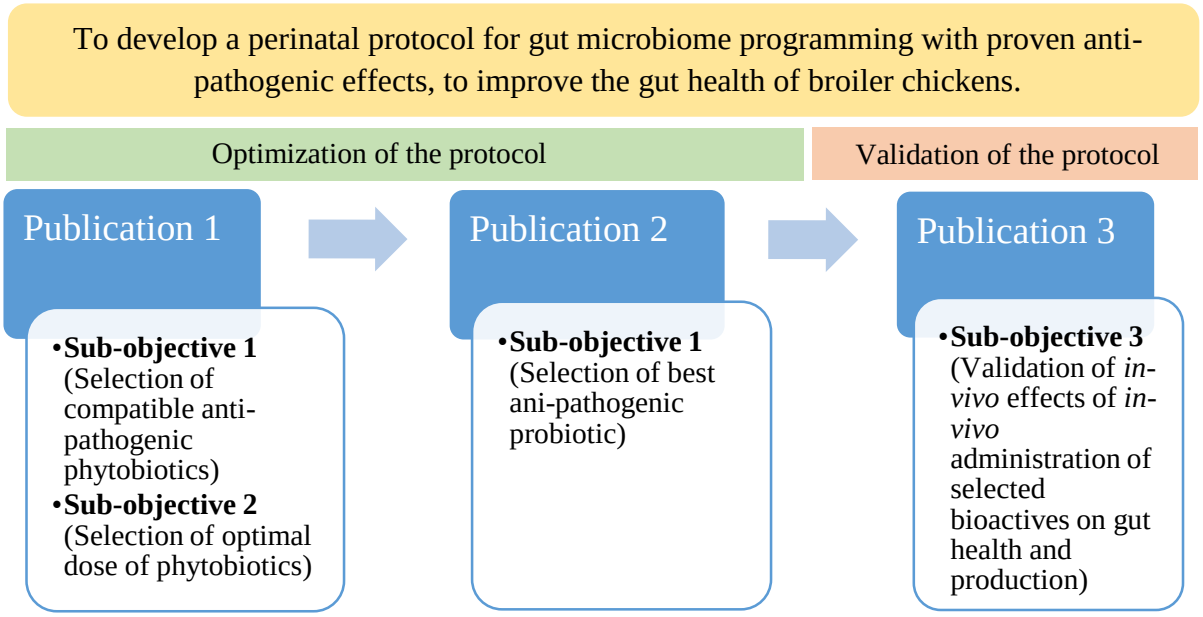

Figure 2: Thematic coherence of the publication series of the PhD thesis

3.3 Materials and research methods

Bioactive substances used for screening

Probiotics:

The following probiotics strains (Table 1) were obtained for the experiments of this PhD project from JHJ Sp Z.o.o, Nowa Wieś, Poland. These strains were lactic acid producing bacteria (LAB) identified using 16s rRNA sequencing and deposited at the Polish collection of Microorganisms in Wrocław.

Table 1: Probiotic strains used for screening

Species	Strain identification No.	Origin	Abbreviation
Lactiplantibacillus plantarum	B/00166		LP
Lacticaseibacillus casei	B/00164		LC
Limosilactobacillus reuteri	B/00281	Swine	LR
Lacticaseibacillus rhamnosus	B/00279		LRh
Leuconostoc mesenteroides	B/00288		LM
Pediococcus pentosaceus	B/00165	Chicken	PP

Prebiotics:

Three commercial prebiotics were used for the screening namely,

- Raffinose (RAF), VWR J392
- Inulin Orafti® HPX (INU) Mannheim, Germany
- Saccharicterpenin (SAC), Hubei, China

Phytobiotics:

Three antimicrobial plant extracts were used for screening as follows (Table 2).

Table 2: Plant extracts used for screening

Plant extract	Abbreviation	Protocol of preparation	Source of the product
Turmeric	CUR	The spray-dried product of alcoholic extract of turmeric rhizomes was re-suspended in sterile distilled water in required percentages and the solution was then filter sterilized using 0.2 µm syringe filters (Merck WHA69012502).	Kaesler Gmbh, Cuxhaven, Germany.
Green tea	GT	The spray-dried product of aqueous extract of green tea was re-suspended in sterile distilled water in required percentages and the solution was then filter sterilized using 0.2 µm syringe filters (Merck WHA69012502).	
Garlic	G	The garlic (cultivar: Thermodrome) was organically grown in the 2021 season in Aarhus University, Department of Food Science at Research Centre at Årslev, Funen, Denmark. The fresh garlic bulbs were chopped (in to 3–5 mm slices), air-dried (2 days at 40°C and 5 days at 50°C) and then, milled into powder which was subsequently sieved with a 1 mm sieve. This powder was stored at −20 °C until usage. The required amount of garlic powder was mixed and allowed to react with sterile distilled water at room temperature to activate the alliinase enzyme reaction to produce allicin from alliin (vortex for 20 s, shaking for 8 min at 550 rpm and stationary for 2 minutes). The supernatant after centrifuging at 10,000 rpm for 5 min was then filter sterilized using 0.2 µm syringe filters (Merck WHA69012502).	Aarhus University, Aarhus, Denmark

In-vitro selection of compatible bioactives (Synbiotics and Prophybiotics) (Experiment 1)

Pre-handling of the probiotic strains:

The stock cultures of all strains were stored at −80°C until use. Isolated single colonies of each culture were obtained by streaking a loop full (approximately 10 uL) of thawed stock cultures on MRS agar (Merck 1.10660, Germany) plates and incubating at 37°C for 48 h. A single colony was used to inoculate a 10 mL of MRS broth (Merck 1.10661, Germany) and this was incubated for 24 h at 37°C. To regain the viability of the bacterial cells after long-term storage at −80°C, two subsequent sub-culturing steps were performed by transferring 100 µL of overnight cultures to 10 mL of new MRS broth. The second subculture was incubated for 20 h and 1 mL from that culture was then, centrifuged at 13,000 rpm for 20 min to obtain the bacterial pellet. This pellet was re-suspended in 1 mL of Ringer's solution (Merck 1.15525, Germany) to prepare the inoculum for the experiment.

Preparation of media for prebiotic assays:

In order to determine which prebiotic was the preferred sole carbohydrate source for the probiotic strains, a carbohydrate-free MRS (cfMRS) broth was prepared from first principles according to the formula listed on Table 3. The selected prebiotics, RAF, SAC, and INU were dissolved in the cfMRS medium separately, at a concentration of 18 g/L. The same amount of D+ glucose (Merck G8270) was dissolved in cfMRS media and acted as the control for the prebiotic assays. Each supplemented medium was then filter sterilized using 0.2 µm syringe filters (Merck WHA69012502).

Table 3: Formula for carbohydrate free MRS (cfMRS) media preparation

Ingredient	Amount per liter
Oxoid peptone	10 g
Yeast extract	5 g
Tween 80	1 mL
K_2HPO_4	2 g
Sodium acetate	5 g
Triammonium citrate	2 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.05 g

Experimental design:

Growth kinetic assays for the six probiotic strains after supplementation of each with the prebiotics (in cfMRS media) and plant extracts (in MRS media) were performed as described on Table 4. Growth kinetics for each treatment were performed in triplicate with a negative control (supplemented media without bacterial inoculation) in 96-well plates (TPP B-0683). Plates were incubated at 37°C for 30 h under aerobic conditions and the absorbance of each at an optical density 600 nm (OD600) was measured at hourly intervals using a Hidex Sense microplate reader. Before each OD600 reading, plates were shaken orbitally at 300 rpm speed for 10 s.

Table 4: Experimental design of the Experiment 1

	Treatment	Annotation	Concentration (w/v)
Prebiotics	Raffinose	RAF	1.8%
	Saccharicter-penin	SAC	
	Inulin	INU	
Plant extracts	Turmeric	CUR1	0.06%
		CUR2	0.1%
		CUR3	0.6%
	Green tea	GT1	0.06%
		GT2	0.1%
		GT3	0.6%
	Garlic	G1	0.25%
		G2	0.5%
		G3	1%

Data illustration and statistical analysis:

The average absorbance from triplicate wells, was used to graph the growth curves using GraphPad Prism 9.5.0. The triplicate data of each treatment was applied to the Gompertz model using “nls” function in R software (4.3.1 version) to obtain maximum OD/growth, maximum growth rate, and lag time. The growth parameters of each treatment group were compared using one-way ANOVA test followed by Tukey’s honest significant difference (HSD) test in R software (4.3.1 version) to identify significant differences at *P*- value <0.05.

In-vitro selection of the most potent anti-pathogenic (anti-Salmonella and anti-Campylobacter) probiotic strain (Experiment 2)

Pathogenic strains:

The pathogenic strains (Table 5) were used in the anti-pathogenic assays of this experiment.

Table 5: Pathogenic strains used in the anti-pathogenic assays

Pathogenic strain	Origin
Salmonella enterica subsp. Enterica serovar Typhimurium (DPC6463)	Chicken
Salmonella enterica subsp. Enterica serovar Typhimurium (ATCC 14028)	
Salmonella enterica subsp. Enterica serovar Braenderup (NRL-IE-22)	
Campylobacter jejuni DVI-SC181	

Anti-Salmonella - Spot overlay assays:

Probiotic strains were cultured in MRS broth (BD Difco 288130, Fisher Scientific, Ireland) as described in the "pre-handling of the probiotics strains" section. MRS agar (1.5%) plates were spotted with 5 mL of each probiotic culture, labelled and allowed to air dry. These plates were then, incubated at 37°C overnight. Simultaneously, the cultures of the three *Salmonella enterica* strains were cultured. Briefly, a loop full from the stocks was streaked on Brain Heart Infusion (BHI) (1.10493 Merck) agar plates and these plates were then, incubated aerobically at 37°C for 24 h. Single colonies were then, inoculated into 10 mL of BHI broth and incubated for 16 h at 37°C under aerobic conditions. Once the overnight probiotic spots were ready, 30 mL of BHI molten and then, cooled (at 50°C) agar (0.75%) was inoculated with 15 µL of the *Salmonella* cultures prepared as above. The molten agar inoculated with the separate *Salmonella strains* was then, gently mixed and was used to overlay the plates containing the overnight probiotic spots. These plates were further, incubated at 37°C overnight. Four measurements of the radius of the zone of inhibition around the probiotic spots were measured perpendicularly (in mm) and averaged. The spot overlay assay was performed in triplicate.

Anti-Salmonella - Well diffusion assays (WDAs):

The three probiotic strains which displayed the highest inhibition of all three *Salmonella* strains were used in the WDAs. Briefly, the overnight cultures of the selected strains were prepared as described in the "pre-handling of the probiotic strains" section. These cultures were then, centrifuged at 4,000 g for 15 min at 4°C. The supernatant was carefully obtained and filter sterilized using 0.2 μm syringe filters. Simultaneously, *Salmonella* Typhimurium (DPC6463) culture was prepared as described in "anti-*Salmonella* - spot overlay assays" section and 25 μL of this culture was then, inoculated in 50 mL of BHI molten and cooled (at 50°C) agar (1%). The inoculated molten agar was gently mixed and poured into a sterile petri dish and allowed to set for 20 min. Through the use of sterile pipette tips (1000 μL), wells of approximately 7 mm in diameter were created in the inoculated agar aseptically. Each well was filled with 100 μL of probiotic culture supernatants and labelled. As a negative control, sterile MRS broth (pH adjusted to 4) was used in one well. The plates were kept stationary on the laminar flow hood approximately for 30 minutes until no displacement of the liquid in the wells was observed upon moving to the incubator. These plates were then, incubated at 37°C for 16 h. The radius of the zone of inhibition around the wells were recorded as described in the "anti-*Salmonella* - spot overlay assay" section. The WDA was performed in triplicate.

Anti-Salmonella - Co-culture assays:

To co-culture each probiotic strain and *Salmonella* Typhimurium DPC6463, a special culture medium was prepared by mixing double strength (prepared by dissolving twice the recommended amount of the media in the required volume of water) BHI and MRS broths in equal volume. The probiotic (three strains selected from the spot overlay assays) and *Salmonella* Typhimurium inoculums were prepared as described in the "pre-handling of the probiotic strains" and the "anti-*Salmonella* - spot overlay assays" sections, respectively. The co-culture medium (10 mL) was then, inoculated with 100 μL of the respective probiotic and *Salmonella* Typhimurium culture and labelled accordingly. Each co-culture was performed in triplicate and all the cultures were incubated for 24 h at 37°C, aerobically. Through the use of the spot plate method, selective enumeration of *Salmonella* in the co-cultures was performed on *Salmonella* chromogen selective agar (CM1007, Oxoid, UK) at 0, 5, 10 and 24 h time points.

Anti-Salmonella - Co-aggregation assays:

Three selected probiotic strains and *Salmonella* Typhimurium DPC6463 culture were prepared as described in the "pre-handling of the probiotic strains" and the "anti-*Salmonella* - spot overlay assay" sections, respectively. These cultures were then, centrifuged at 4000 g for 15 min at 4°C to obtain the bacterial pellet. The pellet was washed with sterile phosphate buffered saline (PBS) twice and then, re-suspended in PBS to a concentration of 0.5 OD600. The OD600 measurements were obtained using BioTek Synergy HT microplate reader. An aliquot of 500 µL from each of those bacterial suspensions was individually, added into the wells of sterile flat bottom 48 well microtiter plate in triplicate. Additionally, 250 µL of each probiotic suspension was mixed with 250 µL of *Salmonella* suspension in triplicate. These suspension mixtures were mixed well by pipetting. The plate was then, incubated at 37°C, aerobically. At the end of 24 h of incubation, the OD600 reading of the wells was recorded using the microplate reader without shaking the plate. The co-aggregation ability of each probiotic strain was determined using the following formula (Balakrishna, 2013).

$$\text{Co} - \text{aggregation ability} = \left[1 - \left((2 \times \text{Am}) \div (\text{Al} + \text{As})\right)\right] \times 100$$

Where;
Am = OD_{600} of the probiotic and *Salmonella* suspension mixture

Al = OD_{600} of probiotic suspension alone

As = OD_{600} of *Salmonella* suspension alone

Anti-Campylobacter – Well Diffusion Assays (WDAs):

Campylobacter jejuni was inoculated in Mueller Hinton broth (BD 275730, Fisher Scientific, Ireland) supplemented with *Campylobacter* selective supplement (Skirrow) (SR0069E, Fisher Scientific, Ireland) according the manufacturer's directions. This inoculated broth was incubated under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) at 42°C for 48 h. The microaerophilic atmosphere was created inside a gas jar (volume: 2.5 L) by using a CampyGen™ 2.5 L Sachet (CN0025A, Oxoid). This *Campylobacter jejuni* culture (100 µL) was then, spread on Mueller Hinton agar (1.5%) plates (90 mm circular plates) and allowed to dry. Wells of approximately 5 mm in diameter were created, aseptically in *Campylobacter* spread agar using a sterile 200 µL pipette tip. Culture supernatants of all six probiotic strains (prepared as described in the "Anti-*Salmonella* - WDAs" section) were added to these wells (50 µL/well) in triplicate. After drying for approximately 30 min as described in the "Anti-

Salmonella - WDAs" section, the plates were incubated at 42°C for 24 h under microaerophilic conditions. The radius of the zone of inhibition by each probiotic was determined as described in the "Anti-*Salmonella* - WDAs" section.

Statistical analysis of data:

The ANOVA followed by Tukey's HSD test was performed using Statistica software (Version 14.0.0.15) to identify statistically significant differences among the means of the triplicate data. The significant differences between the treatments (with probiotic strains) were identified at *P*- value <0.05.

In-vivo validation of effects of in-ovo administration of selected probiotic and prophybiotic on gut health and production of broiler chickens (Experiment 3)

Preparation of probiotic (PB) injection:

Based on the anti-pathogenic assays (Experiment 2), *Leuconostoc mesenteroides* (LM) was selected as the probiotic strain for this protocol. Based on the growth curve assays (Experiment 1), LM reached its peak growth and entered the stationary phase of growth approximately 15 h after incubation. Therefore, in order to obtain maximum number of metabolically active cells for the *in-ovo* injection, LM culture was incubated for 15 h in MRS broth media (BD Difco 288130, Fisher Scientific, Ireland). The culture was then, centrifuged at 4,200 rpm for 20 min at 4˚C to obtain the bacterial pellet. The pellet was washed twice with and re-suspended in 0.9% NaCl physiological saline solution (Natrium Chloratum 0.9% Fresenius KabiPac, Fresenius Kabi, Poland) adjusting the OD600 to 0.0311 (using Thermo Scientific Multiskan FC plate reader) to obtain a cell density of approximately 5×10^6 CFU/mL. A 0.2mL amount from this suspension was used to inject into each egg of the probiotic (PB) treatment group delivering a 10^6 CFU/egg dose.

Preparation of prophybiotic (PPB) injection:

Based on the Experiment 1, garlic aqueous extract was selected as the phytobiotic and used at a dose of 0.5% (w/v) in combination with the selected probiotic, LM. Accordingly, the prophybiotic (PPB) injection was prepared by mixing the LM suspension and garlic aqueous extract at a 2:1 ratio. It was intended to deliver approximately the same amount of bacteria (10^6 CFU/egg) from the PPB treatment similar to the PB treatment. As the volume of injection

material was intended to be a constant (0.2 mL/egg) across all treatments and the volume of bacterial suspension in PPB injection mixture was 2/3 of the volume, the OD600 of the bacterial suspension for the PPB treatment was adjusted to a higher cell density (OD600 = 0.0326; corresponding to 7.5×10^6 CFU/mL) to deliver approximately 10^6 CFU of LM per an egg (similar dose to PB treatment). Similarly, in order to obtain 0.5% concentration (the selected dose from Experiment 1) of garlic in the final injection mixture (in which garlic extract is 1/3 of the total volume), a 1.5% garlic aqueous extract was prepared following the protocol described on the Table 2. A volume of 0.2mL for the final injection mixture was used to inject each egg in the PPB treatment group.

In-ovo experiment:

The incubation of ROSS 308 broiler chicken eggs (n = 400) was performed under standard conditions (Temperature: 37.5˚C and Relative Humidity: 55%) (Midi series I, Fest Incubators, Gostyń, Poland). On the 12^{th} day of incubation, equal number of eggs were randomly allocated into four groups (Table 6) after removing infertile eggs and dead embryos by candling.

Table 6: Experimental design - *in-ovo* experiment

Experimental group	*In-ovo* injection	Dose	Volume
Negative control (NC)	No injection	None	0.2 mL
Positive control (PC)	0.9% NaCl physiological saline solution	None	
Probiotic (PB)	*Leuconostock mesenteroides* B/00288	10^6 CFU/egg	
Prophybiotic (PPB)	*Leuconostock mesenteroides* B/00288 + garlic aqueous extract (in 2:1 ratio in the final volume)	LM: 10^6 CFU/egg + Garlic: 0.5% (w/v)	

Prior to all injections, eggs were disinfected with 70% ethanol and a hole in the egg shell at the site of the air cell (located by candling) was made using 20 G needles manually. Then,

the respective injections were performed with a 26 G needle inserted into the air cell space through this hole without damaging the inner membranes of the eggs. A drop of non-toxic glue (Elmer's school glue, Elmer's Products Inc., Ohio) was then, used to seal the hole in the egg shell. Incubation of these eggs was then, continued under standard conditions until the chicks were hatched.

Hatching and data collection:

Upon hatching, the hatchability of each group was recorded and the chicks were wing tagged for identification. When the chicks were dried well, 25 birds were randomly selected per group to measure the weight, length and the Pasgar score. The length from the tip of the beak to the tip of the middle toe of the straightened right leg was recorded as the chick length by placing the chick face down on a flat surface, as previously described by Sozcu and Ipek, (2015). Pasgar scoring was performed to assess the quality of ten out of the 25 randomly selected birds from each group, as described in the Lohmann breeder guide ("Lohmann Hatchery Guide,").

Rearing and data/sample collection:

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

The chickens were reared in four separate floor pens with deep litter, allocated to each *in-ovo* treatment group (one floor pen/group). All the pens were provided with electronically controlled uniform environmental conditions (temperature, lighting regime, air humidity). During the rearing period, broilers had unlimited access to water and were fed ad libitum with three feeds namely, starter (from day 1-21), containing 22.3% crude protein and 12.45 MJ/Kg metabolizable energy and grower (from day 22–28) and finisher (from day 29-35) both containing 20.2% crude protein and 13.01 MJ/Kg metabolizable energy. The individual body weights (32 birds/group) and feed disappearance per group were recorded weekly, and feed conversion ratio (FCR = Total feed consumed/Total weight gained) was calculated for each

group. Feces samples from each group (n= 8/group) were collected in early (one week old) and late (five weeks old) life stages to quantify the relative abundance of selected bacterial communities as a reflection of the gut microbiome.

On the 35th day, chickens (n=8/group; excluding the NC group) were slaughtered (after 10 h of fasting) by decapitation and then, suspended to bleed for approximately 90 s. As majority of gut microbiome of chickens is harbored in the ceca, the investigation on gut health was mainly focused on the microbiome, gene expression and histomorphology of the ceca. Accordingly, upon dissecting the carcasses, the luminal content of ceca was collected into sterile 5 mL micro-centrifuge tubes for microbial quantification. These tubes were transported in dry ice and frozen at −80°C until use. Additionally, the mucosal layer of the ceca was scraped using a glass slide and collected into tubes containing stabilization buffer (fix RNA: E0280, EURx, Poland) for gene expression analysis. These tubes were transported at room temperature and frozen (after removing fix RNA) at −80°C until use. The middle part of the cecum was directly preserved in Bouin's solution (HT101128, Sigma-Aldrich, Poland) for histology analysis.

Slaughter and meat quality analysis:

In order to investigate the effects of *in-ovo* stimulation protocols on the production parameters, a slaughter and meat quality analysis was performed after cooling the carcasses for 24 h. The ratio of the weight of chilled carcass including neck, abdominal fat, and edible giblets (gizzard, liver, and heart) to the live body weight was calculated as the carcass dressing percentage with giblets. The carcass dressing percentage without giblets was estimated using the same formula but without including the weight of edible giblets in the weight of the chilled carcass. The percentages of the breast muscle, leg muscle (thigh and drumstick), leg bones, giblets, and abdominal fat were calculated as a percentage of the chilled carcass weight including giblets. For meat quality analysis, the physiochemical properties such as pH, color, drip loss, thawing loss, cooking loss, shear force, and texture of the breast and thigh muscles were evaluated. This evaluation was carried out following the methods described by Połtowicz et al. (2015).

DNA and RNA extraction:

A GeneMATRIX Stool DNA Purification Kit (E3575, EURx, Poland) was used to extract DNA from the feces and cecal content samples by optimizing the manufacturer's protocol. To extract RNA from the cecal mucosa, the samples were homogenized in 1 mL of RNA extracol solution (E3700, EURx, Poland) using a TissueRuptor II homogenizer (990890, Qiagen, Poland). The tissue homogenates were then, mixed with 0.2 mL of chloroform (112344305, Chempur, Poland) and centrifuged (12000 g for 15 minutes at 4˚C) to isolate RNA in the supernatant. The isolated RNA was further purified using a Universal RNA purification kit (E3598, EURx, Poland) following the manufacturer's protocol. The quality and quantity of the extracted DNA and RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The integrity of the DNA and RNA was confirmed by performing electrophoresis on a 2% agarose gel. The DNA and RNA samples were stored at −80˚C until use.

Analysis of the relative abundance of bacteria:

The relative abundance of the selected bacterial communities (important biomarkers of gut health) was determined using a quantitative PCR (qPCR) method. In the fecal samples, *Lactobacillus* sp., *Bifidobacterium* sp. *Faecalibacterium* sp. and *Escherichia coli* and in the cecal contents, *Lactobacillus* sp., *Bifidobacterium* sp., *Prevotella* sp., *Akkermansia* sp., *Fecalibacterium* sp. and *Escherichia coli* were quantified. The relative abundance of each bacterial community was calculated relative to the quantity of universal bacteria in each sample.

The qPCR reaction mixture consisted of 6.25 μL of SG qPCR Master Mix (2x) (0401, EURx, Poland), 1 μM of each (forward and reverse) primer (Sigma-Aldrich, Germany) and 20 ng of DNA in a total volume of 12.5 μL. The qPCR reaction for each sample in duplicates was performed in 96 well plates (4TI-0955, AZENTA, Poland) using a LightCycler 480 II (Roche-Diagnostics, Switzerland). The protocol for qPCR included an initial denaturation at 95˚C for 5 min, followed by 40 amplification cycles of denaturation (at 95˚C for 10 s), annealing (at 58˚C for 15 s) and elongation (at 72˚C for 30 s) steps. The average Ct values of the 2 duplicate reactions were used for data analysis. Five dilutions (1x, 0.5x, 0.25x, 0.125x, and 0.0625x) of the pooled bacterial DNA (all the samples of a particular sample type were pooled across the treatment groups) were used to run a standard curve analysis for each primer pair using the

LightCycler 480 II software (Roche-Diagnostics). Then, the PCR efficiency for each primer pair was determined using the software. The primer sequences are listed in the Table 7. The relative abundances calculation was performed using the following formula as described in Slawinska et al. (2019);

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

E universal: qPCR Efficiency of universal bacteria primers
Ct universal: Ct value of qPCR reaction for universal bacteria
E target: qPCR Efficiency of target bacteria primers
Ct target: Ct value of qPCR reaction for target bacteria

Table 7: Primer sequences for determining the relative abundance of bacterial communities in the feces and luminal content of ceca via qPCR

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

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

Analysis of the expression of immune related genes:

The quantitative reverse transcription PCR (RT-qPCR) method was used to quantify the expression of immune related genes (coding for pro-inflammatory cytokines: *IL1-β*, *IL2*, *IL4*, *IL6*, pro-inflammatory chemokine: *IL8*, anti-inflammatory cytokine: *IL10*, free fatty acid receptor 2 (*FFAR2*), host defense peptides: *AVBD1* and *CATHL2* and barrier function related components: *MUC6* and *CLDN1*) in the cecal mucosa. The smART First Strand cDNA Synthesis Kit (0804, EURx, Poland) was used to reverse transcribe the RNA samples according to the manufacturer's protocol. The resulting complementary DNA (20 ng) was then used to perform qPCR in a reaction mixture as described in "Analysis of the relative abundance of bacteria" section. The protocol for qPCR included an initial denaturation at 95˚C for 15 min, followed by 40 amplification cycles of denaturation (at 95˚C for 15 s), annealing (at 58˚C for 30 s) and elongation (at 72˚C for 30 s) steps. All reactions were carried out in duplicate and the average Ct values were used in the data analysis. The gene expression of each target gene was calculated relative to the expression of the reference genes (*ACTB* and *G6PDH*) using ΔΔCt method (Livak and Schmittgen, 2001). The primer details are listed on Table 8.

Table 8: Primer sequences for determining the relative gene expression in cecal mucosa via RT-qPCR

Gene name	Gene symbol	Primer sequence[1] (5'→ 3')	Reference
Actin, beta	*ACTB*	F: CACAGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	(Sevane et al., 2014)
Glucose-6-Phosphate Dehyfrogenase	*G6PDH*	F: CGGGAACCAAATGCACTTCGT R: GGCTGCCGTAGAGGTATGGGA	(Sevane et al., 2014)
Avian beta-defensin 1	*AVBD1*	F: AAACCATTGTCAGCCCTGTG R: TTCCTAGAGCCTGGGAGGAT	(Sławinska et al., 2019)
Cathelicidin 2	*CATHL2*	F: AGGAGAATGGGGTCATCAGG R: GGATCTTTCTCAGGAAGCGG	(Sławinska et al., 2019)
Claudin 1	*CLDN1*	F: TCTTCATCATTGCAGGTCTGTC R: AACGGGTGTGAAAGGGTCAT	(Sławinska et al., 2019)
	FFAR2	F: GCTCGACCCCTTCATCTTCT	

Free fatty acid receptor 2		R: ACACATTGTGCCCCGAATTG	(Sławinska et al., 2019)
Interleukin 1 beta	*IL1-β*	F: GGAGGTTTTTGAGCCCGTC R: TCGAAGATGTCGAAGGACTG	(Dunislawska et al., 2017)
Interleukin 2	*IL2*	F: GCTTATGGAGCATCTCTATCATCA R: GGTGCACTCCTGGGTCTC	(Pietrzak et al., 2020)
Interleukin 6	*IL6*	F: AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT	(Chiang et al., 2009)
Interleukin 8	*IL8*	F: AAGGATGGAAGAGAGGTGTGCTT R: GCTGAGCCTTGGCCATAAGT	(Sławinska et al., 2014)
Interleukin 10	*IL10*	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG	(Rothwell et al., 2004)
Mucin 6	*MUC6*	F: TTCAACATTCAGTTCCGCCG R: TTGATGACACCGACACTCCT	(Sławinska et al., 2019)

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

Analysis of Cecal Histology:

Assessment of the cecal histomorphology was performed using the paraffin technique as described by Bogucka et al. (2016). Briefly, the middle part of the ceca which were preserved in Bouin's solution were sliced into approximately 1 cm lengths. These tissue pieces were subsequently, dehydrated, cleared, and infiltrated with paraffin by incubating in an automatic tissue processor (Microm STP 120, Thermo Shandon, United Kingdom) overnight. Next, the processed tissues were manually embedded into paraffin blocks in a transfer station (TES 99, Medite, Burgdorf, Germany). Sections (7 µm) of these paraffin embedded tissues were then, cut using a rotational microtome (Finesse ME+, Thermo Shandon, United Kingdom) and adhered to glass slides which were covered with egg white and glycerin. These slides were then, de-waxed and hydrated before the staining. The periodic acid-Schiff (PAS) staining was performed on microscopic preparations as described by Dubowitz et al. (1973). Microscopic

images of the stained tissues were captured by using Evolution 300 microscope (Delta Opitcal, Poland) equipped with a digital camera (ToupCam, TP605100A, ToupTek, China). Ten measurements of villus height and width and crypt depth were taken per a chicken using the Multiscan 18.03 microscopic images software (Computer Scanning Systems II, Poland). The villus height to crypt depth ratio (VH/CD) was calculated for each bird. The surface area of the villi was also calculated according to the formula of Sakamoto et al. (2000).

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

VW= villus width

VH = villus height.

Statistical analysis of data:

The outliers of data (values which are greater than Quartile 3 + 1.5 × interquartile range and below Quartile 1 + 1.5 × interquartile range) were removed before the statistical analysis. Assumption of normality was tested by the Shapiro-Wilk test and equal variances were tested by Levene's test. A linear mixed model was used to analyze the production, meat quality, bacterial abundance and histology data where treatment effect was the fixed effect and the sex of the bird was a random effect (to account for possible confounding variation due to sex). The model was fitted using the "lmer" function of the "lme4" package in R (version 4.3.1) and Wald chi square test was performed to test the significance of the fixed effect. Tukey's HSD test was then, performed to identify the significantly different means (*P*- value < 0.05). In the cases where the assumptions were not met, non-parametric analysis, Kruskal-Wallis test followed by Dunn's test was performed to identify the significantly different means (*P*- value < 0.05). For the gene expression analysis, two sample T test was performed in R (version 4.3.1) to compare the changes in expression of each treatment group compared to the positive control group (*P*-value < 0.05).

3.4 Results

In-vitro selection of compatible bioactives (Synbiotics and Prophybiotics) (Experiment 1)

Selection of compatible symbiotics:

The results of the growth kinetics studies on the six selected probiotics after individual supplementation with three selected prebiotics as the sole energy source in cfMRS media are displayed on Figure 3 and Table 9. *L. casei, L. rhamnosus,* and *P. pentosaceus* exhibited poor growth on Raffinose as the sole energy source, indicating it is not a preferred substrate for these strains. In contrast, *L. plantarum, L. reuteri,* and *L. mesenteroides* demonstrated significant growth with Raffinose, albeit with reduced maximum growth and growth rates, and increased lag time compared to the control. Additionally, Inulin was efficiently utilized by *L. casei,* with all growth parameters statistically similar to the control. However, the other five probiotic strains showed significantly lower growth with Inulin compared to the control. Additionally, *L. mesenteroides,* when supplemented with Saccharicterpenin, exhibited statistically similar maximum growth/OD and lag time to the control and a higher maximum growth rate when compared with the control. *L. plantarum* also showed a higher maximum growth rate with Saccharicterpenin, although it did not reach the control's maximum growth/OD. Other strains did not utilize Saccharicterpenin effectively.

These findings suggest that Saccharicterpenin with *L. plantarum* or *L. mesenteroides*, and Inulin with *L. casei*, could be compatible combinations for complementary synbiotics. In contrast, *L. rhamnosus* and *P. pentosaceus* did not show compatibility with any of the tested prebiotics.

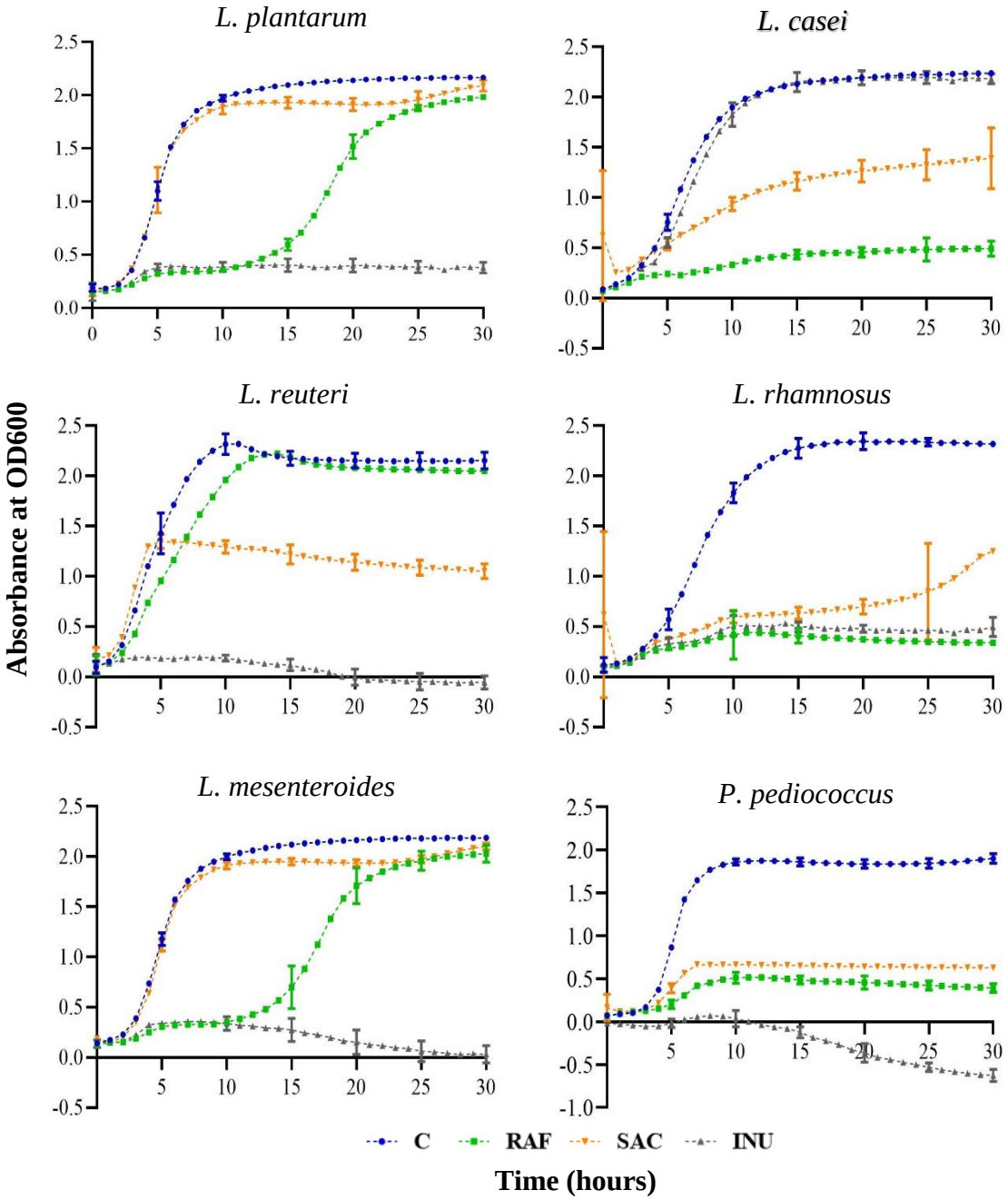

Figure 4**:** Growth kinetics for six probiotic strains in the cfMRS media supplemented with different commercial prebiotics (1.8% w/v). C: Control supplemented with glucose. RAF: Supplemented with Raffinose. SAC: Supplemented with Saccharicterpenin. INU: Supplemented with Inulin. Error bars: 95% confidence interval

Table 9: The growth parameters (mean ± SD) of probiotic strains when different prebiotics were used (C: Glucose - Control, RAF: Raffinose, INU: Inulin and SAC: Saccharicterpenin) as the sole energy source.

Probiotic strain	C	RAF	INU	SAC	Significance
	Max OD/Growth				
L. plantarum	2.13 ± 0.01^{a}	2.03 ± 0.01^{b}	NA	1.95 ± 0.02^{c}	***
L. casei	2.21 ± 0.01^{a}	NA	2.19 ± 0.03^{a}	1.36 ± 0.09^{b}	***
L. reuteri	2.12 ± 0.03^{a}	2.11 ± 0.00^{b}	NA	NA	*
L. mesenteroides	2.15 ± 0.01^{a}	2.05 ± 0.03^{b}	NA	1.97 ± 0.01^{a}	***
	Max growth rate				
L. plantarum	1.07 ± 0.03^{b}	0.52 ± 0.01^{c}	NA	1.14 ± 0.02^{a}	***
L. casei	0.80 ± 0.01^{a}	NA	0.83 ± 0.04^{a}	0.22 ± 0.01^{b}	***
L. reuteri	1.29 ± 0.06^{a}	0.84 ± 0.0^{b}	NA	NA	***
L. mesenteroides	1.07 ± 0.01^{b}	0.60 ± 0.02^{c}	NA	1.20 ± 0.01^{a}	***
	Lag time				
L. plantarum	2.68 ± 0.08^{b}	13.58 ± 0.27^{a}	NA	2.75 ± 0.12^{b}	***
L. casei	2.70 ± 0.14	NA	3.68 ± 0.05	2.32 ± 1.6	NS
L. reuteri	1.96 ± 0.04^{b}	2.39 ± 0.02^{a}	NA	NA	***
L. mesenteroides	2.41 ± 0.04^{b}	12.89 ± 0.56^{a}	NA	2.86 ± 0.05^{b}	***

Notes: Significance codes: *P*- value >0.0001 '***' / > 0.001 '**' / >0.01 '*' / Not Significant 'NS'
The alphabetical order of superscripts indicate the statistically different (Tuckey HSD) means of treatment levels in descending order. 'NA' indicates the treatments where growth of the probiotic was not observed (growth data were not fitted to Gompertz model)

Selection of compatible prophybiotics:

Supplementation of Turmeric extract:

The results of growth kinetics of the six probiotic strains supplemented with different concentrations of the Turmeric extract in the MRS media are shown on Table 10 and Figure 4. The turmeric extract exhibited prebiotic effects on *L. mesenteroides*. A higher maximum growth/OD and growth rate and lower lag time was observed for *L. mesenteroides* when supplemented with turmeric extract at the highest concentration. Overall, 0.06% and 0.1% turmeric extract did not substantially impair the growth parameters of the six probiotic strains.

Thus, turmeric extract at a 0.1% concentration was identified as a suitable candidate and dose for prophybiotic formulations with all six probiotic strains studied, while a 0.6% dose can be used with *P. pentosaceus* and *L. mesenteroides*.

Table 10: The growth parameters (mean ± SD) of probiotic strains when different levels of turmeric extract (C: Zero turmeric - Control, CUR1: 0.06%, CUR2: 0.1% and CUR3: 0.6%) were supplemented to the MRS broth media.

Probiotic strain	C	CUR1	CUR2	CUR3	Significance
	Max OD/Growth				
L. plantarum	2.19 ± 0.01^{a}	2.17 ± 0.01^{a}	2.17 ± 0.01^{a}	2.11 ± 0.02^{b}	***
L. casei	2.5 ± 0.04^{a}	2.37 ± 0.04^{b}	2.32 ± 0.02^{b}	2.35 ± 0.08^{b}	**
L. reuteri	1.96 ± 0.06	1.91 ± 0.03	1.93 ± 0.02	1.88 ± 0.01	NS
L. rhamnosus	2.25 ± 0.03^{a}	2.26 ± 0.01^{a}	2.28 ± 0.00^{a}	2.08 ± 0.06^{b}	***
L. mesenteroides	0.85 ± 0.03^{b}	0.86 ± 0.00^{b}	0.92 ± 0.12^{ab}	1.06 ± 0.00^{a}	**
P. pentosaceus	1.78 ± 0.09	1.77 ± 0.07	1.85 ± 0.01	1.85 ± 0.03	NS
	Max growth rate				
L. plantarum	0.94 ± 0.04^{a}	0.97 ± 0.03^{a}	0.97 ± 0.05^{a}	0.59 ± 0.01^{b}	***
L. casei	0.24 ± 0.01^{b}	0.26 ± 0.01^{a}	0.27 ± 0.01^{a}	0.20 ± 0.01^{c}	***
L. reuteri	0.24 ± 0.06^{a}	0.26 ± 0.01^{a}	0.27 ± 0.05^{a}	0.20 ± 0.01^{b}	**
L. rhamnosus	0.62 ± 0.01	0.62 ± 0.00	0.59 ± 0.03	0.64 ± 0.02	NS
L. mesenteroides	0.26 ± 0.01^{b}	0.27 ± 0.00^{ab}	0.27 ± 0.01^{ab}	0.28 ± 0.00^{a}	*
P. pentosaceus	1.04 ± 0.07	0.99 ± 0.02	1.03 ± 0.03	1.11 ± 0.61	NS
	Lag time				
L. plantarum	1.10 ± 0.06^{b}	1.43 ± 0.09^{b}	1.43 ± 0.20^{b}	2.49 ± 0.20^{a}	***
L. casei	5.76 ± 0.62^{a}	5.63 ± 0.24^{a}	5.92 ± 0.51^{a}	1.57 ± 1.23^{b}	***
L. reuteri	1.87 ± 0.08	2.10 ± 0.11	2.17 ± 0.39	2.10 ± 0.01	NS
L. rhamnosus	2.82 ± 0.37^{b}	3.39 ± 0.18^{b}	2.99 ± 0.7^{b}	4.71 ± 0.10^{a}	**
L. mesenteroides	8.68 ± 0.30	8.48 ± 0.00	8.51 ± 0.04	8.56 ± 0.00	NS
P. pentosaceus	2.65 ± 0.13^{a}	2.70 ± 0.03^{a}	2.67 ± 0.12^{a}	2.40 ± 0.11^{b}	*

Notes: Significance codes: *P*- value >0.0001 '***' / > 0.001 '**' / >0.01 '*' / Not Significant 'NS'

The alphabetical order of superscripts indicate the statistically different (Tuckey HSD) means of treatment levels in descending order

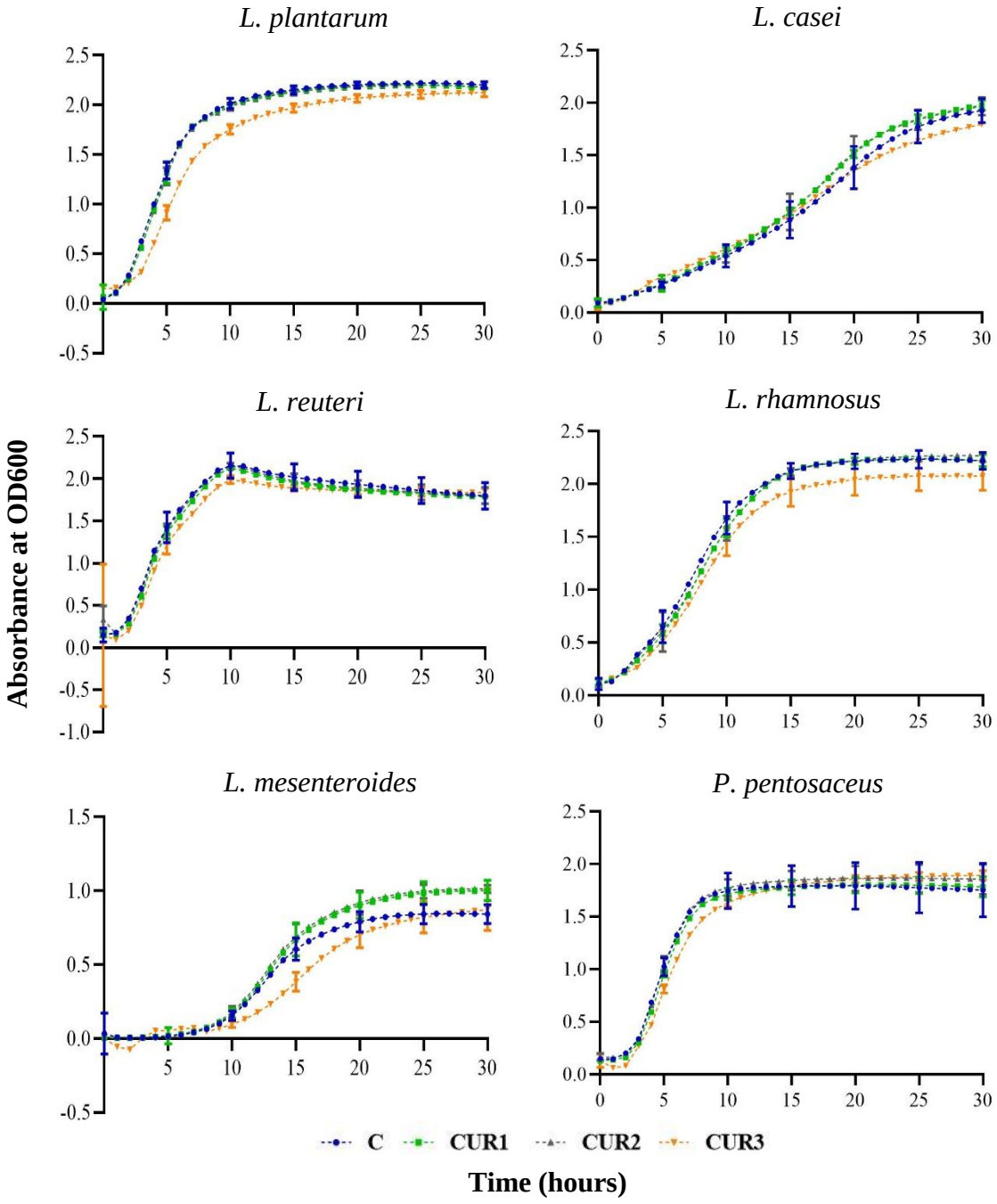

Figure 4: Growth kinetics for six probiotic strains in MRS media supplemented with different concentrations of turmeric extract. C: Control media without any turmeric extract. CUR1: Supplemented with 0.06% (w/v) turmeric extract. CUR2: Supplemented with 0.1% (w/v) turmeric extract. CUR3: Supplemented with 0.6% (w/v) turmeric. Error bars: 95% confidence interval

Supplementation of Green tea extract:

The results of the growth kinetics of six probiotic strains supplemented with different doses of green tea extract are shown on Figure 5 and Table 11.

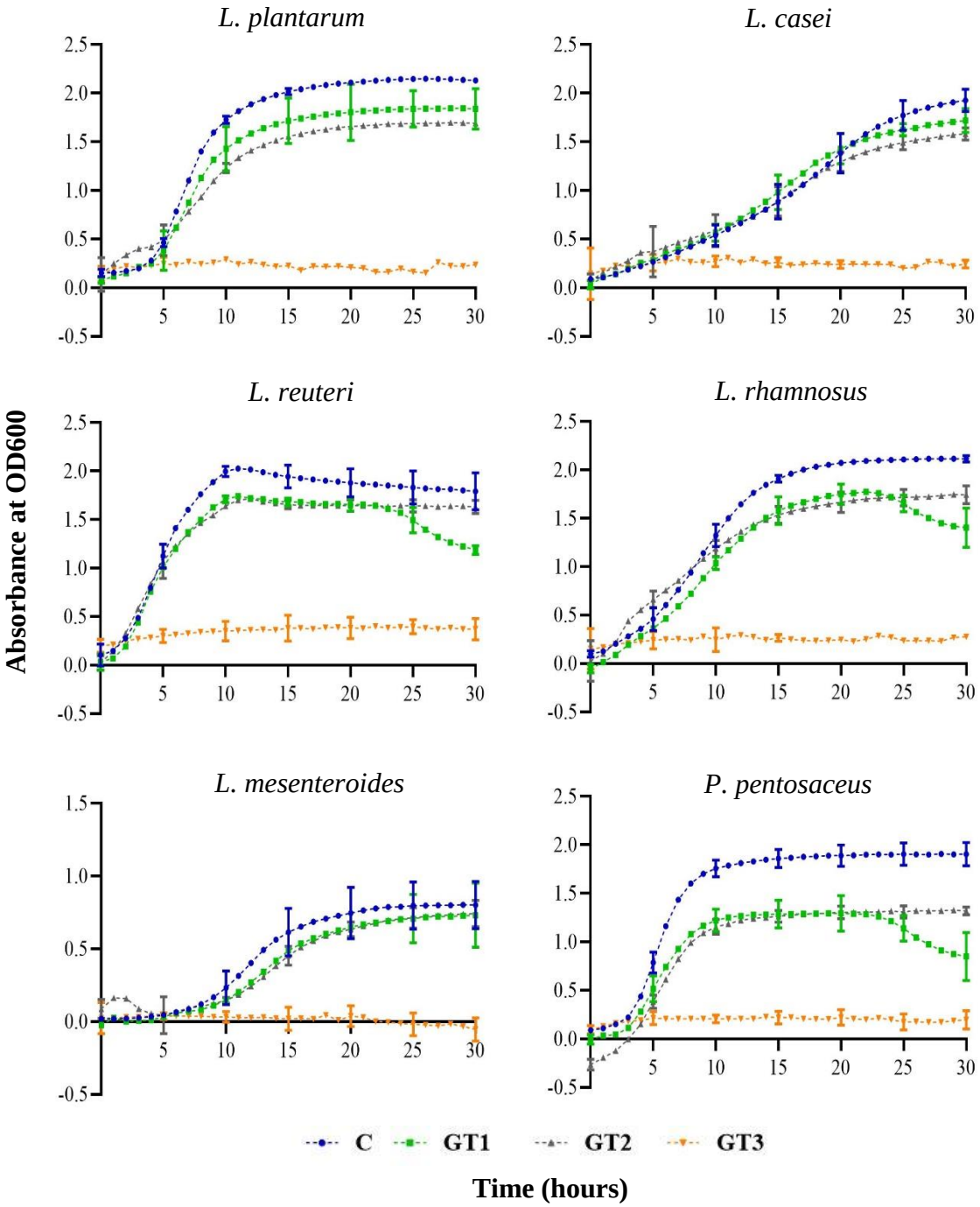

Figure 5: Growth kinetics for six probiotic strains in MRS media supplemented with different concentrations of green tea extract. C: Control media without any green tea extract supplementation. GT1: Supplemented with 0.06% (w/v) green tea extract. GT2: Supplemented with 0.1% (w/v) green tea extract. GT3: Supplemented with 0.6% (w/v) green tea extract. Error bars: 95% confidence interval

36:30853681

Table 11: The growth parameters (mean ± SD) of probiotic strains when different levels of green tea extract (C: Zero green tea – Control, GT1: 0.06%, GT2: 0.1%) were supplemented to the MRS broth media.

LAB	C	GT1	GT2	Significance
	Max OD/Growth			
L. plantarum	2.12 ± 0.01^{a}	1.82 ± 0.00^{b}	1.70 ± 0.01^{c}	***
L. casei	2.50 ± 0.04^{a}	1.93 ± 0.03^{b}	1.93 ± 0.09^{b}	***
L. reuteri	1.9 ± 0.06^{a}	1.57 ± 0.04^{b}	1.66 ± 0.02^{b}	***
L. rhamnosus	2.13 ± 0.01^{a}	1.66 ± 0.01^{c}	1.77 ± 0.03^{b}	***
L. mesenteroides	0.76 ± 0.00	0.74 ± 0.04	0.74 ± 0.04	NS
P. pentosaceus	1.88 ± 0.04^{a}	1.19 ± 0.03^{c}	1.31 ± 0.02^{b}	***
	Max growth rate			
L. plantarum	0.79 ± 0.01^{a}	0.62 ± 0.01^{b}	0.41 ± 0.03^{c}	***
L. casei	0.24 ± 0.01^{a}	0.24 ± 0.00^{a}	0.18 ± 0.02^{b}	**
L. reuteri	1.06 ± 0.06^{a}	0.94 ± 0.04^{a}	0.75 ± 0.03^{b}	***
L. rhamnosus	0.54 ± 0.01^{a}	0.48 ± 0.01^{b}	0.37 ± 0.01^{c}	***
L. mesenteroides	0.76 ± 0.00^{a}	0.74 ± 0.00^{b}	0.74 ± 0.01^{b}	***
P. pentosaceus	0.97 ± 0.2^{a}	0.78 ± 0.02^{b}	0.63 ± 0.01^{c}	***
	Lag time			
L. plantarum	3.80 ± 0.02	3.67 ± 0.21	3.10 ± 0.69	NS
L. casei	3.70 ± 2	4.56 ± 0.65	2.54 ± 1.75	NS
L. reuteri	2.31 ± 0.25^{a}	2.01 ± 0.12^{a}	0.93 ± 0.61^{b}	*
L. rhamnosus	3.79 ± 0.45^{a}	3.90 ± 0.71^{a}	-1.92 ± 0.20^{b}	**
L. mesenteroides	7.62 ± 0.00^{b}	7.99 ± 0.86^{b}	9.65 ± 1.13^{a}	*
P. pentosaceus	3.06 ± 0.05^{b}	3.28 ± 0.10^{a}	2.28 ± 0.16^{c}	***

Notes: Significance codes: *P*- value >0.0001 '***' / > 0.001 '**' / >0.01 '*' / Not Significant 'NS'
The alphabetical order of superscripts indicate the statistically different (Tuckey HSD) means of treatment levels in descending order.

The green tea extract displayed inhibitory effects on the growth of all six probiotic strains, with the highest concentration (0.6%) causing a significant inhibition (*L. plantarum* − 89%, *L. casei* − 70%, *L. reuteri* − 80%, *L. rhamnosus* − 86%, *L.*

mesenteroides − 97%, and *P. pentosaceus* − 88% at 15 h). Therefore, the growth data from GT3 treatment (the highest concentration: 0.6%), did not fit the Gompertz curve thus, have been excluded from the statistical analysis. *L. casei* however, showed the highest resistance to inhibition with 12% more growth on 0.06% green tea supplementation, compared to the control. Nevertheless, due to the overall inhibitory effects, green tea extract was not considered suitable for prophybiotic formulations.

Supplementation of Garlic aqueous extract:

The results of growth kinetics of the six probiotic strains with the supplementation of different concentrations of garlic aqueous extract in MRS media are shown on Figure 6 and Table 12. The results demonstrate the promising potential of garlic aqueous extract in prophybiotic formulations with the probiotic strains studied. In particular, it exhibited prebiotic effects on *L. reuteri* and *P. pentosaceus*, enhancing their maximum growth when supplemented at the two higher doses (0.5% and 1%) compared to the controls. However, the highest dose (1%) displayed a significant lower maximum growth of several strains (*L. plantarum, L. casei, L. rhamnosus* and *L. mesenteroides*). Importantly, a 0.5% concentration displayed overall non-inhibitory effects across all strains. Therefore, garlic extract at a concentration of 0.5% was identified as a suitable candidate and dose for potential prophybiotic formulation with all six probiotic strains studied.

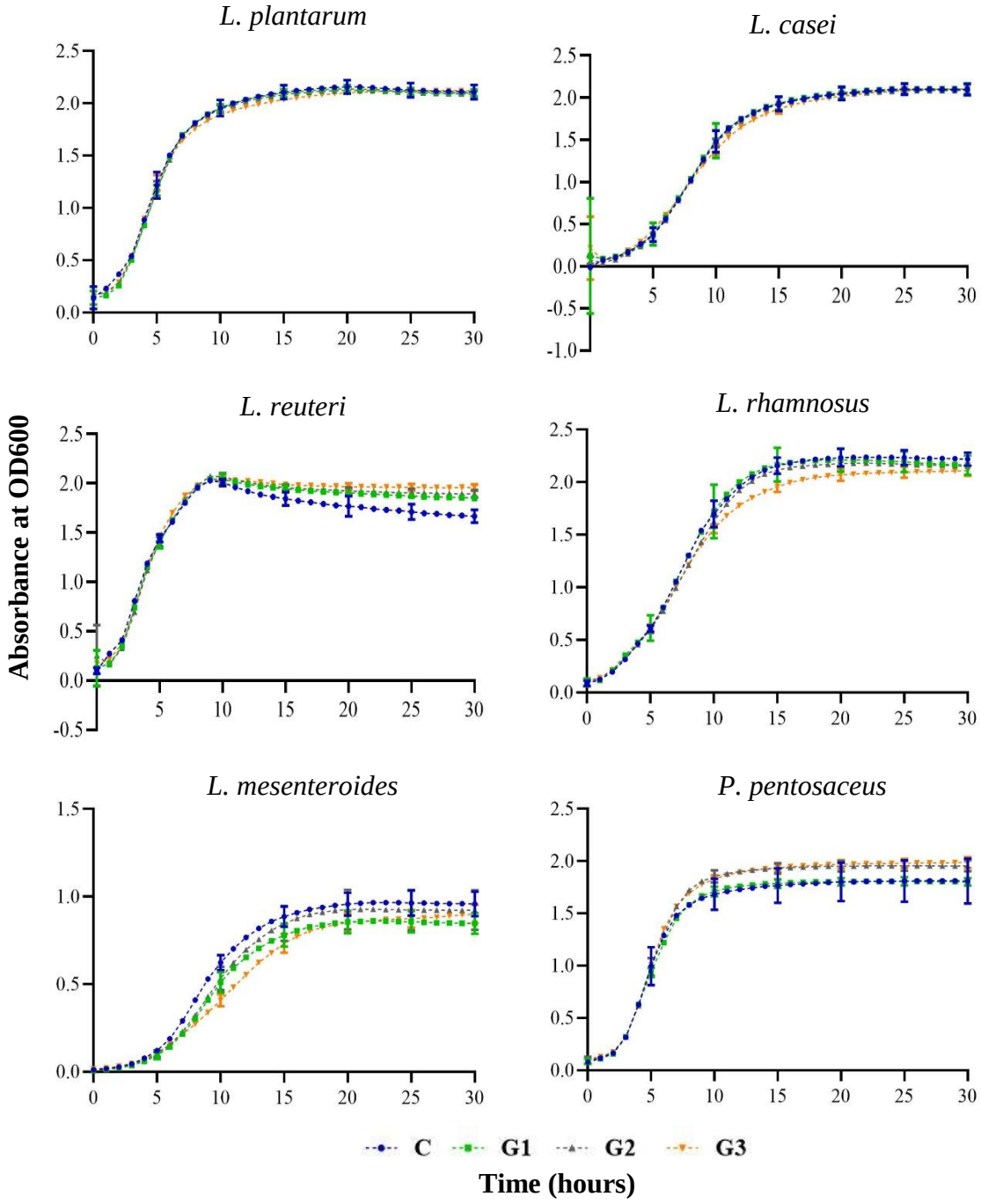

Figure 6: Growth kinetics for six probiotic strains in MRS media supplemented with different concentrations of garlic extract. C: Control media without any garlic extract supplementation. G1: Supplemented with 0.25% (w/v) garlic extract. G2: Supplemented with 0.5% (w/v) garlic extract. G3: Supplemented with 1% (w/v) garlic extract. Error bars: 95% confidence interval

Table 12: The growth parameters (mean ± SD) of probiotic strains when different levels of garlic extract (C: Zero garlic- Control, G1: 0.25%, G2: 0.5% and G3: 0.1%) were supplemented to the MRS broth media.

LAB	C	G1	G2	G3	Significance
	Max OD/Growth				
L. plantarum	2.13 ± 0.03	1.95 ± 0.28	2.10 ± 0.01	2.09 ± 0.02	NS
L. casei	2.09 ± 0.03	2.10 ± 0.02	2.08 ± 0.01	2.09 ± 0.02	NS
L. reuteri	1.81 ± 0.03^{c}	1.92 ± 0.01^{b}	1.95 ± 0.01^{ab}	1.99 ± 0.01^{a}	***
L. rhamnosus	2.25 ± 0.03^{a}	2.22 ± 0.02^{a}	2.19 ± 0.01^{a}	2.11 ± 0.03^{b}	***
L. mesenteroides	0.97 ± 0.03^{a}	0.86 ± 0.02^{ab}	0.94 ± 0.05^{a}	0.90 ± 0.03^{a}	*
P. pentosaceus	1.79 ± 0.07^{b}	1.80 ± 0.01^{b}	1.94 ± 0.02^{a}	1.96 ± 0.02^{a}	***
	Max growth rate				
L. plantarum	0.86 ± 0.01^{b}	0.93 ± 0.01^{a}	0.90 ± 0.01^{a}	0.82 ± 0.01^{c}	***
L. casei	0.64 ± 0.01^{a}	0.65 ± 0.01^{a}	0.65 ± 0.01^{a}	0.55 ± 0.01^{b}	***
L. reuteri	1.25 ± 0.04	1.21 ± 0.02	1.24 ± 0.06	1.23 ± 0.03	NS
L. rhamnosus	0.68 ± 0.02^{a}	0.71 ± 0.00^{a}	0.66 ± 0.02^{a}	0.57 ± 0.04^{b}	***
L. mesenteroides	0.31 ± 0.01^{a}	0.27 ± 0.01^{b}	0.29 ± 0.02^{ab}	0.21 ± 0.01^{c}	***
P. pentosaceus	0.93 ± 0.03^{b}	0.90 ± 0.01^{bc}	1.00 ± 0.01^{a}	0.97 ± 0.01^{ab}	***
	Lag time				
L. plantarum	1.62 ± 0.01^{b}	1.91 ± 0.06^{a}	1.92 ± 0.11^{a}	1.45 ± 0.04^{b}	***
L. casei	3.81 ± 0.10	4.01 ± 0.38	3.93 ± 0.06	3.59 ± 0.35	NS
L. reuteri	1.65 ± 0.08	1.71 ± 0.03	2.01 ± 0.29	1.86 ± 0.12	NS
L. rhamnosus	3.16 ± 0.06^{b}	3.37 ± 0.04^{a}	3.39 ± 0.09^{a}	2.65 ± 0.08^{c}	***
L. mesenteroides	4.58 ± 0.06^{b}	5.08 ± 0.02^{a}	5.19 ± 0.17^{a}	4.89 ± 0.16^{ab}	**
P. pentosaceus	2.37 ± 0.08^{c}	2.42 ± 0.03^{bc}	2.62 ± 0.05^{a}	2.53 ± 0.05^{ab}	**

Notes: Significance codes: *P*- value >0.0001 '***' / > 0.001 '**' / >0.01 '*' / Not Significant 'NS'
The alphabetical order of superscripts indicate the statistically different (Tuckey HSD) means of treatment levels in descending order

Based on above results, the effect of prebiotics seemed strain specific whereas the phytobiotics (turmeric and garlic extracts) seemed to be non-inhibitory for all the probiotics studied. Considering these results and the inherent benefits of phytobiotics on gut health, prophybiotic application was selected for the *in-ovo* stimulation protocol.

In-vitro selection of the most potent anti-pathogenic probiotic strain (Experiment 2)

Anti-Salmonella assays:

Spot overlay assays:

The results of the probiotic spot overlay assays against the three *Salmonella enterica* serovars are displayed on Figure 7. The probiotic strains *L. rhamnosus*, *L. mesenteroides*, and *P. pentosaceus* exhibited the most significant anti-Salmonella activity, overall. Therefore, these three probiotic strains were selected for further anti-Salmonella assays.

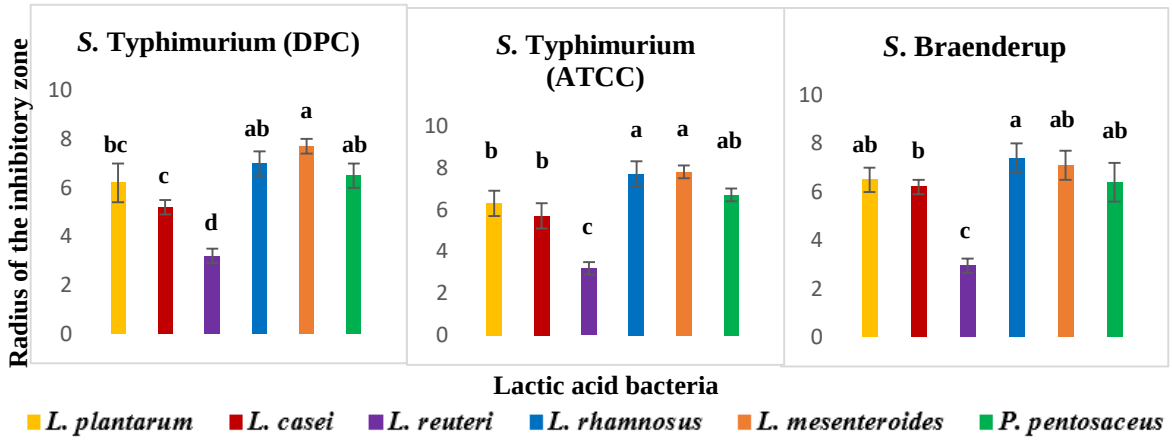

Figure 7: Radius of the inhibitory zone (mm) observed in spot overlay assays against three *Salmonella enterica* serovars. Error bars: ± SD. Homogenous means have been indicated by similar letters identified by Tukey's HSD test (*P*- value <0.05)

Well diffusion assay:

The results of the well diffusion assay against *Salmonella* Typhimurium DPC6463 strain, are displayed in Figure 8. The three selected probiotic strains displayed equally potent inhibition against *Salmonella* Typhimurium DPC6463.

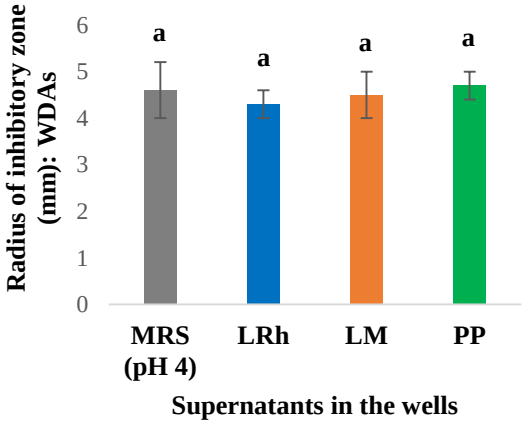

Figure 8: Radius of inhibitory zone (mm) observed in well diffusion assays against *Salmonella* Typhimurium DPC6463. LRh: *L. rhamnosus*, LM: *L. mesenteroides*, PP: *P. pentosaceus* Error bars: ±SD. Homogenous means indicated by similar letters identified by Tukey's HSD test (*P*- value <0.05)

Co-culture assay:

The results of the co-culture assay of each of the three probiotic strains selected with *Salmonella* Typhimurium DPC6463 is displayed in Figure 9. The number of colony forming units (CFUs) of *Salmonella* Typhimurium in the presence of each of the probiotic was markedly reduced compared to those in the control medium. Remarkably, no *Salmonella* Typhimurium colonies were detected in the co-culture at 24 hours, indicating a complete eradication of *Salmonella* Typhimurium by these probiotics. The three probiotic strains displayed equally potent bactericidal properties against *Salmonella* Typhimurium DPC6463.

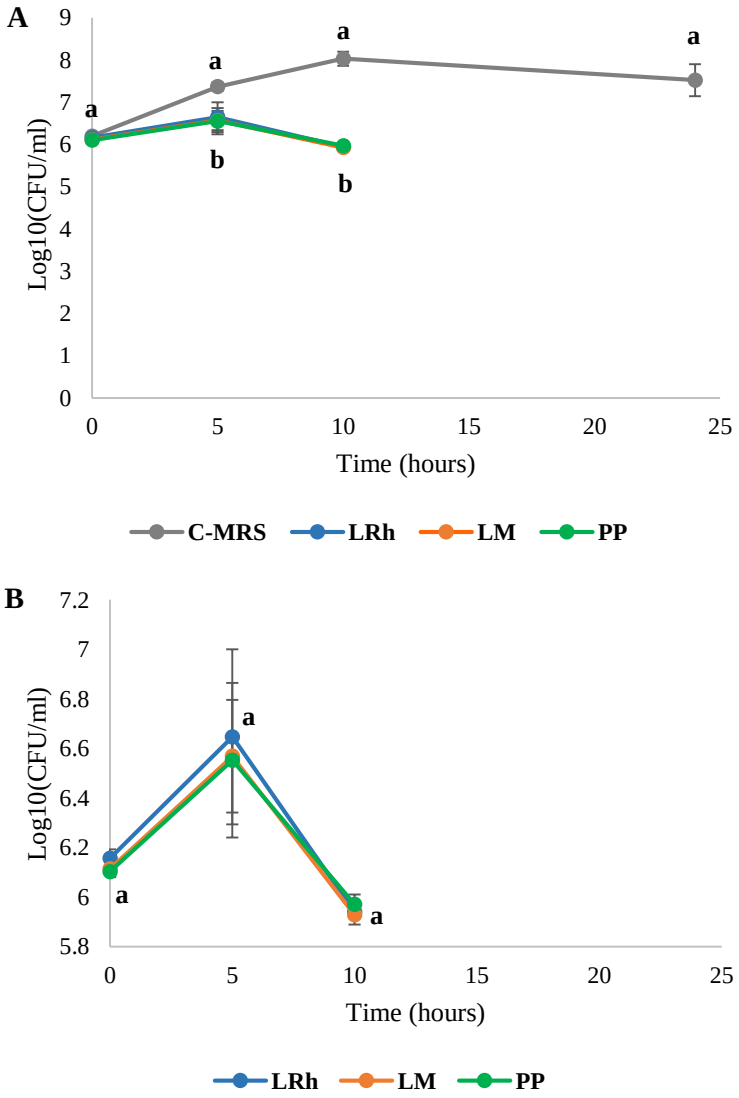

Figure 9: Selective enumeration of *Salmonella* Typhimurium DPC6463 in co-culture. A: Comparison of growth of *Salmonella* in the presence and absence of LABs. B: Comparison of growth of *Salmonella* in co-culture with different LAB. C-MRS: Control media (MRS+BHI), LRh: *L. rhamnosus*, LM: *L. mesenteroides*, PP: *P. pentosaceus*. Error bars: ±SD. Homogenous means indicated by similar letters: Tukey's HSD test (*P*- value <0.05)

Co-aggregation assay:

The results of the co-aggregation assay of the three selected probiotics with *Salmonella* Typhimurium DPC6463 are shown on Figure 10. Among the three probiotics selected, *L. mesenteroides* displayed the highest co-aggregation potential with *Salmonella* Typhimurium followed by *P. pentosaceous.*

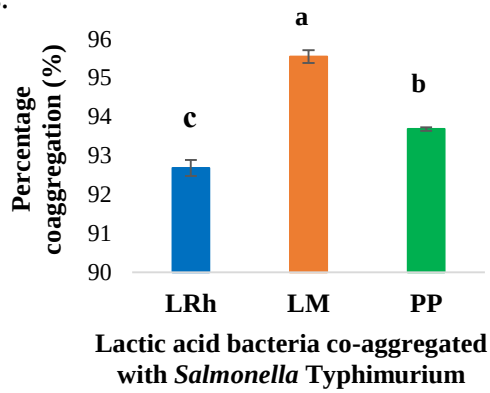

Figure 10: Results of co-aggregation assays of selected probiotic strains with *Salmonella* Typhimurium DPC6463. LRh*: L. rhamnosus*, LM: *L. mesenteroides*, PP: *P. pentosaceus.* Error bars: ±SD. Homogenous means indicated by similar letters: Tukey's HSD test (*P*- value <0.05)

Anti-Campylobacter well diffusion assay:

The results of the well diffusion assay conducted against *Campylobacter jejuni* are shown on Figure 11. Among the six probiotic strains studied, *L. casei*, *L. mesenteroides* and *P. pentosaceus* displayed equally potent inhibition of *Campylobacter jejuni* followed by *L. plantarum.*

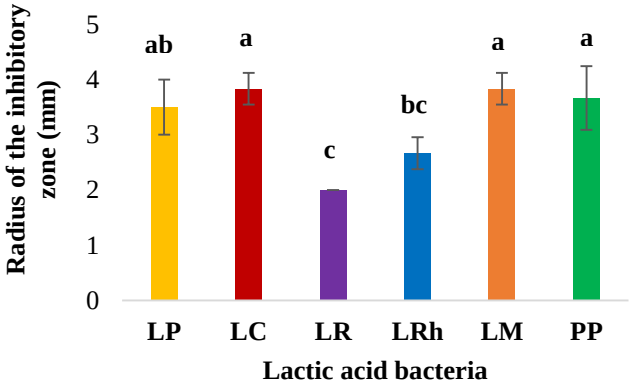

Figure 11: Inhibition of *Campylobacter jejuni* by probiotic culture supernatants in Well diffusion assays. LP: *L. plantarum*, LC: *L. casei*, LR: *L. reuteri*, LRh: *L. rhamnosus*, LM: *L. mesenteroides*, PP: *P. pentosaceus*. Error bars: ±SD. Homogenous means indicated by similar letters: Tukey's HSD test (*P*- value s<0.05)

and co-aggregation potential against *Salmonella* and bacteriostatic potential against *Campylobacter jejuni*. Considering the results of the growth of *L. mesenteroides* from

Experiment 1, 0.5% garlic aqueous extract was selected as the phytobiotic component, to be used in combination with *L. mesenteroides* for the *in-ovo* stimulation protocol.

In-vivo validation of effects of in-ovo administration of selected probiotic (PB) and prophybiotic (PPB) on gut health and production of broiler chickens (Experiment 3)

Hatch data:

The NC group achieved the highest hatchability (91.7%), while the PPB group exhibited the highest hatchability among the *in-ovo* injected groups (89.5%), surpassing both the PC (86.9%) and PB (85.5%) groups. There were no statistically significant differences observed in chick length and chick quality (Pasgar score) among the groups (*P*- value > 0.05). However, chick weight was notably higher in the PB and PPB groups compared to the control groups (*P*-value < 0.05), as illustrated in Figure 12.

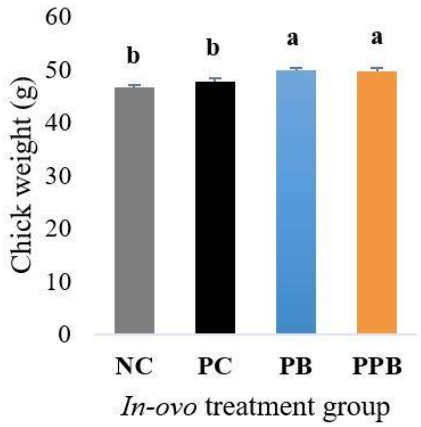

Figure 12: The weight of chicks at hatch across *in-ovo* treatment groups. Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending order). Abbreviations: NC: negative control group, PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group

Body weight and feed conversion ratio:

The chickens from both the PB and PPB groups exhibited higher body weights compared to those from the control groups from hatch to two weeks of age (Table 13), indicating the beneficial effects of these treatments in the early life of the broilers. However, no significant differences in body weight were observed among the groups from the third week onwards. Furthermore, both the weekly feed conversion ratio (FCR) (Figure 13A) and the

overall FCR for the entire production period (Figure 13B) did not reveal definitive evidence that any group had a higher FCR compared to the others.

Table 13: Body weight of chickens of *in-ovo* treatment groups

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

[1]NC: Negative control, PC: Positive control, PB: Probiotic group, PPB: Prophybiotic group

[2]Data are represented as mean ± SD. Homogenous means have been indicated by similar letters (in descending order)

[3]Significant codes: *P*- values < 0.0001: ***, < 0.001: **, < 0.05: *, <0.1: T, >0.1: NS

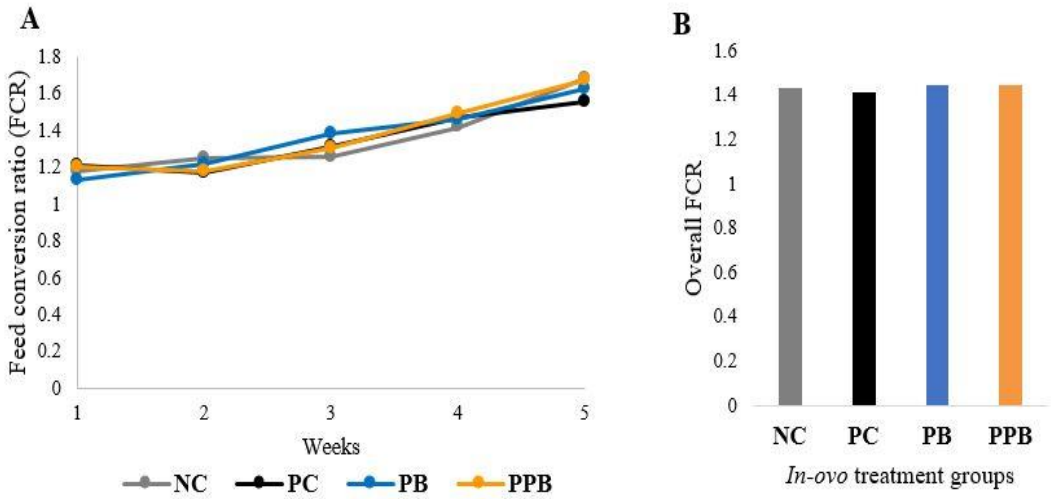

Figure 13. Feed conversion ratio (FCR) of the chickens of *in-ovo* treatment groups. (A) Weekly FCR. (B) Overall FCR. Abbreviations: NC: Negative control group, PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group.

Slaughter and meat quality assays:

The results of the slaughter analysis has been summarized on Table 14. Most of the carcass parameters studied were not statistically different among the *in-ovo* treatment groups. However, both PB and PPB groups displayed a statistically lower cooling loss compared to that in the PC group. In addition, the PB group displayed a statistically higher leg bone percentage and lower breast muscle percentage compared to those in the PC group.

Table 14: Slaughter analysis of the chickens of *in-ovo* treatment groups

Parameter	Slaughter analysis[2]			Treatment effect[3]
	PC[1]	PB[1]	PPB[1]	
Cooling losses (%)	1.79 ± 0.21^{a}	1.35 ± 0.29^{b}	1.547 ± 0.09^{b}	***
Dressing percentage with giblets (%)	79.81 ± 1.14	79.51 ± 1.25	79.82 ± 1.24	NS
Dressing percentage without giblets (%)	76.83 ± 1.19	76.49 ± 1.25	76.70 ± 1.3	NS
Breast muscle (%)	31.35 ± 2.05^{a}	29.39 ± 1.53^{b}	30.77 ± 2.37^{ab}	*
Leg muscles (%)	19.19 ± 1.47	19.39 ± 1.27	18.89 ± 2.07	NS
Giblets (%)	3.75 ± 0.42	3.93 ± 0.24	3.91 ± 0.3	NS
Liver (%)	2.23 ± 0.3	2.42 ± 0.3	2.34 ± 0.19	NS
Gizzard (%)	0.96 ± 0.2	0.92 ± 0.19	0.97 ± 0.12	NS
Heart (%)	0.53 ± 0.06	0.55 ± 0.07	0.53 ± 0.05	NS
Leg bones (%)	3.98 ± 0.48^{b}	4.44 ± 0.49^{a}	4.17 ± 0.4^{ab}	T (*P*- value:0.06)
Abdominal fat (%)	1.83 ± 0.3	1.94 ± 0.46	1.7 ± 0.34	NS

[1]PC: Positive control, PB: Probiotic group, PPB: Prophybiotic group

[2]Data are represented as mean ± SD. Homogenous means have been indicated by similar letters (in descending order)

[3]Significant codes: *P*- values < 0.0001: ***, < 0.001: **, < 0.05: *, <0.1: T, >0.1: NS

Meat quality analysis:

The significant changes in meat quality are summarized in Table 14. In the PPB treated chickens, the thawing loss (%) was lower in both breast and leg muscles whereas, the drip loss (%) in leg muscles was higher compared to those in other groups. The breast meat of chickens in PB and PPB groups was more acidic than that in the PC group. Additionally, some changes in the sensory properties of the breast meat (higher chewiness and gumminess in PB group and higher springiness in PPB group) were also noticed.

Table 15: Significant changes in the meat quality of the chickens of *in-ovo* treatment groups

Parameter	Meat quality analysis[2]			Treatment effect[3]
	PC[1]	PB[1]	PPB[1]	
Breast muscle				
Chewiness	10.255 ± 2.83^{b}	12.642 ± 3.24^{a}	11.191 ± 1.35^{ab}	T (*P*- value: 0.077)
Gumminess	29.316 ± 6.96^{b}	35.018 ± 8.79^{a}	29.925 ± 3.11^{ab}	T (*P*- value: 0.071)
Springiness	0.348 ± 0.03^{b}	0.361 ± 0.02^{ab}	0.372 ± 0.03^{a}	T (*P*- value: 0.092)
Thawing loss (%)	5.373 ± 1.32^{a}	4.271 ± 1.98^{ab}	3.150 ± 1.1^{b}	**
pH15 min	6.597 ± 0.14^{a}	6.338 ± 0.13^{b}	6.361 ± 0.18^{b}	***
Leg muscle				
Drip loss 24h (%)	0.57 ± 0.12^{b}	0.60 ± 0.08^{b}	0.65 ± 0.05^{a}	T (*P*- value: 0.06)
Thawing loss (%)	3.05 ± 1^{a}	3.60 ± 1.39^{a}	2.29 ± 0.64^{b}	*

[1]PC: Positive control, PB: Probiotic group, PPB: Prophybiotic group

[2]Data are represented as mean ± SD. Homogenous means have been indicated by similar letters (in descending order)

[3]Significant codes: *P*- values < 0.0001: ***, < 0.001: **, < 0.05: *, <0.1: T

Relative abundance of bacteria in feces:

Feces samples of 7 days old chickens displayed a statistically higher relative abundance of *Bifidobacterium* sp. in the PPB group (Figure 14A) and higher abundance of *Faecalibacterium* sp. in both the PB and PPB groups (Figure 14B) compared to those in the PC group. Towards the end of the production life (day 34), no significant difference in these bacterial genera were observed among the groups while a significant reduction in the relative abundance of *E. coli* was observed in the PB and PPB groups (Figure 14C) compared to the PC group.

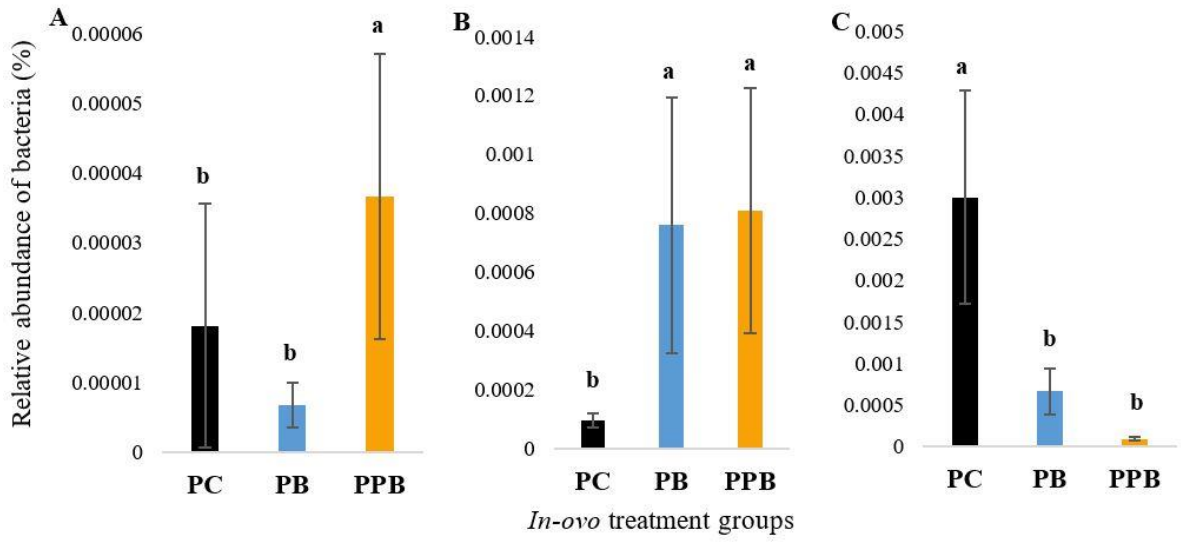

Figure 14: The relative abundance of bacterial communities in the feces of chickens of different *in-ovo* treated groups. (A) D 7 – *Bifidobacterium* sp. (B) D 7 – *Faecalibacterium* sp. (C) D 34 – *E. coli*. Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending order). Abbreviations: PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group

Relative abundance of bacteria in cecal content:

The cecal content of the *in-ovo* treated chickens displayed modulation of short chain fatty acid (SCFA) producing beneficial bacteria. The PPB treatment reduced the relative abundance of *Faecalibacterium* sp. (Figure 15A) while both PB and PPB treatments increased the relative abundance of *Akkermansia* sp. (Figure 15B) compared to those in the PC group. Additionally, similar to day 34 feces samples, the relative abundance of *E. coli* was reduced in the PPB group (Figure 15C).

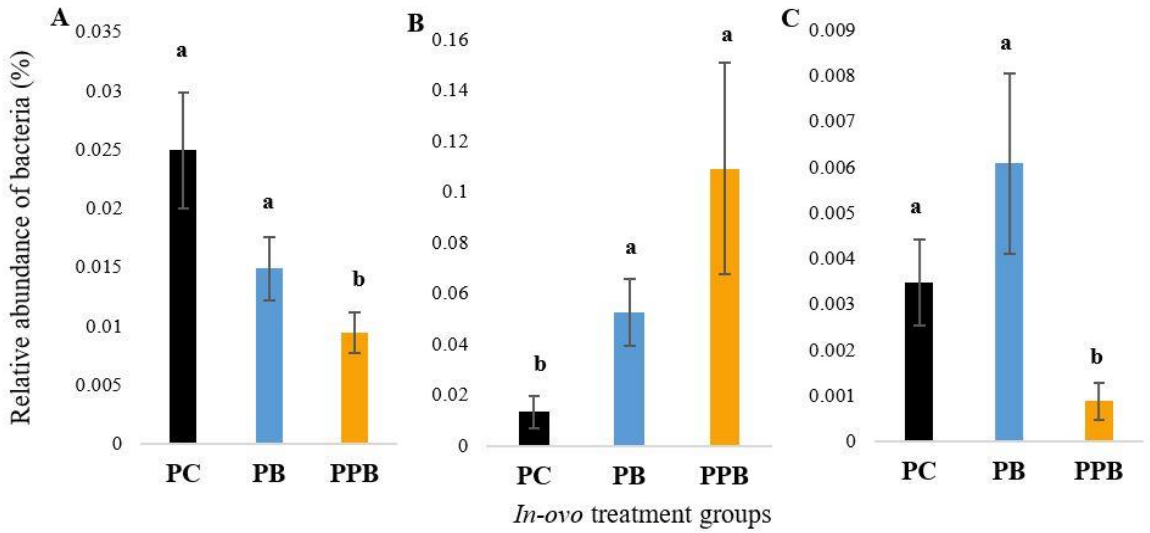

Figure 15. The relative abundance of bacterial communities in the luminal content of ceca of chickens of different *in-ovo* treated groups. (A) *Faecalibacterium sp*. (B) *Akkermansia sp*. (C) *Escherichia coli*. Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending order). Abbreviations: PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group

Expression of immune related genes in the cecal mucosa:

The gene expression analysis displayed no significant differences in the expression of *IL1-β, IL2, IL6, IL8, IL10, CLDN* and *CATHL2* in the mucosa of the ceca among the *in-ovo* treatment groups. Interestingly, the chickens in the PPB group displayed an over expression of *AVBD1* and *FFAR2* (Figure 16A and B) while, both PB and PPB groups displayed an over expression of *MUC6* (Figure 16C) in the cecal mucosa when compared to those in the PC group.

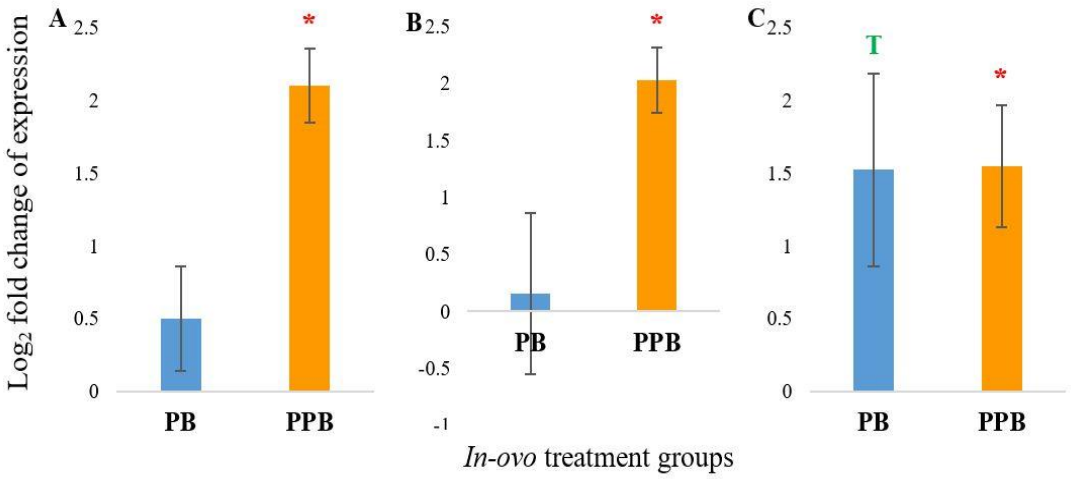

Figure 16. Immune-related gene expression in the cecal mucosa of chickens of different *in-ovo* treated groups. (A) *AVBD1* (B) *FFAR2* (C) *MUC6*. Error bars: ± SE. Red color asterick (*) indicates significant changes (*P*- value < 0.05). The letter T in green indicates there is a tendency (*P*- value = 0.0637). Abbreviations: PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group

Histomorphological changes in the ceca:

The villus height and villus width measurements in the ceca were not statistically different among the *in-ovo* treatment groups (Figure 17A and B). However, due to the slight (statistically insignificant) reduction of both the width and the height of the villi in the PPB group, a statistically significant reduction in the villus surface area was observed (Figure 17C). Similarly, statistically significant and insignificant increases in the crypt depth of the ceca in the PPB and PB groups, respectively (Figure 17D) resulted in statistically significant decrease in the Villus height:Crypt depth ratio (Figure 17E) when compared to the PC group.

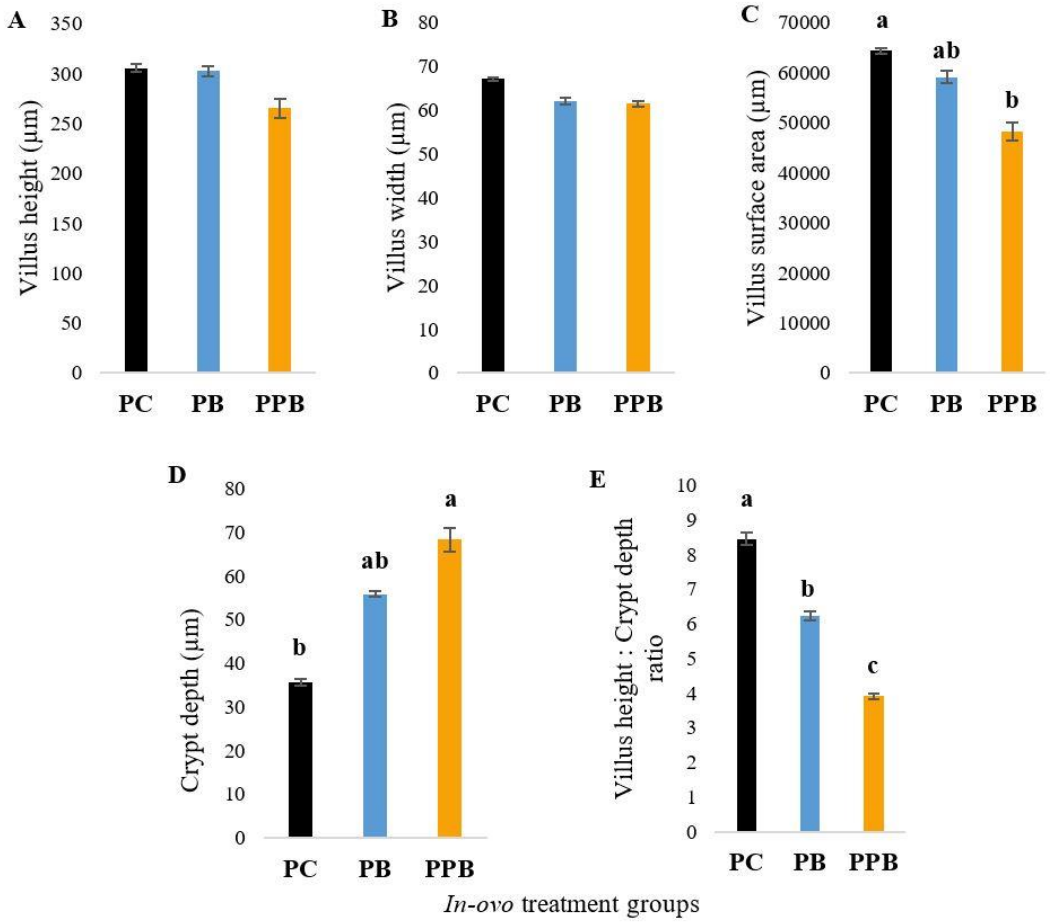

Figure 17. The analysis of histomorphological parameters of the cecal tissue of the *in-ovo* treated chickens. (A) Villus Height. (B) Villus Width. (C) Villus Surface Area. (D) Crypt depth. (E) Villus Height to Crypt Depth Ratio. Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending order). Abbreviations: PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group

3.5 Discussion

As we are progressing through an antibiotic stewardship era, it is the responsibility of livestock scientists to develop novel approaches to improve the health of livestock, mitigating the use of antibiotics in livestock production. Poultry particularly, carry zoonotic pathogens such as *Campylobacter* which are naturally found in poultry production systems and difficult to eradicate (Hakeem and Lu, 2021). Not all farmers around the world are able to afford the strict biosecurity measures and continuous feed supplements to maintain appropriate gut health in poultry. *In-ovo* intervention strategies which confer long term benefits related to gut microbiome and immune system functioning in the chickens therefore, can be an excellent approach to overcome above mentioned challenges in the poultry production. However, the efficacy of this application largely depends on careful optimization of the protocol. Therefore, this PhD project was carried out to develop an *in-ovo* stimulation protocol for gut microbiome programming with proven anti-pathogenic effects, to improve the gut health of broiler chickens.

In-ovo stimulation is performed on embryonic day 12 when the chorioallantoic membrane is highly vascularized. The aim of *in-ovo* stimulation with a prebiotic therefore, is to deliver it to the blood circulatory system of the developing embryo (through the vascularized chorioallantoic membrane) to stimulate the native microbiome in the developing embryo and start immune system development (Siwek et al., 2018). The prebiotic assays performed in experiment 1 showed that the prebiotic potential of the commercial prebiotics used was very specific, indicating their limited potential in stimulating an overall beneficial microbiome profile in the gut. Sharma and Padwad (2020) also claimed that conventional oligosaccharides may stimulate the growth of non-beneficial bacteria, leading to inconsistent observations in clinical endpoints and lack inherent bioactivity for improving the gut health. These authors then proposed that plant-based polyphenolic substrates would be a more suitable companion for probiotics to achieve synergistic effects. Therefore, as the main goal of this protocol was to confer anti-pathogenic effects and the prebiotics used seemed to have a limited prebiotic potential, it was decided to proceed with the use of anti-pathogenic plant extracts in combination with the probiotics (as a prophybiotic) in this protocol. Interestingly, to the best of our knowledge, this project is the first to investigate the effects of *in-ovo* stimulation of broiler chickens with a prophybiotic combination.

In this protocol, the probiotic and the phytobiotic components were expected to act separately on the host. The anti-pathogenic phytobiotic component was expected to boost the

immune system development in the embryo while the probiotic component was intended to reprogram the gut microbiome of the newly hatched chicks. Therefore, it was necessary to use a phytobiotic which neither promotes nor inhibits the growth of the selected probiotic, so it can be entirely used on the immunomodulation of the host without interacting with the probiotic. The selected phytobiotic, garlic is well known for immunomodulatory properties (Arreola et al., 2015) and 0.5% dose of garlic aqueous extract displayed non-inhibitory and non-prebiotic properties with the selected probiotic, *Leuconostoc mesenteroides,* meeting all the requirements necessary for the expected outcome of the protocol. Similarly, the probiotic strains used in this project are currently used in a multi-strain commercial probiotic supplement for poultry and proven to be safe and effective against *Salmonella* (Smialek et al., 2019) and *Campylobacter* (Smialek et al., 2018) pathogens. *Leuconostoc mesenteroides* species have displayed antimicrobial (Zhang et al., 2021, 2023) and probiotic (de Paula et al., 2015) properties in previous studies. Moreover, oligosaccharide by-products of *L. mesenteroides* fermentation are known to stimulate the growth of beneficial bacteria, both *in-vitro* and *in-vivo* (Chung and Day, 2004; Miyamoto et al., 2023, respectively) but not harmful pathogens such as *Salmonella* and *E. coli, in-vitro* (Chung and Day, 2004). To the best of my knowledge, this study is the first investigating the potential use of *L. mesenteroides* in *in-ovo* stimulation of broiler chickens.

As it was hypothesized that this protocol will reprogram the gut microbiome and boost the immune system which will last until the end of the production lifetime, the relative abundance of selected bacterial communities and immune related gene expression in the *in-ovo* treated chickens were investigated at 35 days of age. The results of bacterial quantification in feces suggested that the effects of *in-ovo* stimulation on beneficial microbes was evident during early life (1 week of age) and in adult chickens (at 5 weeks of age), the treatment effect may be confounded with other biological and management factors such as feed and genetics. However, as significant reduction of potentially harmful *E. coli* was observed in both the feces and cecal content in the prophybiotic treated adult chickens, it can be suggested that the prophybiotic protocol imparted long lasting protection against *E.coli* in the chicken gut.

The cecal microbiome further, displayed significant changes in the beneficial bacteria even at the end of 5 weeks of age. *Akkemansia* sp. which displayed an increased abundance in the ceca in both probiotic and prophybiotic treated chickens, function mainly as mucin degraders (particularly, *Akkermansia muciniphila*) producing SCFA necessary for the metabolism of the epithelial cells, immune reactions (Yang et al., 2022) and production of

antimicrobial peptides in the gut (Paone and Cani, 2020). Therefore, an increased abundance of this genera indicates a stimulated immune status in the ceca. In compliance with this result, *MUC6* expression was increased in the cecal mucosa in both probiotic and prophybiotic treated chickens indicating a possible higher mucin production to cope with higher mucin turnover by *Akkermansia* sp.

The prophybiotic treatment further, resulted in a decrease of the abundance of *Faecalibacterium* sp. As both *Akkermansia* and *Faecalibacteria* are SCFA producers, an increase of one and decrease of the other by the prophybiotic treatment may indicate a differential modulation of SCFA production in ceca of the broilers when compared to probiotic alone treatment. SCFAs are known to recruit immune cells particularly, leucocytes in the gut epithelium and the free fatty acid receptor 2 (*FFAR2*) is the key receptor found in these cells (Schlatterer et al., 2021). Moreover, these immune cells are known to secrete antimicrobial peptides such as avian beta defensing 1 (*AVBD1)* (Flaherty, 2012). Interestingly, an increased expression of *AVBD1* and *FFAR2* in the cecal mucosa was observed in the prophybiotic group but not in the probiotic group possibly due to this differential modulation of SCFA production in the ceca.

Moreover, the cecal histomorphology displayed that the crypt depth of the prophybiotic treatment was higher when compared to the probiotic and positive control group. As crypts generally, function as the villus factories and their depth determines the cell renewal rate in the mucosa (Sobolewska et al., 2017), it can be suggested that the prophybiotic treatment resulted in a higher cell renewal in the cecal mucosa which can be justified by the increased immune status of the tissue.

In addition to above results, the gene expression analysis in the other immune related organs such as cecal tonsils, spleen and liver of the same birds was performed (Wishna-Kadawarage et al., 2024d – not included in the publication series of the thesis). The spleen displayed an up-regulation of *AVBD1* by both probiotic and prophybiotic treatments and an up-regulation of *IL1-β* only by the probiotic treatment. Moreover, a down-regulation of pro- (*IL1-β* and *IL8*) and and anti-inflammatory (*IL10*) cytokines in the cecal tonsils was observed in the probiotic treated chickens whereas a down-regulation of pro- (*IL1-β, IL6* and *IL6*) and anti-inflammatory (*IL10*) cytokines in the liver was observed with the prophybiotic treatment. The differential regulation of the immune related genes in the cecal tonsils and liver by the two

treatments may be due to the differences in the metabolites (such as SCFA produced in the ceca) reaching the respective organs.

These results together indicate that all in all, the *in-ovo* stimulation with the protocols optimized in this PhD project (probiotic and prophybiotics), conferred long term benefits to the cecal microbiome, resulted in differential expression of immune related genes in important immune organs and modulated the cecal histomorphology of the broiler chickens. Interestingly, according to the results of hatch parameters, body weights, slaughter and meat quality analysis of the same chickens, no adverse effects on the production of these broilers was observed as a result of induced immune status.

A limitation of this protocol might be the requirement of handling eggs on day 12 at the hatcheries. It is also possible that the composition of the garlic extract is different between the cultivars or origin and therefore, differences in the effects on chickens at the molecular level can be observed if a different garlic cultivar was used. Changes in factors such as maternal flock age, management practices and the genotype can also influence the outcome of the protocol. Moreover, the current validation study quantified the selected bacterial communities and gene expression at the mRNA level. However, a detailed microbiome profiling (such as 16s rRNA sequencing) and an investigation of the immune components at the protein level, will provide further insights to potential mode of action of the bioactive treatments validated in this study. All in all, this PhD project demonstrates the potential of *in-ovo* stimulation with a novel approach, a prophybiotic combination as a tool to improve gut health of broiler chickens and opens up a new line of knowledge on the topic.

3.6 Summary

This PhD project was conducted to optimize a protocol to administer bioactive substances with anti-pathogenic effects, into chicken eggs on the 12th day of incubation (*in-ovo* stimulation) conferring long term benefits on the gut microbiome and immune system of the broiler chickens without impairing their production. Two *in-vitro* experiments were conducted to screen the most efficacious bioactive substances out of a variety of prebiotics, probiotics and phytobiotics, followed by an *in-vivo* experiment to validate the effects of the optimized protocol on gut health and production of broiler chickens. The following are the main findings of the above experiments;

- The prebiotics selectively supported the growth of the probiotics studied. Accordingly, the following compatible synbiotic pairs were identified;
  - Raffinose and *L. reuteri*
  - Inulin and *L. casei*
  - Saccharicterpenin and *L. plantarum* or *L. mesenteroides*
- Green tea significantly inhibited the growth of all six probiotics studied indicating it's unsuitability in prophybiotic (probiotic + phytobiotic) combinations. However, turmeric and garlic extracts did not display inhibitory effects against the probiotics studied indicating their potential to be used in following prophybiotic combinations;
  - Turmeric (0.1%) (w/v) with one of the four probiotics, *L. plantarum, L. casei, L. reuteri* and *L. rhamnosus*
  - Turmeric (0.6%) (w/v) together with *L. mesenteroides* or *P. pentosaceus*
  - Garlic (0.5%) (w/v) with all six probiotics studied.
- *L. rhamnosus, L. mesenteroides* and *P. pentosaceus* displayed equally efficient bacteriostatic and bactericidal properties while *L. mesenteroides* displayed the highest co-aggregation ability against *Salmonella.*
- *L. plantarum, L. casei, L. mesenteroides* and *P. pentosaceus* were the most effective against *Campylobacter*.
- *In-ovo* stimulation with the selected prophybiotic (10^6 CFU of *L. mesenteroides* with 0.5% garlic aqueous extract) and its probiotic component alone (10^6 CFU of *L. mesenteroides*) resulted in no impairment of hatchability, chick quality and chick length. Interestingly, both treatments resulted in a higher chick weight compared to the controls. Moreover, both treatments resulted in higher body weights during the first 2 weeks of age without

compromising the feed efficiency of the broiler chickens. The overall meat quality and carcass parameters were not adversely affected by these *in-ovo* treatments.

- Interestingly, the treatments resulted in higher abundance of beneficial bacteria (*Faecalibacterium* sp. in both groups and *Bifidobacterium* sp. in prophybiotic group) in the feces at one week age and lower abundance of potentially harmful *E. coli* in the feces and cecal content at 5 weeks age. Moreover, both treatments resulted in a higher abundance of *Akkermansia* sp. while the prophybiotic treatment resulted in a lower abundance of *Faecalibacterium* sp. in the cecal content.
- Both treatments resulted in the up-regulation of *MUC6* expression while only the prophybiotic treatment resulted in an up-regulation of *AVBD1* and *FFAR2* genes in the cecal mucosa. No changes in pro- or anti-inflammatory interleukins were observed in the cecal mucosa resulting from the treatments.
- The prophybiotic treated chickens displayed a higher crypt depth in the ceca.

Based on the above findings, this PhD thesis concludes that *in-ovo* stimulation with *Leuconostoc mesenteroides* (10^6 CFU/egg) alone (as a probiotic) and in combination with garlic aqueous extract (0.5%) (as a prophybiotic) confers long term benefits to the gut microbiome, gene expression and histomorphology of ceca providing prophylactic effects in ROSS308 broiler chickens. This validated protocol encourages more research on *in-ovo* stimulation with different prophybiotic combinations to gain optimal benefits for future applications.

3.7 References of the thesis

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

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

Appleton, J. 2018. The Gut-Brain Axis: Influence of Microbiota on Mood and Mental Health. Integr. Med. Clin. J. 17:28–32.

Arreola, R., S. Quintero-Fabián, R. I. López-Roa, E. O. Flores-Gutiérrez, J. P. Reyes-Grajeda, L. Carrera-Quintanar, and D. Ortuño-Sahagún. 2015. Immunomodulation and Anti-Inflammatory Effects of Garlic Compounds. J. Immunol. Res. 2015:401630.

Ashonibare, V. J., B. A. Akorede, P. J. Ashonibare, T. M. Akhigbe, and R. E. Akhigbe. 2024. Gut microbiota-gonadal axis: the impact of gut microbiota on reproductive functions. Front. Immunol. 15 Available at https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2024.1346035/full (verified 16 April 2024).

Authority, E. F. S. and European Centre for Disease Prevention and Control. 2022. The European Union One Health 2021 Zoonoses Report. EFSA J. 20:e07666.

Balakrishna, A. 2013. *In vitro* evaluation of adhesion and aggregation abilities of four potential probiotic strains isolated from guppy (*Poecilia reticulata*). Braz. Arch. Biol. Technol. 56:793–800.

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

Campagnoli, L. I. M., A. Varesi, A. Barbieri, N. Marchesi, and A. Pascale. 2023. Targeting the Gut–Eye Axis: An Emerging Strategy to Face Ocular Diseases. Int. J. Mol. Sci. 24:13338.

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

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

De Pessemier, B., L. Grine, M. Debaere, A. Maes, B. Paetzold, and C. Callewaert. 2021. Gut–Skin Axis: Current Knowledge of the Interrelationship between Microbial Dysbiosis and Skin Conditions. Microorganisms 9:353.

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 Available at https://www.frontiersin.org/journals/veterinary-science/articles/10.3389/fvets.2021.658301/full (verified 3 July 2024).

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

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

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

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

Enaud, R., R. Prevel, E. Ciarlo, F. Beaufils, G. Wieërs, B. Guery, and L. Delhaes. 2020. The Gut-Lung Axis in Health and Respiratory Diseases: A Place for Inter-Organ and Inter-Kingdom Crosstalks. Front. Cell. Infect. Microbiol. 10 Available at https://www.frontiersin.org/articles/10.3389/fcimb.2020.00009 (verified 16 April 2024).

Flaherty, D. K. (Ed). 2012. Chapter 2 - Innate Immunity.Pages 15–22 in Immunology for Pharmacy. Mosby, Saint Louis.

Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, R. Gast, T. J. Humphrey, and F. Van Immerseel. 2009. Mechanisms of egg contamination by *Salmonella Enteritidis*. FEMS Microbiol. Rev. 33:718–738.

Hakeem, M. J., and X. Lu. 2021. Survival and Control of Campylobacter in Poultry Production Environment. Front. Cell. Infect. Microbiol. 10 Available at https://www.frontiersin.org/articles/10.3389/fcimb.2020.615049 (verified 10 April 2024).

Huang, N., Y. Ma, J. Chai, Z. Li, X. You, X. Wang, Y. Huang, and H. Shi. 2024. *In ovo* injection dosage of *Lactobacillus rhamnosus* on intestinal health and microbial composition of yellow broilers with or without *Eimeria* challenge. J. Appl. Poult. Res. 33:100411.

Kizerwetter-Świda, M., and M. Binek. 2008. Bacterial microflora of the chicken embryos and newly hatched chicken. J. Anim. Feed Sci. 17:224–232.

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

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

Kpodo, K. R., and M. Proszkowiec-Weglarz. 2023b. Physiological effects of *in ovo* delivery of bioactive substances in broiler chickens. Front. Vet. Sci. 10 Available at https://www.frontiersin.org/articles/10.3389/fvets.2023.1124007 (verified 18 April 2024).

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

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

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

Martin, F. E., M. A. Nadkarni, N. A. Jacques, and N. Hunter. 2002. Quantitative Microbiological Study of Human Carious Dentine by Culture and Real-Time PCR: Association of Anaerobes with Histopathological Changes in Chronic Pulpitis. J. Clin. Microbiol. 40:1698–1704.

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

OECD. 2023. OECD-FAO Agricultural Outlook 2023-2032. Organisation for Economic Co-operation and Development, Paris.

Oladokun, S., and D. I. Adewole. 2020a. *In ovo* delivery of bioactive substances: an alternative to the use of antibiotic growth promoters in poultry production—a review. J. Appl. Poult. Res. 29:744–763.

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

Pabst, O., M. W. Hornef, F. G. Schaap, V. Cerovic, T. Clavel, and T. Bruns. 2023. Gut–liver axis: barriers and functional circuits. Nat. Rev. Gastroenterol. Hepatol. 20:447–461.

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

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

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

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

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

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

Rothwell, L., J. R. Young, R. Zoorob, C. A. Whittaker, P. Hesketh, A. Archer, A. L. Smith, and P. Kaiser. 2004. Cloning and Characterization of Chicken IL-10 and Its Role in the Immune Response to *Eimeria maxima*1. J. Immunol. 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. J. Surg. Res. 94:99–106.

Schlatterer, K., A. Peschel, and D. Kretschmer. 2021. Short-Chain Fatty Acid and FFAR2 Activation – A New Option for Treating Infections? Front. Cell. Infect. Microbiol. 11 Available at https://www.frontiersin.org/articles/10.3389/fcimb.2021.785833 (verified 27 October 2023).

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

Sharma, R., and Y. Padwad. 2020. Plant-polyphenols based second-generation synbiotics: Emerging concepts, challenges, and opportunities. Nutrition 77:110785.

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. Anim. Open Access J. MDPI 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 Vet. Res. 14:402.

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

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

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

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

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

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

Staudacher, H. M., and A. Loughman. 2021. Gut health: definitions and determinants. Lancet Gastroenterol. Hepatol. 6:269.

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.

Wickramasuriya, S. S., I. Park, K. Lee, Y. Lee, W. H. Kim, H. Nam, and H. S. Lillehoj. 2022. Role of Physiology, Immunity, Microbiota, and Infectious Diseases in the Gut Health of Poultry. Vaccines 10:172.

Wishna-Kadawarage, R. N., R. M. Hickey, and M. Siwek. 2024a. *In-vitro* selection of lactic acid bacteria to combat *Salmonella enterica* and *Campylobacter jejuni* in broiler chickens. World J. Microbiol. Biotechnol. 40:133.

Wishna-Kadawarage, R. N., M. Jensen, S. Powałowski, R. M. Hickey, and M. Siwek. 2024b. *In-vitro* screening of compatible synbiotics and (introducing) “prophybiotics” as a tool to improve gut health. Int. Microbiol. 27:645–657.

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

Wishna-Kadawarage, R. N., K. Połtowicz, R. M. Hickey, and M. Siwek. 2024d. Modulation of gene expression in immune-related organs by *in ovo* stimulation with probiotics and prophybiotics in broiler chickens. J. Appl. Genet. Available at https://doi.org/10.1007/s13353-024-00891-y (verified 11 July 2024).

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

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

Zhang, H., H. HuangFu, X. Wang, S. Zhao, Y. Liu, H. Lv, G. Qin, and Z. Tan. 2021. Antibacterial Activity of Lactic Acid Producing *Leuconostoc mesenteroides* QZ1178 Against Pathogenic *Gallibacterium anatis*. Front. Vet. Sci. 8 Available at https://www.frontiersin.org/articles/10.3389/fvets.2021.630294 (verified 31 October 2023).

Zhu, Q., P. Sun, B. Zhang, L. Kong, C. Xiao, and Z. Song. 2021. Progress on Gut Health Maintenance and Antibiotic Alternatives in Broiler Chicken Production. Front. Nutr. 8:692839.

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

ENGLISH ABSTRACT

In-ovo stimulation as a tool to improve gut health of broiler chickens.

Ramesha Nirmali Wishna Kadawarage, MSc.

Keywords: gut microbiome, immunomodulation, phytobiotic, probiotic, prophybiotic

In-ovo stimulation refers to the administration of bioactive substances into eggs during the 12th day of incubation and aims to modulate the gut microbiome and immune system of chickens thus improving their gut health. The effects of this approach largely depends on the bioactive substances used. Therefore, this PhD thesis was conducted to optimize an *in-ovo* stimulation protocol using anti-pathogenic bioactive substances to improve the gut health of broiler chickens. *In-vitro* growth kinetics assays were performed to identify compatible synbiotic (probiotic + prebiotic) and prophybiotic (probiotic + phytobiotic) pairs to be used in this protocol. The most potent anti-pathogenic probiotic was identified by performing anti-*Salmonella* (spot overlay, well diffusion, co-culture and co-aggregation assays) and anti-*Campylobacter* (well diffusion assay) assays. *In-ovo* stimulation of ROSS308 chickens was performed to validate the *in-vivo* effects of the protocol on the gut microbiome (feces and cecal content), expression of immune-related genes (in the cecal mucosa), histomorphology (in ceca) together with various production parameters (hatchability, hatch quality, body weight, feed conversion ratio, carcass and meat quality). The results show that prebiotics selectively promoted the growth of probiotics studied. Turmeric and garlic extracts did not inhibit the growth of all the probiotics studied indicating their broad potential for use in prophybiotic combinations. *Leuconostoc mesenteroides* B/00288 (LM) strain was selected as the most potent anti-pathogenic probiotic based on it's overall anti-*Salmonella* and anti-*Campylobacter* activity. Accordingly, *in-ovo* stimulation with 10^6 CFU/egg LM alone and in combination with 0.5% garlic aqueous extract (the dose at which garlic was non-inhibitory to LM) was performed in ROSS308 broiler chickens. The treatments resulted in beneficial changes on the gut microbiome, gene expression in the cecal mucosa and histomorphology in the ceca. These changes indicated a possible prophylactic effect without compromising the production parameters. In conclusion, this *in-ovo* stimulation protocol can be used as a tool to improve the gut health of broiler chickens.

64:10188776

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

POLISH ABSTRACT

Stymulacja *in-ovo* jako narzędzie do poprawy zdrowia jelit kurcząt brojlerów.

Mgr. Ramesha Nirmali Wishna Kadawarage

Słowa kluczowe: mikrobiom jelitowy, immunomodulacja, fitobiotyk, probiotyk, profibiotyk

Stymulacja *in-ovo* odnosi się do podawania substancji bioaktywnych do jaja w 12 dniu inkubacji i ma na celu modulację mikrobiomu jelitowego i układu immunologicznego kurcząt, a także poprawę zdrowia jelit. Efekty tej stymulacji w dużej mierze zależą od zastosowanych substancji bioaktywnych. Dlatego też celem tej dysertacji doktorskiej była optymalizacja protokołu stymulacji *in-ovo* z wykorzystaniem antypatogennych substancji bioaktywnych w kierunku poprawy zdrowia jelit kurcząt brojlerów. Przeprowadzone zostały testy kinetyki wzrostu *in-vitro* w celu zidentyfikowania kompatybilnych par synbiotyków (probiotyk + prebiotyk) i probiotyków (probiotyk + fitobiotyk). Probiotyk o najsilniejszych właściwościach antypatogennych został zidentyfikowany poprzez wykonanie testów anty-*Salmonella* (punktowy, dyfuzja w studzience, ko-kultura i ko-agregacja) i anty-*Campylobacter* (test dyfuzji w studzience). Waliidacja efektywności wytypowanych substancji bioaktywnych została przepwoadzona w doświadczeniu *in-vivo* w którym ocenie poddany został wpływ iniekcji *in-ovo* na mikrobiom jelitowy (oznaczone w kałomoczu i jelicie ślepym), ekspresję genów związanych z odpowiedzią immunologiczną (w błonie śluzowej jelita ślepego), histomorfologię (w jelicie ślepym) wraz z niektórymi parametrami produkcyjnymi (wylęgowość, jakość piskląt, masa ciała, współczynnik konwersji paszy, jakość tuszy i jakość mięsa). Wyniki wykazały, że prebiotyki selektywnie promowały wzrost badanych probiotyków. Ekstrakty z kurkumy i czosnku nie hamowały wzrostu badanych probiotyków, co wskazuje na ich szeroki potencjał do stosowania w kombinacjach profilaktycznych. Szczep *Leuconostoc mesenteroides* B/00288 (LM) został wybrany jako najsilniejszy antypatogenny probiotyk w oparciu o ogólną aktywność przeciw *Salmonella* i *Campylobacter*. W związku z tym u kurcząt brojlerów ROSS308 przeprowadzono stymulację in-ovo z zastosowaniem 10^6 CFU/jajo *Leuconostoc mesenteroides* oraz w połączeniu *Leuconostoc mesenteroides* z 0,5%

wodnym ekstraktem z czosnku (dawka, przy której czosnek nie hamował działania LM). Stymulacja *in-ovo* wykazała korzystne zmiany w mikrobiomie jelitowym, ekspresji genów w błonie śluzowej jelita ślepego i histomorfologii jelita ślepego. Zmiany te wskazywały na możliwy efekt profilaktyczny bez kompensowania parametrów produkcyjnych. Podsumowując, opracowany protokół stymulacji *in-ovo* może być stosowany jako narzędzie do poprawy zdrowia jelit kurcząt brojlerów.

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

ATTACHMENTS (MANDATORY)

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

67:21736435

International Microbiology (2024) 27:645–657
https://doi.org/10.1007/s10123-023-00417-2

RESEARCH

In-vitro screening of compatible synbiotics and (introducing) "prophybiotics" as a tool to improve gut health

Ramesha N. Wishna-Kadawarage[1] · Martin Jensen[2] · Szymon Powałowski[3] · Rita M. Hickey[4] · Maria Siwek[1]

Received: 11 April 2023 / Revised: 27 July 2023 / Accepted: 3 August 2023 / Published online: 22 August 2023
© The Author(s) 2023

Abstract

Synbiotics have been intensively studied recently to improve gut health of humans and animals. The success of synergistic synbiotics depends on the compatibility of the prebiotic and probiotic components. Certain plant extracts possess both antimicrobial and prebiotic properties representing a potential use in combination with probiotics to improve the gut health. Here, we coined the term "prophybiotics" to describe this combined bioactivity. The current study aimed to select prebiotics that are preferred as an energy source and antimicrobial plant extracts which do not inhibit the growth, of six strains of lactic acid bacteria (LAB namely; *Lactiplantibacillus plantarum*, *Lacticaseibacillus casei*, *Limosilactobacillus reuteri*, *Lacticaseibacillus rhamnosus*, *Leuconostoc mesenteroides*, and *Pediococcus pentosaceus*) in-vitro to identify compatible combinations for potential synbiotic/prophybiotic use, respectively. Their growth kinetics were profiled in the presence of prebiotics: Inulin, Raffinose, and Saccharicterpenin with glucose, as the control, using carbohydrate free MRS broth media. Similarly, their growth kinetics in MRS broth supplemented with turmeric, green tea, and garlic extracts at varying concentrations were profiled. The results revealed the most compatible pairs of prebiotics and LAB. Turmeric and garlic had very little inhibitory effect on the growth of the LAB while green tea inhibited the growth of all LAB in a dose-dependent manner. Therefore, we conclude that turmeric and garlic have broad potential for use in prophybiotics, while the prebiotics studied here have limited use in synbiotics, with these LAB.

Keywords Antimicrobial · Gompertz model · Growth kinetics · Plant extracts · Prebiotics · Probiotics

Introduction

A healthy gut microbiome is largely responsible for maintaining innate immunity, gut barrier functioning as well as direct and indirect exclusion of pathogens (Diaz Carrasco et al. 2019). The use of probiotics, prebiotics, and synbiotics (prebiotics + probiotics) to improve gut health of humans and animal species has been studied and reported intensively in literature (as reviewed by Yadav et al. 2022). Lactic acid bacteria (LAB) have been intensively studied and are widely used as probiotics with a wide range of beneficial properties (Ljungh and Wadström 2006). Indeed, many species of LAB are listed in the updated list of qualified presumption of safety (QPS) recommended microorganisms by European Food Safety Authority (EFSA Biohaz Panel (EFSA Panel on Biological Hazards) et al. 2023) indicating the potential use of LAB in humans and animals safely.

According to the latest consensus statement by international scientific association for probiotics and prebiotics (ISAPP), based on the components and their functional role, synbiotics are divided into two main categories namely: complementary and synergistic (Swanson et al. 2020). A complementary synbiotic is a mixture of a probiotic and prebiotic chosen to act individually to improve gut health of the host while a synergistic synbiotic is a combination of live microorganisms which have beneficial effects on a host and a substrate which can selectively stimulate the growth and activity of the chosen

✉ Ramesha N. Wishna-Kadawarage
ramesha.wishna-kadawarage@pbs.edu.pl

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

2 Department of Food Science, Aarhus University, AgroFoodPark 48, 8200 Århus N, Denmark

3 Univeristy of Humanities Król Stanisław Leszczyński, Królowej Jadwigi 10, 64-100 Leszno, Poland

4 Teagasc Food Research Centre, Moorepark, Fermoy P61 C996, Co. Cork, Ireland

68:54306275

microorganism. The selection of components in a complementary synbiotic is relatively easier given the fact that they are expected to affect the host individually. However, the selection of a components for a synergistic synbiotic requires more carefully planned studies to select the most compatible prebiotic that effectively improves the growth and functioning of the probiotic of choice (Quintero et al. 2022). Therefore, careful screening of components in a synbiotic development is crucial for its successful application. Thus, the first objective of the current study was to determine the effect of commercial oligosaccharide-based prebiotics (Inulin, Raffinose, and Sachcharicter penin) on the growth of six strains of LAB to identify the best combinations for potential synbiotic use in terms of in-vitro growth.

As an innovative approach to synergistic synbiotics with oligosaccharide based prebiotics, plant-based second-generation synbiotics have been reviewed by Sharma and Padwad (2020). Here, the authors address the problems of using conventional oligosaccharides including, supporting growth of non-beneficial bacteria, inconsistent observations in clinical endpoints, and lack of inherent bioactivity for improving the gut health (Bindels et al. 2015; Krumbeck et al. 2016) and propose plant-based polyphenolic substrates as a better companion for probiotics in synergistic synbiotics. Among these plant-based bioactives, turmeric (*Curcuma longa*) (Scazzocchio et al. 2020), green tea (*Camellia sinensis*) (Jung et al. 2017), and garlic (*Allium sativum*) (Chen et al. 2020) have shown pronounced effects in modulating the gut microbiome and improving gut-associated immunity and overall gut health in many species. Moreover, the literature indicates that these plant extracts could also display prebiotic properties on LAB (Lu et al. 2021; Sunu et al. 2019; Yazdi et al. 2019). We coined the term **Prophybiotics** (probiotic + phytobiotic) to describe this approach where we aim to utilize these beneficial health effects of these phytobiotics and probiotics synergistically in improving gut health of human and animal species. However, as these plant extracts also contain antimicrobial compounds (turmeric: Adamczak et al. 2020, green tea: Gopal et al. 2016, and garlic: Bhatwalkar et al. 2021), it is important to confirm that the growth of probiotics used in combination might not be inhibited by these antimicrobial phytobiotics. Thus, the second objective of the current study was to assess the growth of six LAB in the presence of the turmeric, green tea, and garlic extracts to assess their effects on the growth.

Materials and methods

Probiotics, prebiotics, and plant extracts

A total of six LAB, namely, *Lactiplantibacillus plantarum* B/00166 (LP), *Lacticaseibacillus casei* B/00164 (LC), *Limosilactobacillus reuteri* B/00281 (LR), *Lacticaseibacillus rhamnosus* B/00279 (LRh), *Leuconostoc mesenteroides* B/00288 (LM), and *Pediococcus pentosaceus* B/00165 (PP) provided by JHJ sp. z o.o., Nowa Wieś, Poland, were used. All the strains were identified using 16S rRNA sequencing and deposited at the Polish Collection of Microorganisms located in Wrocław.

Three commercial prebiotics, namely, Raffinose VWR J392 (RAF), Saccharicterpenin (SAC) (Hubei, China), and Inulin Orafti® HPX (INU) (Mannheim, Germany), were used to determine the substrate preference of the LAB. Three plant extracts, namely, turmeric, green tea, and garlic, were used in the current study to determine their effects on probiotic growth. Green tea (spray-dried aqueous extract) and turmeric (spray-dried product of alcoholic extract of turmeric rhizomes) extracts were provided by Kaesler Gmbh, Cuxhaven, Germany. Approximately 67.5% polyphenols and 0.4% caffeine were present in the green tea extract while 2% curcumin was present in the turmeric extract used in the current study. The garlic (cultivar: Thermodrome) used in experiment was organically grown in the 2021 season in Aarhus University, Department of Food Science at Research Centre at Årslev, Funen, Denmark.

Pre-handling of bacterial strains

All strains were retrieved from the stock cultures stored at −80°C. A loop of stock cultures were streaked on MRS agar (Merck 1.10660, Germany) plates and incubated at 37°C for 48 h to obtain isolated single colonies. A single colony was then inoculated in 10 ml of MRS broth (Merck 1.10661, Germany) and incubated for 24 h at 37°C. Two steps of subculturing were performed transferring 100 µl of overnight cultures to 10 ml of MRS broth to regain the viability after long-term storage at −80°C, and 1 ml of the second subculture incubated for 20 h was centrifuged at 13,000 rpm for 20 min to remove spent media. The cell pellet was re-suspended in 1 ml of ringer's solution (Merck 1.15525, Germany) to prepare the inoculum for the experiment.

Preparation of media for prebiotic assays

In order to determine the substrate preference of different LAB, a carbohydrate-free MRS (cfMRS) broth was prepared according to the formula listed in Table 1, from first principles. RAF, SAC, and INU were supplemented to the cfMRS medium separately, at 18 g/l concentration. The same concentration of D + glucose (Merck G8270) was supplemented as the control of the prebiotic assays. Each supplemented medium was then filter sterilized using 0.2-µm syringe filters (Merck WHA69012502).

Table 1 Formula of cfMRS preparation for prebiotic assays

Ingredient	Amount per liter
Oxoid peptone	10 g
Yeast extract	5 g
Tween 80	1 ml
K_2HPO_4	2 g
Sodium acetate	5 g
Triammonium citrate	2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$MnSO_4 \cdot 4H_2O$	0.05 g

Preparation of plant extracts for growth curve analysis

Turmeric and green tea extracts (CUR and GT)

Turmeric and green tea extracts (spray-dried products in fine powder form) were measured in required quantities and directly dissolved in MRS broth at respective concentrations of supplementation. Finally, the supplemented broth media were filter sterilized using 0.2 µm syringe filters.

Garlic extract (G)

Fresh garlic bulbs cv. Thermodrome were chopped in to 3–5 mm slices and air-dried for 2 days at 40°C and 5 days at 50°C. Then, air-dried garlic chips were milled into powder and subsequently sieved with a 1 mm sieve. This powder was stored at −20 °C until usage. Of sieved garlic powder, 1.25 g was incubated with 10 ml of distilled water at room temperature to activate the alliinase enzyme reaction to produce allicin from alliin. First, the mixture was mixed using a vortex mixer briefly for 20 s and then shaken for 8 min at 550 rpm. After, the mixture was left for sedimentation for another 2 min. Finally, the mixture was centrifuged at 10,000 rpm for 5 min, and the supernatant was obtained. This was filter sterilized using a 0.2 µm syringe filter before it was added to MRS broth for the growth kinetic assays.

Experimental design

Growth kinetic assays for the six LAB with respective prebiotic and plant extract supplementation (Table 2) were performed in 96-well plates (TPP B-0683). Plates were incubated at 37°C for 30 h under aerobic conditions in Hidex Sense microplate reader, and absorbance at an optical density 600 (OD600) was measured at hourly intervals. Plates were shaken orbitally at 300 rpm speed for 10 s before taking each reading. For each treatment, a negative control (without bacteria) was used as a blank. The average absorbance from triplicate wells/LAB/treatment was used to graph the growth curves using GraphPad Prism 9.5.0.

Table 2 Prebiotic and plant extract supplements used in the experiment

Treatment		Annotation	Concentration (w/v)
Prebiotics	Raffinose	RAF	1.8%
	Saccharicterpenin	SAC	
	Inulin	INU	
Plant extracts	Turmeric	CUR1	0.06%
		CUR2	0.1%
		CUR3	0.6%
	Green tea	GT1	0.06%
		GT2	0.1%
		GT3	0.6%
	Garlic	G1	0.25%
		G2	0.5%
		G3	1%

Data illustration and statistical analysis

The average values of triplicate growth curves were plotted using GraphPad Prism 9.0 version. The growth curve data of the triplicates was applied in to the Gompertz model using "nls" function in R software 4.3.1 version to obtain maximum OD/growth, maximum growth rate, and lag time. The growth parameters extracted from triplicate growth curves of different levels of each supplement were compared using one-way ANOVA test in R software 4.3.1 version. The mean comparison was performed using the Tukey's honest significant difference (HSD) test.

Results

Growth of lactic acid bacteria strains supplemented with different energy sources

The growth of the six LAB strains when supplemented with different commercial prebiotics as the sole energy source is shown in Fig. 1 and Table 3. The growth data of *L. casei*, *L. rhamnosus*, and *P. pentosaceus* when supplemented with Raffinose did not fit the standard Gompertz model where they displayed poor growth as compared to the control group (− 80%, − 82%, and − 72%, respectively). This indicates that these LAB strains did not prefer RAF as their energy source. Nevertheless, the remaining LAB strains (*L. plantarum*, *L. reuteri*, and *L. mesenteroides*) displayed a considerable growth when supplemented with

70:82092817

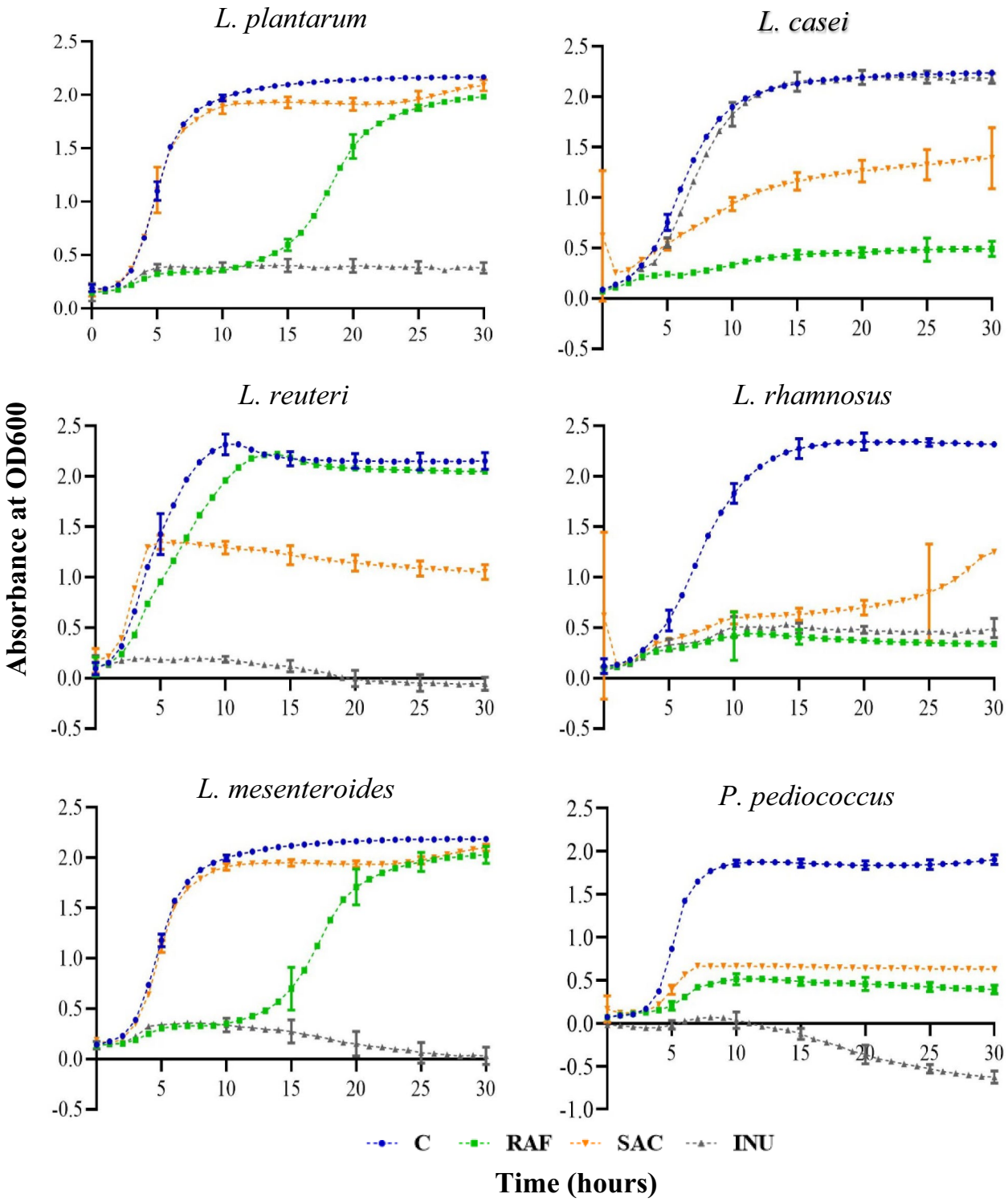

Fig. 1 Growth kinetics (OD600 absorbance vs time (h)) for six LAB in the cfMRS media supplemented with different commercial prebiotics (1.8% w/v). C: control supplemented with glucose is indicated in blue color. RAF: supplemented with Raffinose is indicated in green color. SAC: supplemented with Saccaricterpenin is indicated in orange color. INU: supplemented with Inulin is indicated in ash color (error bars: 95% confidence interval)

Table 3 The growth parameters (mean ± SD) of lactic acid bacteria (LAB) when different carbohydrate sources were used (C: glucose/RAF: Raffinose/ INU: Inulin and SAC: Saccharicterpenin) as the sole energy source

LAB	C	RAF	INU	SAC	Significance
	Max OD/growth				
L. plantarum	2.13 ± 0.01^{a}	2.03 ± 0.01^{b}	NA	1.95 ± 0.02^{c}	***
L. casei	2.21 ± 0.01^{a}	NA	2.19 ± 0.03^{a}	1.36 ± 0.09^{b}	***
L. reuteri	2.12 ± 0.03^{a}	2.11 ± 0.00^{b}	NA	NA	*
L. mesenteroides	2.15 ± 0.01^{a}	2.05 ± 0.03^{b}	NA	1.97 ± 0.01^{a}	***
	Max growth rate				
L. plantarum	1.07 ± 0.03^{b}	0.52 ± 0.01^{c}	NA	1.14 ± 0.02^{a}	***
L. casei	0.80 ± 0.01^{a}	NA	0.83 ± 0.04^{a}	0.22 ± 0.01^{b}	***
L. reuteri	1.29 ± 0.06^{a}	0.84 ± 0.0^{b}	NA	NA	***
L. mesenteroides	1.07 ± 0.01^{b}	0.60 ± 0.02^{c}	NA	1.20 ± 0.01^{a}	***
	Lag time				
L. plantarum	2.68 ± 0.08^{b}	13.58 ± 0.27^{a}	NA	2.75 ± 0.12^{b}	***
L. casei	2.70 ± 0.14	NA	3.68 ± 0.05	2.32 ± 1.6	NS
L. reuteri	1.96 ± 0.04^{b}	2.39 ± 0.02^{a}	NA	NA	***
L. mesenteroides	2.41 ± 0.04^{b}	12.89 ± 0.56^{a}	NA	2.86 ± 0.05^{b}	***

The alphabetical order of superscripts indicate the statistically different (Tukey HSD) means of treatment levels in descending order. "NA" indicates the treatments where growth of the LAB was not observed (growth data were not fitted to Gompertz model)

NS not significant

***p value > 0.0001, **p > 0.001, *p > 0.01

Raffinose although the maximum growth/OD and maximum growth rate were lower and lag time was higher than the respective values of the control.

Interestingly, Inulin was efficiently utilized as an energy source by *L. casei* where all the growth parameters when supplemented with Inulin were statistically similar to the control. Surprisingly, INU was not preferred as an energy source by the remaining five LAB stains where they displayed a considerably lower growth compared to the control (*L. plantarum* – 81%, *L. reuteri* – 94%, *L. rhamnosus* – 78%, *L. mesenteroides* – 87% and *P. pentosaceus* – 100%) at 15 h of incubation.

Moreover, *L. mesenteroides* displayed statistically similar maximum growth/OD and lag time and higher maximum growth rate compared to the respective values of the control when supplemented with Saccharicterpenin. Similarly, the Saccharicterpenin-supplemented *L. plantarum* showed a higher maximum growth rate compared to that of the control although it did not reach the maximum growth/OD of the control. The remaining strains did not show promise on utilizing Saccharicterpenin as an energy source successfully.

Given these results, Saccharicterpenin with *L. plantarum* or *L. mesenteroides* and Inulin with *L. casei* can be selected as compatible pairs for potential synergistic synbiotic production. Interestingly, *L. rhamnosus* and *P. pentosaceus* strains tested in the current study did not show a compatibility with any of the prebiotics studied as potential synbiotic products.

Growth of lactic acid bacteria strains supplemented with turmeric extract

Growth of six LAB strains supplemented with varying concentrations of turmeric extract is shown in Table 4 and Fig. 2. Interestingly, *P. pentosaceus* and *L. mesenteroides* displayed the prebiotic effects with the turmeric extract supplementation at all three concentrations studied. *P. pentosaceus* displayed a statistically similar maximum growth/OD and maximum growth rate at all the concentrations tested compared to the control. Interestingly, it further showed a lower lag time when supplemented with the highest concentration (CUR3 0.6%) of the turmeric extract. Moreover, *L. mesenteroides* displayed higher maximum growth/OD and maximum growth rate compared to other treatment levels and the control when supplemented with the highest concentration of the turmeric extract. Furthermore, *L. reuteri* displayed a statistically similar maximum growth/OD and lag time with the supplementation of all three levels of turmeric extract although the maximum growth rate of the highest concentration was statistically lower than that of the other treatment levels and the control. All in all, the results indicate that 0.06% and 0.1% turmeric extract supplementation did not cause any inhibition (which could be expected due to the curcumin effects) to all six LAB strains studied in the current study. Therefore, according to our results, 0.1% turmeric extract can be selected as a suitable candidate for potential prophybiotic formulation in combination with all six LAB studied while with *P. pentosaceus* and *L.*

Table 4 The growth parameters (mean ± SD) of lactic acid bacteria (LAB) when different levels of turmeric (C: zero turmeric, control/CUR1 0.06%/CUR2 0.1% and CUR3 0.6%) were supplemented to the MRS broth media

LAB	C	CUR1	CUR2	CUR3	Significance
	Max OD/growth				
L. plantarum	2.19 ± 0.01^{a}	2.17 ± 0.01^{a}	2.17 ± 0.01^{a}	2.11 ± 0.02^{b}	***
L. casei	2.5 ± 0.04^{a}	2.37 ± 0.04^{b}	2.32 ± 0.02^{b}	2.35 ± 0.08^{b}	**
L. reuteri	1.96 ± 0.06	1.91 ± 0.03	1.93 ± 0.02	1.88 ± 0.01	NS
L. rhamnosus	2.25 ± 0.03^{a}	2.26 ± 0.01^{a}	2.28 ± 0.00^{a}	2.08 ± 0.06^{b}	***
L. mesenteroides	0.85 ± 0.03^{b}	0.86 ± 0.00^{b}	0.92 ± 0.12^{ab}	1.06 ± 0.00^{a}	**
P. pentosaceus	1.78 ± 0.09	1.77 ± 0.07	1.85 ± 0.01	1.85 ± 0.03	NS
	Max growth rate				
L. plantarum	0.94 ± 0.04^{a}	0.97 ± 0.03^{a}	0.97 ± 0.05^{a}	0.59 ± 0.01^{b}	***
L. casei	0.24 ± 0.01^{b}	0.26 ± 0.01^{a}	0.27 ± 0.01^{a}	0.20 ± 0.01^{c}	***
L. reuteri	0.24 ± 0.06^{a}	0.26 ± 0.01^{a}	0.27 ± 0.05^{a}	0.20 ± 0.01^{b}	**
L. rhamnosus	0.62 ± 0.01	0.62 ± 0.00	0.59 ± 0.03	0.64 ± 0.02	NS
L. mesenteroides	0.26 ± 0.01^{b}	0.27 ± 0.00^{ab}	0.27 ± 0.01^{ab}	0.28 ± 0.00^{a}	*
P. pentosaceus	1.04 ± 0.07	0.99 ± 0.02	1.03 ± 0.03	1.11 ± 0.61	NS
	Lag time				
L. plantarum	1.10 ± 0.06^{b}	1.43 ± 0.09^{b}	1.43 ± 0.20^{b}	2.49 ± 0.20^{a}	***
L. casei	5.76 ± 0.62^{a}	5.63 ± 0.24^{a}	5.92 ± 0.51^{a}	1.57 ± 1.23^{b}	***
L. reuteri	1.87 ± 0.08	2.10 ± 0.11	2.17 ± 0.39	2.10 ± 0.01	NS
L. rhamnosus	2.82 ± 0.37^{b}	3.39 ± 0.18^{b}	2.99 ± 0.7^{b}	4.71 ± 0.10^{a}	**
L. mesenteroides	8.68 ± 0.30	8.48 ± 0.00	8.51 ± 0.04	8.56 ± 0.00	NS
P. pentosaceus	2.65 ± 0.13^{a}	2.70 ± 0.03^{a}	2.67 ± 0.12^{a}	2.40 ± 0.11^{b}	*

The alphabetical order of superscripts indicate the statistically different (Tukey HSD) means of treatment levels in descending order

NS not significant

***p value > 0.0001, **p > 0.001, *p > 0.01

mesenteroides, it could be increased up to 0.6% to maximize the benefits of the combination.

Growth of lactic acid bacteria strains supplemented with green tea extract

The growth of the six LAB strains supplemented with varying concentrations of green tea extract (Table 5 and Fig. 3) revealed that green tea displays inhibitory effect towards the growth of these LAB strains tested in the current study. At the highest concentration (GT3 0.6%), there was a strong inhibition of all six LAB studied (*L. plantarum* – 89%, *L. casei* – 70%, *L. reuteri* – 80%, *L. rhamnosus* – 86%, *L. mesenteroides* – 97%, and *P. pentosaceus* – 88% at 15 h) compared to the control growth. Thus, these growth data did not fit to the Gompertz growth model successfully. Notably, the maximum growth/OD of all LAB strains at all the levels of green tea extract supplementation was statistically lower compared to that of the respective controls except for *L. mesenteroides*. Although statistically similar maximum growth was observed at all levels of green tea extract supplementation, the growth rate of the *L. mesenteroides* was significantly lower compared to the control indicating that the maximum growth was somehow achieved at a slower pace with the green tea supplementation. Additionally, *L. casei* and *L. reuteri* displayed similar maximum growth rate as compared to the respective controls when supplemented with the two lower concentrations (0.06% and 0.1%) of green tea extract despite their lower maximum growth/OD achieved. Visually, *L. casei* displayed the highest resistance to inhibition by GT with 12% more growth observed with GT1 treatment (0.06%) compared to growth in the control media at 15 h although achieved the stationary phase at a lower OD. Given these results, green tea extract (at any concentration studied) was not selected as a suitable candidate for potential prophybiotic formulation as it showed negative effects on the growth of most of LAB strains studied.

Growth of lactic acid bacteria strains supplemented with garlic extract

The growth of the six LAB strains supplemented with varying concentrations of garlic extract is indicated in Table 6 and Fig. 4. Indeed, the results of the garlic extract supplementation showed the promise for prophybiotic formulation with the LAB used in the current study. Interestingly, garlic extract supplementation displayed prebiotic effects on *L. reuteri* and *P. pentosaceus* where they display higher maximum

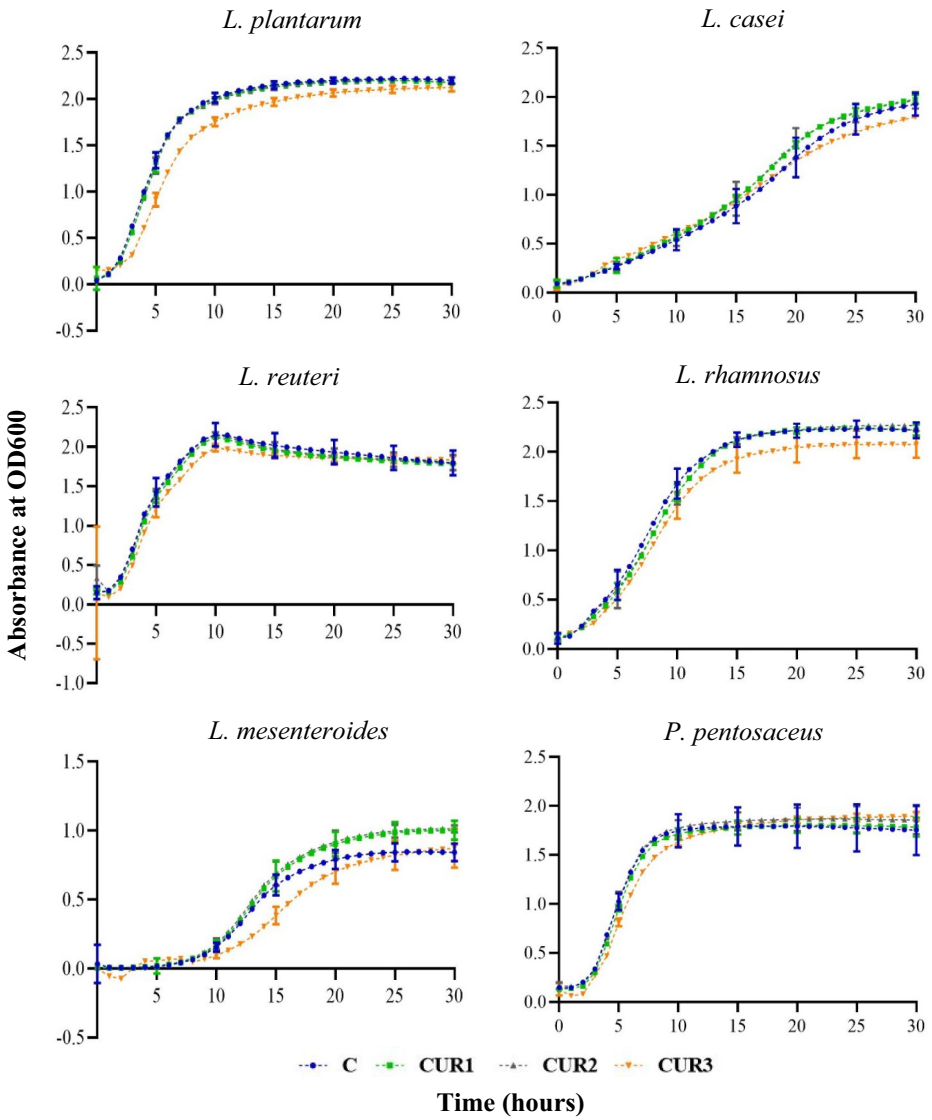

Fig. 2 Growth kinetics (OD600 absorbance vs time (h)) for six LAB in MRS media supplemented with different concentrations of turmeric extract. C: control media without any turmeric extract supplementation is indicated in blue color. CUR1: supplemented with 0.06% (w/v) turmeric extract is indicated in green color. CUR2: supplemented with 0.1% (w/v) turmeric extract is indicated in grey color. CUR3: supplemented with 0.6% (w/v) turmeric extract is indicated in orange color. Error bars: 95% confidence interval

growth with the supplementation of garlic extract as compared to the respective controls. Moreover, all the strains indicated non-inhibitory effects with the supplementation of garlic extract generally, 0.5% being the most beneficial concentration to almost all the strains studied. Therefore, 0.5% garlic extract was selected as a suitable candidate for potential prophybiotic formulation with all six LAB studied.

Discussion

Initial screening of the compatibility of bioactive compounds is extremely important for the success of synergistic synbiotic production (Wu et al. 2017). The current study aided in the identification of compatible bioactive substances, either commercial prebiotics or plant extracts for use in potential formulations of synergistic synbiotics or prophybiotics, respectively, with the six LAB studied. All the LAB strains used in the current study are commercially used in probiotic products for poultry and swine, produced by JHJ sp. z o.o due to their proven beneficial characteristics in improving gut health (Jhj-lavipan-2021; Smialek et al. 2018, 2019).

Commercial prebiotics, Raffinose and Inulin used in the current study, are well known for their prebiotic potential as reviewed by Anggraeni (2022) and Teferra (2021), respectively. However, in the current study, those prebiotics were not compatible with many of the LAB such as *L. rhamosus* and *P. pentosaceus* indicating the limited use of these commercial prebiotics in synergistic synbiotic application with these strains. Saccharicterpenin is a novel feed additive derived from extracts of Theaceae plants (Liu et al. 2019) containing primarily polysaccharides and triterpinoides (Liu et al. 2016) with wide variety of benefits to livestock including antioxidant activity (Liu et al. 2019), intestinal development (Peng et al. 2011), and digestive enzyme activity (Liu et al. 2016).

Table 5 The growth parameters (mean ± SD) of lactic acid bacteria (LAB) when different levels of green tea extract (C: zero green tea, control/GT1 0.06%/GT2 0.1%) were supplemented to the MRS broth media

LAB	C	GT1	GT2	Significance
	Max OD/growth			
L. plantarum	2.12 ± 0.01^{a}	1.82 ± 0.00^{b}	1.70 ± 0.01^{c}	***
L. casei	2.50 ± 0.04^{a}	1.93 ± 0.03^{b}	1.93 ± 0.09^{b}	***
L. reuteri	1.9 ± 0.06^{a}	1.57 ± 0.04^{b}	1.66 ± 0.02^{b}	***
L. rhamnosus	2.13 ± 0.01^{a}	1.66 ± 0.01^{c}	1.77 ± 0.03^{b}	***
L. mesenteroides	0.76 ± 0.00	0.74 ± 0.04	0.74 ± 0.04	NS
P. pentosaceus	1.88 ± 0.04^{a}	1.19 ± 0.03^{c}	1.31 ± 0.02^{b}	***
	Max growth rate			
L. plantarum	0.79 ± 0.01^{a}	0.62 ± 0.01^{b}	0.41 ± 0.03^{c}	***
L. casei	0.24 ± 0.01^{a}	0.24 ± 0.00^{a}	0.18 ± 0.02^{b}	**
L. reuteri	1.06 ± 0.06^{a}	0.94 ± 0.04^{a}	0.75 ± 0.03^{b}	***
L. rhamnosus	0.54 ± 0.01^{a}	0.48 ± 0.01^{b}	0.37 ± 0.01^{c}	***
L. mesenteroides	0.76 ± 0.00^{a}	0.74 ± 0.00^{b}	0.74 ± 0.01^{b}	***
P. pentosaceus	0.97 ± 0.2^{a}	0.78 ± 0.02^{b}	0.63 ± 0.01^{c}	***
	Lag time			
L. plantarum	3.80 ± 0.02	3.67 ± 0.21	3.10 ± 0.69	NS
L. casei	3.70 ± 2	4.56 ± 0.65	2.54 ± 1.75	NS
L. reuteri	2.31 ± 0.25^{a}	2.01 ± 0.12^{a}	0.93 ± 0.61^{b}	*
L. rhamnosus	3.79 ± 0.45^{a}	3.90 ± 0.71^{a}	-1.92 ± 0.20^{b}	**
L. mesenteroides	7.62 ± 0.00^{b}	7.99 ± 0.86^{b}	9.65 ± 1.13^{a}	*
P. pentosaceus	3.06 ± 0.05^{b}	3.28 ± 0.10^{a}	2.28 ± 0.16^{c}	***

The alphabetical order of superscripts indicates the statistically different (Tukey HSD) means of treatment levels in descending order

NS not significant

***p value > 0.0001, **p > 0.001, *p > 0.01

However, its prebiotic potential has not been studied to date. Hence, to our knowledge, our study is the first to show the potential of Saccharicterpenin as a prebiotic for lactic acid bacteria. As we observed that *L. plantarum* and *L. mesenteroides* were able to use Saccharicterpenin as an energy source, Saccharicterpenin displays the potential to be utilized for developing a potential synergistic synbiotic along with these two LAB. However, further investigation is necessary to elucidate the potential of Saccharicterpenin as a prebiotic in terms of its effects on the poultry gut microbiota.

The results of supplementation of turmeric extract suggested that turmeric extract might be a suitable candidate for potential prophybiotic formulation in combination with the LAB studied. In compliance with our results, other studies have shown that turmeric enhanced the growth of probiotics such as *L. rhamnosus* GG ATCC 53103 and *Bifidobacterium animalis* BB12 (Yazdi et al. 2019) and did not inhibit the growth of *L. acidophilus* (Ilham et al. 2018), *L. acidophilus* A001F8, *L. rhamnosus* A001G8, *L. paracasei* A002C5, *L. plantarum* A003A7, and *L. casei* A003D4 (Kim et al. 2020). In addition to that, previous literature has also shown that turmeric in combination with *Lactobacillus* probiotics resulted in enhanced antimicrobial activity (Kim et al. 2020) and anti-allergic inflammatory activity (Yazdi et al. 2020) while improving poultry production parameters (Kinati et al. 2022). These studies along with our current results indicate that turmeric may be a potential candidate to use in combination with *Lactobacillus* species without affecting bacterial growth for potential prophybiotic application.

On the other hand, existing literature has shown that green tea modulates the composition of intestinal microbiota to improve overall gut health (Chen et al. 2019), while green tea in combination with probiotics reduced the high-fat-diet-induced inflammation in mice (Axling et al. 2012) and hepatorenal syndrome in rat model (Al-Okbi et al. 2019), indicating that green tea is an excellent candidate for prophybiotic application. However, our results demonstrate that supplementation of green tea extracts at higher doses can inhibit the growth of the LAB strains used. In contrast, Story et al. (2009) found that the growth of *L. acidophilus* and *L. gasseri* was increased even at higher concentrations of green tea supplementation. Moreover, several studies have reported that the count of *Lactobacillus* starter cultures in yoghurt is increased with green tea supplementation (Lim 2017; Marhamatizadeh et al. 2013; Najgebauer-Lejko 2014). Interestingly, Janiak et al. (2018) claim that the variation of effects of green tea on probiotic growth could be due to the composition of polyphenolic compounds. The authors reported that the catechins (monomeric flavan-3-ols) help to modulate the growth of microorganisms more selectively than the polymeric fraction in green tea. Proanthocyanidins will inhibit microbial growth more generally and efficiently. Therefore, these findings highlight the importance of performing the individual growth curves for selected probiotic strains with a particular green tea extract when selecting the combinations for potential prophybiotic application as the LAB strains used in the current study showed sensitivity to green tea at higher concentrations.

Our results indicated that garlic extract did not inhibit the growth of most LAB strains while it displayed prebiotic effects on some strains. Interestingly, garlic has been

75:90799492

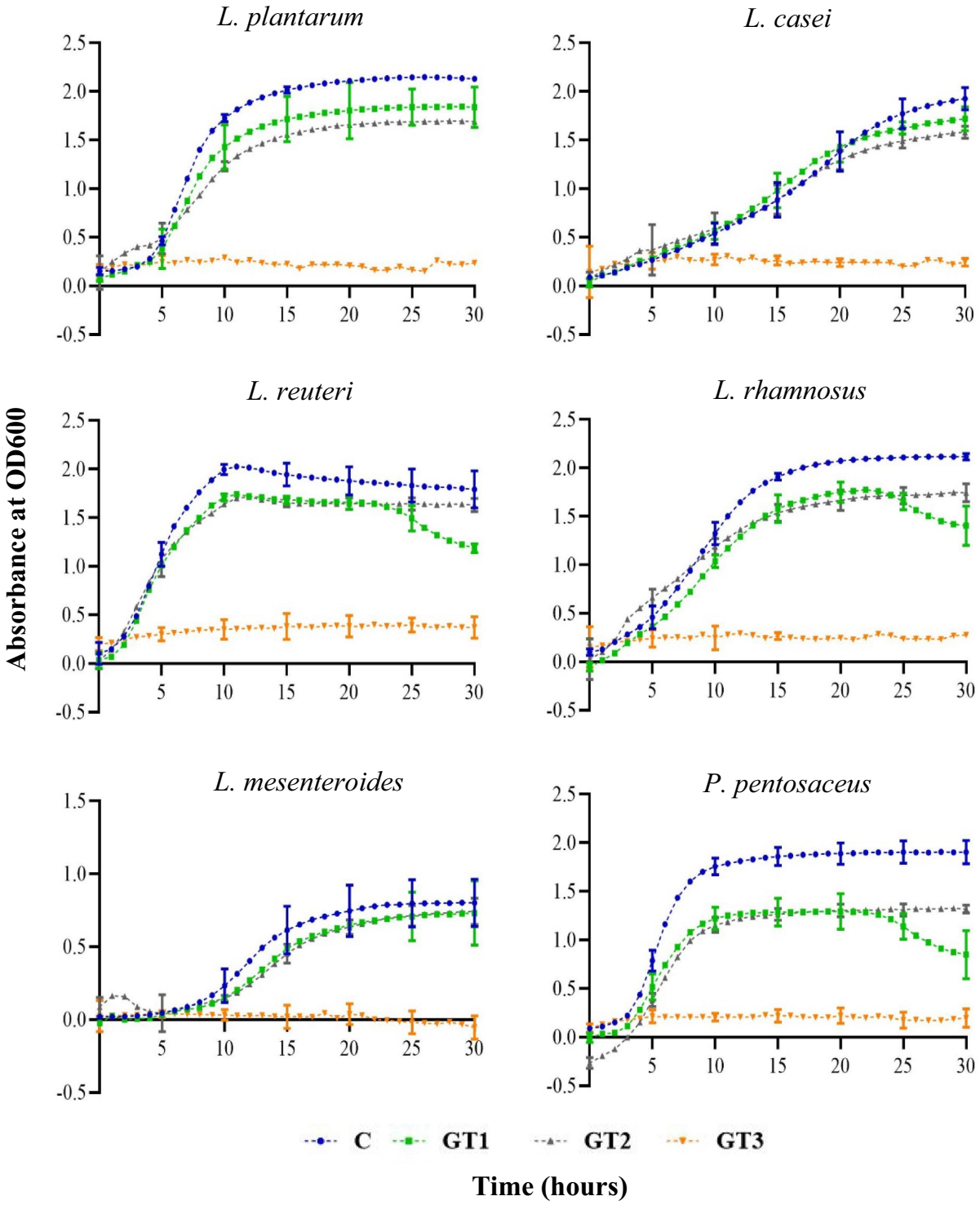

Fig. 3 Growth kinetics (OD600 absorbance vs time (h)) for six LAB in MRS media supplemented with different concentrations of green tea extract. C: control media without any green tea extract supplementation is indicated in blue color. GT1: supplemented with 0.06% (w/v) green tea extract is indicated in green color. GT2: supplemented with 0.1% (w/v) green tea extract is indicated in grey color. GT3: supplemented with 0.6% (w/v) green tea extract is indicated in orange color. Error bars: 95% confidence interval

reported to have prebiotic effects particularly on *Lactobacillus* species (Lu et al. 2021; Sunu et al. 2019; Sutherland et al. 2009) and *Bifidobacterium* species (Zhang et al. 2013). However, some contrasting results have also been found in the literature. Altuntas and Korukluoglu (2019) and Booyens and Thantsha (2013) observed that garlic extracts display

Table 6 The growth parameters (mean ± SD) of lactic acid bacteria (LAB) when different levels of garlic extract (C: zero garlic, control/G1 0.25%/ G2 0.5% and G3 0.1%) were supplemented to the MRS broth media

LAB	C	G1	G2	G3	Significance
	Max OD/growth				
L. plantarum	2.13 ± 0.03	1.95 ± 0.28	2.10 ± 0.01	2.09 ± 0.02	NS
L. casei	2.09 ± 0.03	2.10 ± 0.02	2.08 ± 0.01	2.09 ± 0.02	NS
L. reuteri	1.81 ± 0.03^{c}	1.92 ± 0.01^{b}	1.95 ± 0.01^{ab}	1.99 ± 0.01^{a}	***
L. rhamnosus	2.25 ± 0.03^{a}	2.22 ± 0.02^{a}	2.19 ± 0.01^{a}	2.11 ± 0.03^{b}	***
L. mesenteroides	0.97 ± 0.03^{a}	0.86 ± 0.02^{ab}	0.94 ± 0.05^{a}	0.90 ± 0.03^{a}	*
P. pentosaceus	1.79 ± 0.07^{b}	1.80 ± 0.01^{b}	1.94 ± 0.02^{a}	1.96 ± 0.02^{a}	***
	Max growth rate				
L. plantarum	0.86 ± 0.01^{b}	0.93 ± 0.01^{a}	0.90 ± 0.01^{a}	0.82 ± 0.01^{c}	***
L. casei	0.64 ± 0.01^{a}	0.65 ± 0.01^{a}	0.65 ± 0.01^{a}	0.55 ± 0.01^{b}	***
L. reuteri	1.25 ± 0.04	1.21 ± 0.02	1.24 ± 0.06	1.23 ± 0.03	NS
L. rhamnosus	0.68 ± 0.02^{a}	0.71 ± 0.00^{a}	0.66 ± 0.02^{a}	0.57 ± 0.04^{b}	***
L. mesenteroides	0.31 ± 0.01^{a}	0.27 ± 0.01^{b}	0.29 ± 0.02^{ab}	0.21 ± 0.01^{c}	***
P. pentosaceus	0.93 ± 0.03^{b}	0.90 ± 0.01^{bc}	1.00 ± 0.01^{a}	0.97 ± 0.01^{ab}	***
	Lag time				
L. plantarum	1.62 ± 0.01^{b}	1.91 ± 0.06^{a}	1.92 ± 0.11^{a}	1.45 ± 0.04^{b}	***
L. casei	3.81 ± 0.10	4.01 ± 0.38	3.93 ± 0.06	3.59 ± 0.35	NS
L. reuteri	1.65 ± 0.08	1.71 ± 0.03	2.01 ± 0.29	1.86 ± 0.12	NS
L. rhamnosus	3.16 ± 0.06^{b}	3.37 ± 0.04^{a}	3.39 ± 0.09^{a}	2.65 ± 0.08^{c}	***
L. mesenteroides	4.58 ± 0.06^{b}	5.08 ± 0.02^{a}	5.19 ± 0.17^{a}	4.89 ± 0.16^{ab}	**
P. pentosaceus	2.37 ± 0.08^{c}	2.42 ± 0.03^{bc}	2.62 ± 0.05^{a}	2.53 ± 0.05^{ab}	**

The alphabetical order of superscripts indicates the statistically different (Tukey HSD) means of treatment levels in descending order

NS not significant

***p value > 0.0001, **p > 0.001, *p > 0.01

antimicrobial effects on *L. acidophilus* and *Bifidobacterium* species. However, in the latter study, the inhibition of probiotic growth by fresh garlic extracts (crushing garlic cloves) was significantly higher than that of garlic powder extract. The authors suggest that it is possibly due to the presence of more active allinase enzymes in fresh cloves when compared with the powdered garlic which will produce more allicin (the active antimicrobial compound) during the extraction process. Since powdered garlic has been used in the current study, it is possible that the allicin content in our garlic extract was less than that of the study of Booyens and Thantsha which resulted in inhibition of probiotics. However, in the same study, it was shown that the sensitivity of different probiotics to garlic extract varied. Therefore, it is also possible that the strains that we have tested in the current study are more resistant to antimicrobial effects of garlic. Therefore, it is imperative to focus on the content of the antimicrobial compounds in the phytobiotics when screening for potential prophybiotic combinations. Therefore, growth curve analysis of probiotics in each case is required to develop successful potential prophybiotics.

It is also important to highlight that the effect of the supplementation of these prebiotics and plant extracts may be different in different strains of the same LAB species owing to wide metabolic differences within the strains of LAB species. Nonetheless, considering the results of the strains used in the current study, prophybiotic formulation seemed promising as plant extracts used in the current study did not inhibit the LAB studied in two out of three species. Therefore, it shows the potential to use a mixture of these LAB along with plant extracts (turmeric or garlic) to optimize the beneficial effects on the gut health of the host. However, as the LAB were very selective in their ability to exploit the commercial prebiotics as their energy source, use of a mixture of LAB with commercial prebiotics, as a synergistic symbiotic, might not be possible due to this selectivity. Nevertheless, the main constraint of prophybiotic formulations is the differences among different cultivars or different extraction systems in terms of bioactive composition. Therefore, we suggest that more future research is necessary to elucidate the potential of prophybiotic formulation, minimizing these constraints.

Conclusion

Garlic and turmeric extracts displayed non-inhibitory effects for all LAB strains studied indicating their potential to use in prophybiotic formulations in the future.

77:35116353

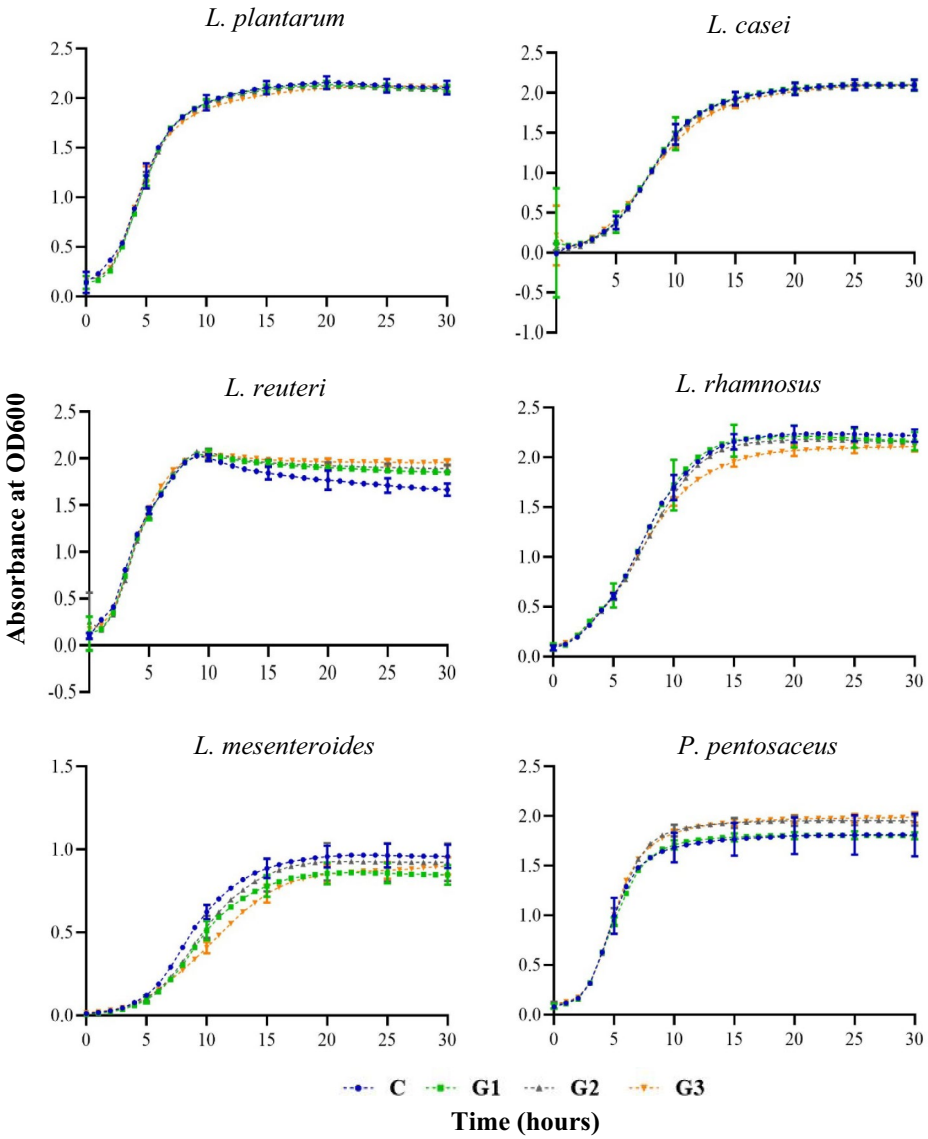

Fig. 4 Growth kinetics (OD600 absorbance vs time (h)) for six LAB in MRS media supplemented with different concentrations of garlic extract. C: control media without any garlic extract supplementation is indicated in blue color. G1: supplemented with 0.25% (w/v) garlic extract is indicated in green color. G2: supplemented with 0.5% (w/v) garlic extract is indicated in grey color. G3: supplemented with 1% (w/v) garlic extract is indicated in orange colr. Error bars: 95% confidence interval

Nevertheless, the commercial prebiotics displayed the potential as an energy substrate limited only to particular LAB indicating a limited use of these prebiotics in synergistic symbiotic formulation with the LAB studied.

Acknowledgements We acknowledge IC Lab, Krakow, Poland, for providing the Hidex Sense microplate reader and JHJ sp z o.o., Poland, for providing lactic acid bacteria for our experiment. We also thank Ovobime project funded by National Science Centre, Poland, and Kaesler Nutrition GmbH, Cuxhaven, Germany for supplying us the prebiotics and plant extracts (turmeric and green tea) for this study.

Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Ramesha N. Wishna Kadawarage. The first draft of the manuscript was written by Ramesha N. Wishna Kadawarage. Funding acquisition, supervision, review, and editing was by Rita M. Hickey and Maria Siwek. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This research was carried out under the funding from the European Union's Horizon 2020 research and innovation program under grant agreement no. 955374.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated

otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Adamczak A, Ożarowski M, Karpiński TM (2020) Curcumin, a natural antimicrobial agent with strain-specific activity. Pharmaceuticals 13(7):153. https://doi.org/10.3390/ph13070153

Al-Okbi YS, Mohamed AD, Hamed EST, Abd El Khalek AB, Mohammed ES (2019) Role of probiotic mixture with and without green tea extract in prevention of hepatorenal syndrome in rat model. Pak J Biol Sci 22(1):21–27. https://doi.org/10.3923/pjbs.2019.21.27

Altuntas S, Korukluoglu M (2019) Growth and effect of garlic (*Allium sativum*) on selected beneficial bacteria. Food Sci Technol 39:897–904. https://doi.org/10.1590/fst.10618

Anggraeni AA (2022) Mini-review: the potential of raffinose as a prebiotic. IOP Conf Ser: Earth and Environmental Science 980(1):012033. https://doi.org/10.1088/1755-1315/980/1/012033

Axling U, Olsson C, Xu J, Fernandez C, Larsson S, Ström K, Ahrné S, Holm C, Molin G, Berger K (2012) Green tea powder and Lactobacillus plantarum affect gut microbiota, lipid metabolism and inflammation in high-fat fed C57BL/6J mice. Nutr Matab 9:105. https://doi.org/10.1186/1743-7075-9-105

Bhatwalkar SB, Mondal R, Krishna SBN, Adam JK, Govender P, Anupam R (2021) Antibacterial properties of organosulfur compounds of garlic (Allium sativum). Front Microbiol 12. https://www.frontiersin.org/articles/https://doi.org/10.3389/fmicb.2021.613077

Bindels LB, Delzenne NM, Cani PD, Walter J (2015) Towards a more comprehensive concept for prebiotics. Nat Rev Gastroenterol Hepatol 12(5):Article 5. https://doi.org/10.1038/nrgastro.2015.47

Booyens J, Thantsha MS (2013) Antibacterial effect of hydrosoluble extracts of garlic (Allium sativum) against Bifidobacterium spp. And Lactobacillus acidophilus. Afr J Microbiol Res 7(8):669–677. https://doi.org/10.5897/AJMR12.1906

Chen X, Zhu W, Liu X, Li T, Geng Z, Wan X (2019) The growth performance, meat quality, and gut bacteria of broilers raised with or without antibiotics and green tea powder. J Appl Poult Res 28(3):712–721. https://doi.org/10.3382/japr/pfz023

Chen K, Nakasone Y, Xie K, Sakao K, Hou DX (2020) Modulation of allicin-free garlic on gut microbiome. Molecules 25(3):682. https://doi.org/10.3390/molecules25030682

Diaz Carrasco JM, Casanova NA, Fernández Miyakawa ME (2019) Microbiota, gut health and chicken productivity: what is the connection? Microorganisms 7(10):374. https://doi.org/10.3390/microorganisms7100374

EFSA Biohaz Panel (EFSA Panel on Biological Hazards), Koutsoumanis K, Allende A et al (2023) Scientific Opinion on the update of the list of qualified presumption of safety (QPS) recommended microorganisms intentionally added to food or feed as notified to EFSA. EFSA J 21(1):7747. https://doi.org/10.2903/j.efsa.2023.7747

Gopal J, Muthu M, Paul D, Kim DH, Chun S (2016) Bactericidal activity of green tea extracts: the importance of catechin containing nano particles. Sci Rep 6(1):Article 1. https://doi.org/10.1038/srep19710

Ilham LA, Herla R, Dwi S, DewiRestuana S (2018) Antimicrobial activity of turmeric leaf extract against Escherichia coli, Staphylococcus aureus, Shigella dysenteriae, and Lactobacillus acidophilus. IOP Conf Ser: Earth and Environmental Science 205(1):012048. https://doi.org/10.1088/1755-1315/205/1/012048

Janiak MA, Amarowicz R, Rostek D (2018) Influence of catechin fraction and high molecular fraction from green tea extract on Lactobacillus, Bifidobacterium and Streptococcus strains. Nat Prod Commun 13(6) https://doi.org/10.1177/1934578X1801300615

Jhj-lavipan-2021-pl_A4.pdf. http://jhj.pl/files/karty_prod/Ulotki_2021/jhj-lavipan-2021-pl_A4.pdf. Accessed July 14 2023

Jung ES, Park HM, Hyun SM, Shon JC, Liu KH, Hwang JS, Lee CH (2017) Modulation of gut microbiome by green tea diet and its correlation with metabolic changes. Drug Metab Pharmacokinet 32(1 Supplement):S62. https://doi.org/10.1016/j.dmpk.2016.10.248

Kim J, Kim H, Jeon S, Jo J, Kim Y, Kim H (2020) Synergistic antibacterial effects of probiotic lactic acid bacteria with curcuma longa rhizome extract as synbiotic against Cutibacterium acnes. Appl sci 10(24):Article 24. https://doi.org/10.3390/app10248955

Kinati C, Ameha N, Girma M, Nurfeta A (2022) Effective microorganisms, turmeric (Curcuma longa), and their combination on performance and economic benefits in broilers. Heliyon 8(6):e09568. https://doi.org/10.1016/j.heliyon.2022.e09568

Krumbeck JA, Maldonado-Gomez MX, Ramer-Tait AE, Hutkins RW (2016) Prebiotics and synbiotics: dietary strategies for improving gut health. Curr Opin Gastroenterol 32(2):110. https://doi.org/10.1097/MOG.0000000000000249

Lim ES (2017) Effect of green tea supplementation on probiotic potential, physico-chemical, and functional properties of yogurt. Misainmurhag Hoiji 53(2):103–117. https://doi.org/10.7845/kjm.2017.7035

Liu J, Zeng T, Du X, Li G, Yu Y, Lu L, Li C (2016) Effects of water supplemented with Saccharicterpenin and photosynthetic bacteria on egg production, egg quality, serum immunoglobulins, and digestive enzyme activities of ducks. Can J Anim Sci 96(3):397–403. https://doi.org/10.1139/cjas-2015-0139

Liu G, Zheng J, Wu X, Jia G, Zhao H, Chen X, Wu C, Wang J (2019) Effects of saccharicterpenin on antioxidant status and urinary metabolic profile of rats. Anim Nutr 5(2):191–195. https://doi.org/10.1016/j.aninu.2018.09.003

Ljungh A, Wadström T (2006) Lactic acid bacteria as probiotics. Curr Issues Intest Microbiol 7(2):73–89

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

Marhamatizadeh MH, Ehsandoost E, Gholami P (2013) The influence of green tea (Camellia sinensis L.) extract on characteristic of probiotic bacteria in milk and yoghurt during fermentation and refrigerated storage. Int J Farming Allied Sci 2(17):599–606. http://www.ijfas.com/wp-content/uploads/2013/09/599-606.pdf

Najgebauer-Lejko D (2014) Effect of green tea supplementation on the microbiological, antioxidant, and sensory properties of probiotic milks. Dairy Sci Technol 94(4):327–339. https://doi.org/10.1007/s13594-014-0165-6

Peng H, ShanShan Z, XueWu Z, TingTing Z, ChuanBin X (2011) Effects of dietary saccharicterpenin on mucosal structure of small intestine in Gushi chickens. Chin J Anim Nutr 23(11):2016–2023. https://www.cabdirect.org/cabdirect/abstract/20123074110

Quintero GDF, Kok CR, Hutkins R (2022) The future of synbiotics: rational formulation and design. Front Microbiol 13. https://www.frontiersin.org/articles/https://doi.org/10.3389/fmicb.2022.919725

Scazzocchio B, Minghetti L, D'Archivio M (2020) Interaction between gut microbiota and curcumin: a new key of understanding for the health effects of curcumin. Nutrients 12(9):2499. https://doi.org/10.3390/nu12092499

Sharma R, Padwad Y (2020) Plant-polyphenols based second-generation synbiotics: emerging concepts, challenges, and opportunities. Nutrition 77:110785. https://doi.org/10.1016/j.nut.2020.110785

Smialek M, Burchardt S, Koncicki A (2018) The influence of probiotic supplementation in broiler chickens on population and carcass contamination with Campylobacter spp. - Field study. Res Vet Sci 118:312–316. https://doi.org/10.1016/j.rvsc.2018.03.009

Smialek M, Kaczorek E, Szczucińska E, Burchardt S, Kowalczyk J, Tykałowski B, Koncicki A (2019) Evaluation of Lactobacillus spp. and yeast based probiotic (Lavipan) supplementation for the reduction of Salmonella Enteritidis after infection of broiler chickens. Pol J Vet Sci 22(1):5–10. https://doi.org/10.24425/pjvs.2018.125616

Story EN, Azcarate-Peril A, Klaenhammer TR, Harris GK (2009) Effect of green tea and EGCG on the growth rate and cell density of the probiotic bacteria Lactobacillus acidophilus and Lactobacillus gasseri. FASEB J 23(S1):719.11-719.11. https://doi.org/10.1096/fasebj.23.1_supplement.719.11

Sunu P, Sunarti D, Mahfudz LD, Yunianto VD (2019) Prebiotic activity of garlic (Allium sativum) extract on Lactobacillus acidophilus. Vet World 12(12):2046–2051. https://doi.org/10.14202/vetworld.2019.2046-2051

Sutherland J, Miles M, Hedderley D, Li J, Devoy S, Sutton K, Lauren D (2009) In vitro effects of food extracts on selected probiotic and pathogenic bacteria. Int J Food Sci Nutr 60(8):717–727. https://doi.org/10.3109/09637480802165650

Swanson KS, Gibson GR, Hutkins R, Reimer RA, Reid G, Verbeke K, Scott KP, Holscher HD, Azad MB, Delzenne NM, Sanders ME (2020) The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. Nat Rev Gastroenterol Hepatol 17(11):Article 11. https://doi.org/10.1038/s41575-020-0344-2

Teferra TF (2021) Possible actions of inulin as prebiotic polysaccharide: a review. Food Front 2(4):407–416. https://doi.org/10.1002/fft2.92

Wu XD, Chen Y, Huang W (2017) A Perspective on the application of pro-/synbiotics in clinical practice. Front Microbiol 8:866. https://doi.org/10.3389/fmicb.2017.00866

Yadav MK, Kumari I, Singh B, Sharma KK, Tiwari SK (2022) Probiotics, prebiotics and synbiotics: safe options for next-generation therapeutics. Appl Microbiol Biotechnol 106:505–521. https://doi.org/10.1007/s00253-021-11646-8

Yazdi GF, Soleimanian-Zad S, Worm VDE, Folkerts G (2019) Turmeric extract: potential use as a prebiotic and anti-inflammatory compound? Plant Foods Hum Nutr 74(3):293–299. https://doi.org/10.1007/s11130-019-00733-x

Yazdi GF, Zakeri A, Ark, IV, Leusink-Muis, T, Braber S, Soleimanian-Zad, S, Folkerts G (2020) Crude turmeric extract improves the suppressive effects of Lactobacillus rhamnosus GG on allergic inflammation in a murine model of house dust mite-induced asthma. Front. immunol 11:1092. https://www.frontiersin.org/articles/https://doi.org/10.3389/fimmu.2020.01092

Zhang N, Huang X, Zeng Y, Wu X, Peng X (2013) Study on prebiotic effectiveness of neutral garlic fructan in vitro. Food Sci Hum Wellness 2(3):119–123. https://doi.org/10.1016/j.fshw.2013.07.001

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

World Journal of Microbiology and Biotechnology (2024) 40:133
https://doi.org/10.1007/s11274-024-03946-8

RESEARCH

In-vitro selection of lactic acid bacteria to combat *Salmonella enterica* and *Campylobacter jejuni* in broiler chickens

Ramesha N. Wishna-Kadawarage[1] · **Rita M. Hickey**[2] · **Maria Siwek**[1]

Received: 26 September 2023 / Accepted: 28 February 2024 / Published online: 14 March 2024
© The Author(s) 2024

Abstract

Campylobacter and *Salmonella* are the two most prominent foodborne zoonotic pathogens reported in the European Union. As poultry is one of the major sources of these pathogens, it is imperative to mitigate the colonization of these pathogens in poultry. Many strains of lactic acid bacteria (LAB) have demonstrated anti-*Salmonella* and anti-*Campylobacter* characteristics to varying degrees and spectrums which are attributed to the production of various metabolites. However, the production of these compounds and consequent antimicrobial properties are highly strain dependent. Therefore, the current study was performed to select a potent LAB and determine its causal attribute in inhibiting *Salmonella enterica* and *Campylobacter jejuni*, *in-vitro*. Six LAB (*Lactiplantibacillus plantarum* (LP), *Lacticaseibacillus casei* (LC), *Limosilactobacillus reuteri* (LR), *Lacticaseibacillus rhamnosus* (LRh), *Leuconostoc mesenteroides* (LM) and *Pediococcus pentosaceus* (PP)) and three serovars of *Salmonella enterica* (Typhimurium, Enterica and Braenderup) and *Campylobacter jejuni* were used in the current study. Spot overlays, well diffusion, co-culture and co-aggregation assays against *Salmonella* and well diffusion assays against *Campylobacter jejuni* were performed. Organic acid profiling of culture supernatants was performed using HPLC. The results indicated that LRh, LM and PP had the most significant anti-*Salmonella* effects while LP, LC, LM and PP displayed the most significant anti-*Campylobacter* effects. Lactic acid and formic acid detected in the culture supernatants seem the most likely source of the anti-*Salmonella* and anti-*Campylobacter* effects exhibited by these LAB. In conclusion, *Leuconostoc mesenteroides* displayed the most significant overall anti-pathogenic effects when compared to the other LAB strains studied, indicating its potential application *in-vivo*.

Keywords Anti-*Campylobacter* · Anti-*Salmonella* · Foodborne pathogens and organic acids

Introduction

Foodborne pathogens are the microorganisms which may transmit to humans via consumption of certain foods (Bintsis 2017). According to the latest reports, *Campylobacter* and *Salmonella* are the two most prominent foodborne zoonotic pathogens reported within the European Union (Authority EFS. & European Centre for Disease Prevention and Control 2022). *Salmonella* is also known as the foodborne pathogen with the highest number of reported human hospitalizations in the United States (Centers for Disease Control and Prevention 2022). Approximately, one million people become sick in the United States each year due to consumption of contaminated poultry products and the Center for Disease Control claims that chicken is one of the major sources of *Salmonella* and *Campylobacter* pathogens in humans. On the other hand, as per the estimations published by European Food Safety Association (EFSA) in 2020 updating the 2011 opinion, a 10^3 reduction of *Campylobacter* contamination in chicken ceca can cause a 58% reduction of the public health risk (Hazards (BIOHAZ) et al. 2020). Therefore, it is imperative to find solutions to mitigate *Salmonella* and *Campylobacter* prevalence in broiler chickens to combat foodborne infections and assure food safety worldwide.

✉ Ramesha N. Wishna-Kadawarage
ramesha.wishna-kadawarage@pbs.edu.pl

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

[2] Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork P61 C996, Ireland

Lactic acid bacteria (LAB) have been intensively studied over the past few decades with the aim of harnessing their antimicrobial properties as alternatives to antibiotics in livestock production. Consequently, many LAB strains have been shown to possess anti-pathogenic effects against *Salmonella* and *Campylobacter* and have been used in the food industry due to their antimicrobial food preservation abilities (Reviewed by Vieco-Saiz et al. 2019 and Ibrahim et al. 2021). Furthermore, many LAB strains are identified by the Food and Drug Administration (FDA) under the status of Generally Recognized As Safe (GRAS) and by EFSA under the status of Qualified Presumption of Safety (QPS) and as such, have been used in the food and feed industry for many years (Webb et al. 2022). LAB consist of diverse genera of bacteria which produce different metabolites or compounds which possess antimicrobial properties. Bacteriocins are the one type of antimicrobial compound that are known to be produced by some of the LAB strains. These are antimicrobial peptides with either a broad or narrow spectrum of antimicrobial ability (Wyszyńska and Godlewska 2021). Their mechanisms include disruption of cell wall synthesis and pore formation in cell wall/membrane of pathogens inhibiting their growth and survival (Kumariya et al. 2019). Another important attribute of LAB associated with anti-pathogenic properties, is the production of organic acids (Cizeikiene et al. 2013). Among these organic acids, lactic acid, acetic acid and formic acid, are the major by-products of LAB that are associated with a broad spectrum anti-pathogenic effects. These organic acids create a low intracellular pH environment where pathogens cannot perform their regular metabolic functions such as replication and protein synthesis (Vieco-Saiz et al. 2019). Apart from bacteriocins and organic acids, some LAB can produce hydrogen peroxide, diacetyl, ethanol and carbon dioxide also providing antimicrobial activity against wide range of pathogens (Vieco-Saiz et al. 2019; Wyszyńska and Godlewska 2021; Webb et al. 2022).

Table 1 Lactic acid bacteria and pathogenic strains used

LAB		Pathogens	
Strain	Origin	Strain	Origin
Lactiplantibacillus plantarum B/00166 (LP)	Swine	*Salmonella enterica* subsp. Enterica serovar Typhimurium (DPC6463)	Chicken
Lacticaseibacillus casei B/00164 (LC)		*Salmonella enterica* subsp. Enterica serovar Typhimurium (ATCC 14028)	
Limosilactobacillus reuteri B/00281 (LR)		*Salmonella enterica* subsp. Enterica serovar Braenderup (NRL-IE-22)	
Lacticaseibacillus rhamnosus B/00279 (LRh)		*Campylobacter jejuni* DVI-SC181	
Leuconostoc mesenteroides B/00288 (LM)			
Pediococcus pentosaceus B/00165 (PP)	Chicken		

Considering the potential of LAB to produce such antimicrobial metabolites against pathogenic bacteria, we selected a number of LAB to screen for the strain with the most broad spectrum of activity in inhibiting different strains of *Salmonella* and *Campylobacter jejuni* in broiler chickens. However, the antimicrobial characteristics are highly dependent both on the probiotic and pathogenic strains chosen (Campana et al. 2017). Therefore, *in-vitro* selection of LAB strains for antimicrobial applications in livestock production required specific focus on certain LAB strains. Accordingly, six commercial LAB strains (homofermentative, obligatory heterofermentative and facultative heterofermentative) belonging to different genera, were chosen for screening against strains of *Salmonella enterica* and *Campylobacter jejuni* under *in-vitro* conditions.

Materials and methods

Bacterial strains

Six LAB strains (which are currently commercially used in multi-strain probiotic supplements for swine and poultry and produced by JHJ Sp Z.o.o, Nowa Wieś, Poland) were selected for anti-pathogenic screening. All the LAB strains had been identified using 16s rRNA sequencing and deposited at the Polish collection of Microorganisms located in Wrocław. The pathogens used in the study included three serovars of *Salmonella enterica* subspecies Enterica and one strain of *Campylobacter jejuni* (Table 1).

Anti-*Salmonella* assays

Spot overlay assays

LAB were inoculated into MRS broth (BD 288130) and incubated aerobically at 37°C for 20 h. Five microliters of each LAB culture were spotted into a labelled MRS agar plate allowed to air dry. These plates were incubated at 37°C overnight. Fifteen microliters of cultures of each *Salmonella* strain (incubated at 37°C for 16 h in BHI broth (1.10493 Merck)) was added to 30 ml of BHI molten cooled (at 50°C) agar (0.75%) and mixed gently. The *Salmonella* inoculated agar was overlaid the plate containing LAB spots grown overnight and was further incubated at 37°C overnight. The zone of inhibition surrounding the LAB spots were

measured in mm (Four measurements of the radius were taken perpendicularly and averaged). The experiment was performed in triplicate. The three most promising LAB which displayed highest inhibition of all three *Salmonella* strains were selected for further assays.

Well diffusion assays (WDAs) against *Salmonella* Typhimurium

The overnight cultures of the selected strains were prepared as described in spot overlay assays section. These cultures were centrifuged at 4000 g for 15 min at 4°C and the supernatant was retained. The pH of the cultures (grown for 20 h) was determined using a pH meter. Supernatant obtained from each culture was neutralized using 1 M NaOH or 1 M HCL, to pH 7 ± 0.2. Untreated and pH neutralized supernantants were filter sterilized using 0.22 µm syringe filters.

Salmonella Typhimurium (DPC6463) overnight culture was prepared as described in spot overlay assays section and 25 µl of the culture was inoculated in 50 ml of BHI molten cooled (at 50°C) agar (1%) and was mixed gently. The inoculated molten agar was poured into a square petri dish and allowed to set for 20 min. Wells of approximately 7 mm in diameter were created in the inoculated agar aseptically, using a sterile pipette tip (1000 µl). Each well was labelled with the names of LAB and 100 µl of the filtered LAB culture supernatants (neat and pH neutralized) was added into the respective wells. For the WDA with neat LAB supernatants, MRS broth (pH = 4) was used as a negative control. The wells were dried at room temperature in a laminar flow hood to the point that when moved to the incubator, the liquid in the wells was not displaced (approximately 30 min). Then the plates were incubated at 37°C for 16 h. Inhibition around the wells were observed and recorded (in mm). The experiment was performed in triplicate.

Co-culture assays

The three LAB which exhibited the strongest inhibition of all three *Salmonella* strains were selected for co-culture experiments. Double strength BHI broth (for *Salmonella*) and MRS broth (for LAB) were prepared. Double strength MRS was mixed in equal volume with double strength BHI for the co-culture experiment of LAB with *Salmonella*. The mixture of double strength media (10 ml) was inoculated with 100 µl of each LAB overnight culture (incubated for 20 h) and 100 µl of *Salmonella* Typhimurium culture (incubated for 16 h) and incubated for 24 h at 37°C. Selective enumeration of *Salmonella* Typhimurium in each coculture was performed at 0, 5, 10 and 24 h time points using spot plate method on *Salmonella* chromogen selective agar (CM1007). Results were graphed to visualize the growth of *Salmonella* in presence and absence of LAB. The experiment was performed in triplicate. The pH of the cultures was also recorded at each time point.

Co-aggregation assay with *Salmonella* Typhimurium

The co-aggregation ability of a bacterium is an indicator of the potential inhibition of the colonization of a pathogen in the gut by a beneficial bacteria which co-aggregates with it. Therefore, the co-aggregation ability of the three LAB selected was tested together with *Salmonella* Typhimurium. All bacterial overnight cultures were prepared as described in the spot overlay assays section. Cultures were centrifuged at 4000 g for 15 min at 4°C. The supernatant was discarded and cell pellet was washed with sterile PBS twice. Then the cell pellet was re-suspended in PBS to a concentration of 0.5 optical density at 600 nm (OD_{600}). OD_{600} measurements were obtained using BioTek Synergy HT microplate reader. Five hundred microliters of each bacterial suspension was aliquoted into a sterile flat bottom 48 well microtiter plate. Additionally, 250 µl of each LAB suspension was added with 250 µl of *Salmonella* suspension into the wells of the same plate and mixed by pipetting. The plate was then incubated at 37°C for 24 h. The OD_{600} reading of the wells was recorded using the microplate reader without shaking the plate. These experiments were performed in triplicate. The co-aggregation ability of each LAB was determined using the following formula (Balakrishna 2013).

$$\text{Co} - \text{aggregation ability} = [1 - ((2 \times \text{Am}) \div (\text{Al} + \text{As}))] \times 100$$

Where;

Am = OD_{600} of mixture of LAB and *Salmonella* suspensions.

Al = OD_{600} of LAB suspension alone.

As = OD_{600} of *Salmonella* suspension alone.

Anti-*Campylobacter* assays

Well diffusion assays against *Campylobacter jejuni.*

Campylobacter jejuni was inoculated in Mueller Hinton broth (BD 275730) supplemented with *Campylobacter* selective supplement (Skirrow) (SR0069E) according the manufacturer's directions. After incubating the inoculated broth at 42°C for 48 h under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) using CampyGen™ 2.5 L Sachet

(CN0025A, Oxoid), Mueller Hinton agar (1.5%) plates (90 mm circular plates) were spread with 100 µl of this culture and were allowed to dry. Then, using a sterile 200 µl pipette tip, wells of approximately 5 mm in diameter were created aseptically in the agar. The LAB culture supernatants (both neat and pH neutralized) were added to each well (50 µl/well) and then the plates were left for approximately 30 min until the supernatants were absorbed into agar (wells were empty). These plates were incubated at 42°C for 24 h under microaerophilic conditions for 24 h. The inhibition zone around the wells was observed and recorded (in mm). The experiment was performed in triplicate.

Organic acids characterization in culture supernatants

The culture supernatants (after 18 h of incubation) were filtered using 0.22 µm syringe filters. Levels of organic acid metabolites were then quantified by HPLC using a Waters Alliance Separations module e2695 coupled to a Waters 2414 refractive index (RI) detector (Waters, Milford MA, USA). Samples or standards at a volume of 20 µl were injected on to a Rezex Organic acids H + column (300 × 7.8 mm) operated at 60°C. The samples were eluted with H_2SO_4 (0.005 N) at a flow rate of 0.6 mL/min. Sample detection was performed by comparing retention times of standards. Analytical grade acetic acid, butyric acid, citric acid, lactic acid, formic acid and propionic acid supplied by Merck were used as standards. The assay was performed in duplicate.

Statistical analysis of the data

The measurements from triplicate assays were used to perform ANOVA followed by Tukey's HSD mean comparison test using Statistica software (Version 14.0.0.15) to identify statistically significant differences among the means.

Results

Anti-*Salmonella*

Spot overlay assays

The results of the spot overlay assays indicated that five out of six LAB strains studied (except *L. reuteri*) are more effective against all three *Salmonella* serovars (Fig. 1). The highest overall anti-*Salmonella* activity was observed for *L. rhamnosus, L. mesenteroides* and *P. pentosaceus*. Therefore, these three LAB were used for further anti-*Salmonella* assays.

Well diffusion assays (WDAs)

The pH of the culture supernatants obtained from the six LAB was approximately 4 (*L. plantarum*- 3.9, *L. casei*- 3.9, *L. reuteri*- 4, *L. rhamnosus*- 4, *L. mesenteroides*- 4.1 and *P. pentosaceus*- 4). In order to determine whether the inhibition observed by LAB in spot overlays was due to pH effect (via organic acid production), the WDAs against *Salmonella* Typhimurium were performed with neat (un-treated) and pH neutralized (pH 7 ± 0.2) culture supernatants of the three LAB selected. Interestingly, no inhibition was observed with the LAB supernatants when pH was neutralized indicating that anti-*Salmonella* effects observed are possibly due to pH effect/action of organic acids produced by the LAB. The neat supernatants however, displayed inhibition of *Salmonella* Typhimurium similar to MRS broth at pH 4 (Fig. 2). Therefore, it can be suggested that the.

Co-culture assays of LAB with *Salmonella*

The results of co-culture assay indicated that the three LAB strains selected (*L. rhamnosus, L. mesenteroides* and *P. pentosaceus*) based on promising inhibition observed with spot overlay assays, are equally efficient in inhibiting *Salmonella* Typhimurium. The number of colony forming units (CFUs) of *Salmonella* Typhimurium observed for in the presence of LAB was significantly lower when compared to the number of CFUs in the control medium (Fig. 3). Intriguingly, no colonies of *Salmonella* Typhimurium were present after plating

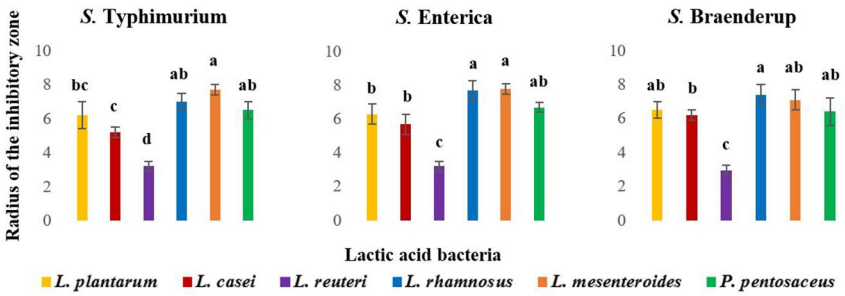

Fig. 1 Radius of inhibitory zone (mm) observed in spot overlay assays against three *Salmonella enterica* serovars. Error bars: ±SD. Homogenous means have been indicated by similar letters identified by Tukey's HSD test (p value < 0.05)

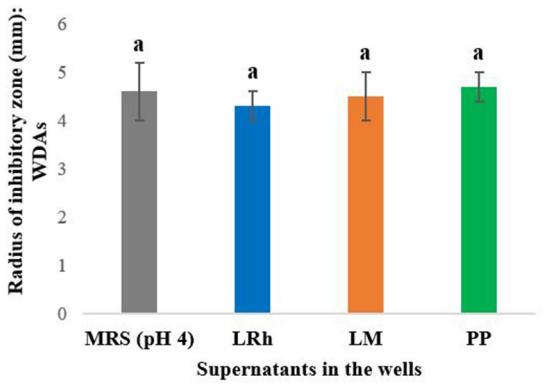

Fig. 2 Radius of inhibitory zone (mm) observed in well diffusion assays (with neat supernatants) against *Salmonella* Typhimurium. **LRh**: *L. rhamnosus*, **LM**: *L. mesenteroides*, **PP**: *P. pentosaceus* Error bars: ±SD. Homogenous means have been indicated by similar letters identified by Tukey's HSD test (p value < 0.05)

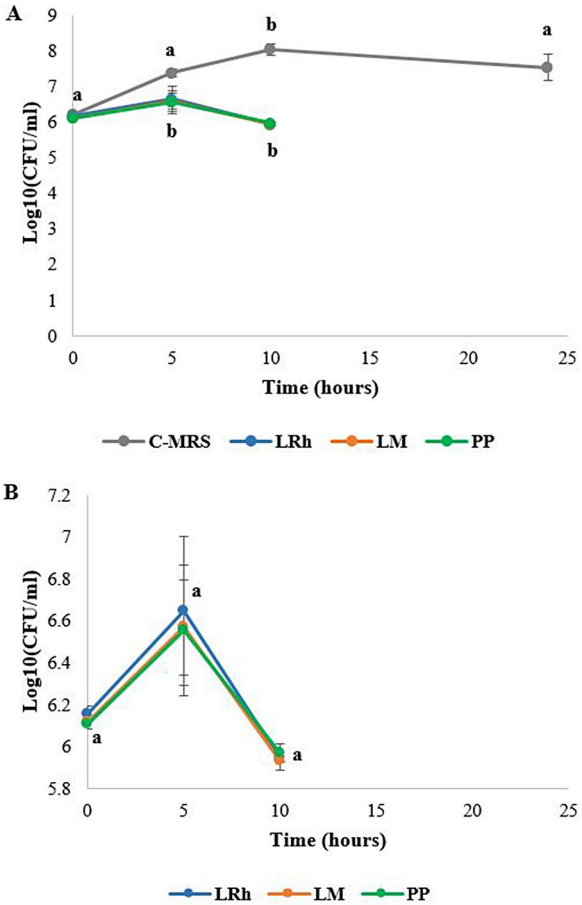

Fig. 3 Selective enumeration of *Salmonella* Typhimurium in co-culture. **A**: Comparison of growth of *Salmonella* with and without LABs. **B**: Comparison of growth of *Salmonella* in co-culture with different LAB. **C-MRS**: Control media (MRS + BHI), **LRh**: *L. rhamnosus*, **LM**: *L. mesenteroides*, **PP**: *P. pentosaceus*. Error bars: ±SD. Homogenous means indicated by similar letters: Tukey's HSD test (p value < 0.05)

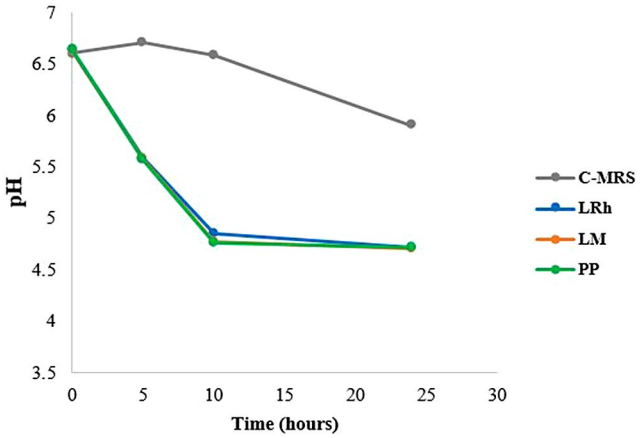

Fig. 4 Changes of pH in the cultures of co-culture assay. **C-MRS**: MRS + BHI media control, **LRh**: *L. rhamnosus*, **LM**: *L. mesenteroides*, **PP**: *P. pentosaceus*

the co-culture at 24 h indicating a complete eradication of *Salmonella* Typhimurium by LAB. These results suggest that the selected LAB strains possess both bacteriostatic and bactericidal properties against *Salmonella* Typhimurium.

The pH of the co-cultures was measured over time (Fig. 4). It was observed that the pH of C-MRS (double strength BHI + MRS media) inoculated only with *Salmonella* Typhimurium gradually dropped to approximately 6 at the end of 24 h of culturing. However, co-culture with LAB strains decreased the pH to approximately 4.7 within first 10 h and remained constant until the end of 24 h. This result also supports the assumed role of organic acids produced by LAB in bactericidal effects on *Salmonella* Typhimurium.

Co-aggregation assays of LAB with *Salmonella*

The co-aggregation assay was performed with the three most promising LAB strains (*L. rhamnosus, L. mesenteroides* and *P. pentosaceus*) together with *Salmonella* Typhimurium. The results (Fig. 5) indicated that highest co-aggregation is observed with *L. mesenteroides*.

Anti-*Campylobacter* well diffusion assays (WDAs)

WDAs against *Campylobacter* was performed with LAB culture supernatants (neat and pH neutralized). The results indicated that *L. mesenteroides*, *P. pentosaceus* and *L. casei*, followed by *L. plantarum* displayed the highest inhibition of *Campylobacter jejuni* (Fig. 6). Similar to anti-*Salmonella* WDAs, no inhibition was observed with pH neutralized supernatants as opposed to the clear inhibitions observed with neat supernatants (Fig. 7) indicating a potential role of organic acids in anti-*Campylobacter* activity also.

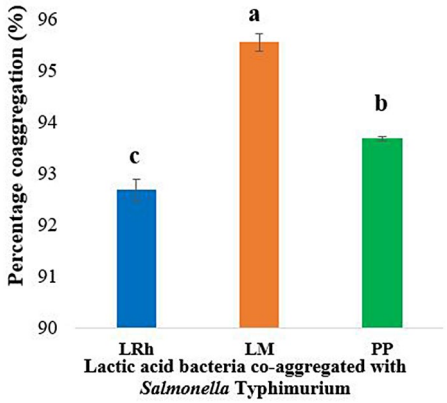

Fig. 5 Results of co-aggregation assays of selected LAB strains with *Salmonella* Typhimurium. **LRh**: *L. rhamnosus*, **LM**: *L. mesenteroides*, **PP**: *P. pentosaceus*. Error bars: ±SD. Homogenous means indicated by similar letters: Tukey's HSD test (p value < 0.05)

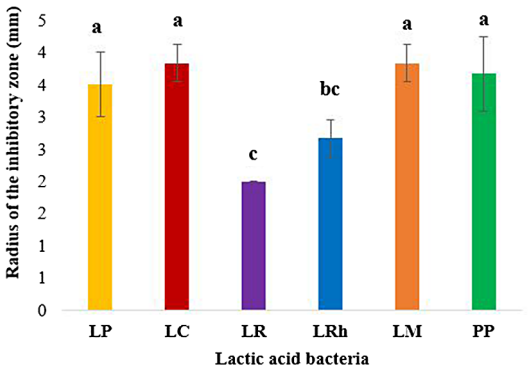

Fig. 6 Inhibition of *Campylobacter jejuni* by neat culture supernatants in Well diffusion assays. **LP**: *L. plantarum*, **LC**: *L. casei*, **LR**: *L. reuteri*, **LRh**: *L. rhamnosus*, **LM**: *L. mesenteroides*, **PP**: *P. pentosaceus*. Error bars: ±SD. Homogenous means indicated by similar letters: Tukey's HSD test (p value < 0.05)

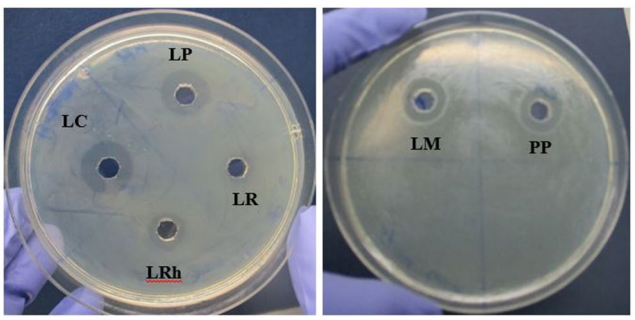

Fig. 7 Anti-*Campylobacter* WDA results for culture supernatants (neat). **PC**: Positive control, **LP**: *L. plantarum*, **LC**: *L. casei*, **LR**: *L. reuteri*, **LRh**: *L. rhamnosus*, **LM**: *L. mesenteroides*, **PP**: *P. pentosaceus*

Organic acid characterization in culture supernatants

The quantification of the organic acids in the culture supernatants is shown in Fig. 8. Propionic or citric acid production was not detected in any supernatants tested. There was significant acetic acid and butyric acid production in the *L. reuteri* while *L. plantarum* displayed a limited acetic acid production. Other LAB did not display significant production of these two organic acids. On the other hand, lactic acid and formic acid were found at high levels in the LAB strains which displayed highest anti-*Salmonella* and anti-*Campylobacter* properties. Limited inhibition of the pathogens was observed by *L. reuteri* while the least lactic acid and formic acid production was observed for the same strain. These results suggest a possible role for lactic and formic acids in the anti-*Salmonella* and anti-*Campylobacter* properties of the LAB studied.

Discussion

Lactic acid bacteria (LAB) are a group of beneficial bacteria that have earned a reputation in inhibiting pathogens both *in-vitro* and *in-vivo* (Ibrahim et al. 2021). It is imperative to select a LAB strain which displays preferably a broad spectrum anti-pathogenic potential for applications to improve the gut health of livestock. The six LAB species that were assessed in the current study are used in multi-strain commercial probiotic supplements for poultry (JHJ Sp. z o.o. 2021) and this product displayed promising results in reduction of *Salmonella enteritidis* (Smialek et al. 2019) and *Campylobacter spp.* (Smialek et al. 2018) in broiler gastrointestinal tract (GIT). The current study evaluated the potential of individual LAB strains against three serovars of *Salmonella enterica* and *Campylobacter jejuni* in terms of bacteriostatic, bactericidal or co-aggregating properties along with their mode of action. *Lecuconostoc mesenteroides* has been identified as the most promising candidate LAB due to its anti-*Salmonella* and anti-*Campylobacter* activity. Moreover, the results of the current study demonstrated a significant role for lactic and formic acid production in this antimicrobial activity.

As the inhibition ability was lost when the culture supernatants of the strains used in the current study, were pH neutralized, the anti-*Salmonella* and anti-*Campylobacter* activity is likely to be associated with a pH effect. LAB are known to impart a pH lowering effect via producing different types of organic acids. Generally, the organic acids demonstrate a non-specific mode of action and thus a broad spectrum antimicrobial activity (Khan et al. 2022). The undissociated form of the organic acids are able to diffuse

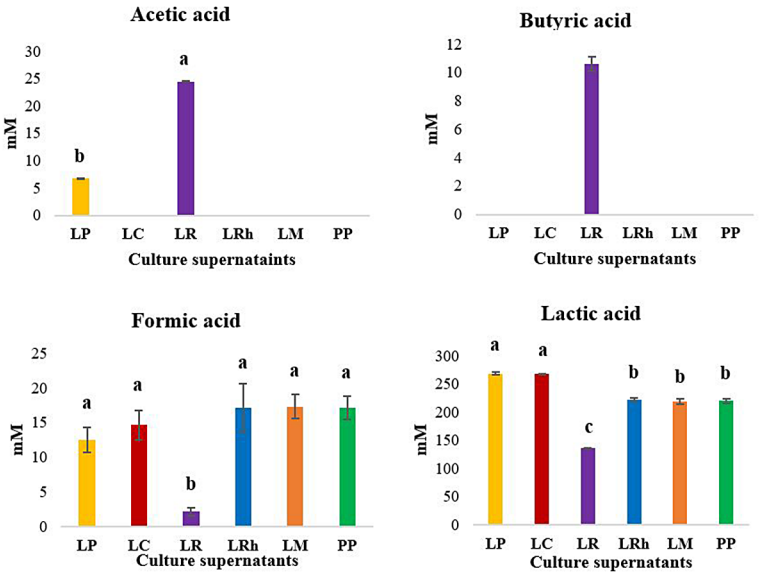

Fig. 8 Organic acid quantification of the culture supernatants of LAB. **LP**: *L. plantarum*, **LC**: *L. casei*, **LR**: *L. reuteri*, **LRh**: *L. rhamnosus*, **LM**: *L. mesenteroides*, **PP**: *P. pentosaceus*. Error bars: ±SD. Homogenous means indicated by similar letters: Tukey's HSD test (p value < 0.05)

into the bacterial cells due to its lipophilic nature. Inside the cytoplasm, they dissociate to release H^+ ions and reduce the intra-cytoplasmic pH of these pathogens. This eventually results in compromised metabolic functions accounted for bacteriostasis or bactericidal activity. Therefore, organic acids produced by LAB seems to be the likely cause for the strains observed inhibitory effects in the current study. Previous studies reported cases where the anti-pathogenic effects from different LAB strains were maintained (De Giani et al. 2019), decreased (Keeratikunakorn et al. 2023) and disappeared (Ołdak et al. 2020), when pH of the cell free supernatant was neutralized. These studies claim that when the antimicrobial activity is maintained, the inhibitory activity is not due to a pH/organic acid effect whereas decreased or no inhibitory activity is partially or completely due to the effects of pH/organic acid production, respectively. These claims are in agreement with our hypothesis that the inhibition observed by our LAB strains is likely to be due to organic acid production.

Further supporting this assumption, interestingly, different degrees of inhibition were observed for the cultures despite having similar pH. This possibly highlights the significance of specific organic acids produced by each LAB which may display different antimicrobial potential at the same pH. According to our results *L. reuteri* displayed almost similar pH to *L. mesenteroides* but displayed much less inhibition of all pathogens studied. It was clear that formic acid and lactic acid content were lowest in the culture supernatant of *L. reuteri* while *L. mesenteroides* displayed great production of these organic acids. Similarly, *L. reuteri* displayed higher production of acetic and butyric acids compared to other LAB studied. Burin et al. (2014) claimed that pathogen inhibition by acetic acid may be higher than lactic acid due to its lower dissociation ability compared to that of lactic acid. The current results suggest that the strains which produce greater amounts of both lactic acid and formic acid appear to cause more inhibition of pathogens compared to *L. reuteri* which produces acetic acid (which is more effective) but production is lower. It might also be possible that the observed antimicrobial properties are due to a synergistic effect of combinations of organic acids (produced by these LAB) as previously documented by Peh et al. (2020) against *Campylobacter* species. These authors observed a synergistic potential of caprylic acid, sorbic acid and caproic acid in inhibiting *Campylobacter jejuni* and *Campylobacter coli, in-vitro.*

LAB ferment sugars yielding mainly lactic acid to produce the energy necessary for their metabolism. Interestingly, LAB consist of diverse species belong to different genera including *Lactobacillus* (recently reclassified in to 25 genera such as *Lactiplantibacillus, Lacticaseibacillus, Limosilactobacillus, etc.*), *Leuconostoc, Pediococcus etc.* Although fermentation ability is a common feature of these bacteria, they are broadly divided into two major groups of fermenters namely, homofermentative and heterofermentative bacteria. The sole by-product of homofermentation is considered to be lactic acid while heterofermentation yields several by-products such as lactic acid, carbon dioxide (CO_2), ethanol and/or acetic acid (Kim et al. 2022). Theoretically, the homofermenters produce 2 moles of lactic acid per 1 mol of glucose while heterofermenters produce less (1 mol) lactic acid per 1 mol of glucose (Kim et al. 2022). Therefore, it is indicative that these differences in fermentation metabolism may attribute to differences in organic acids and their quantities produced by the LAB in the current study. Interestingly, the six LAB were belonged to different fermentation groups. *P. pentosaceus* is considered more a homofermenter while the rest are obligate (*L. reuteri*) and

facultative (*L. plantarum, L. casei. L. rhamnosus* and *L. mesenteroides*) heterofermenters. Therefore, another possibility that these strains displayed different degree of inhibition at the same pH, may be because of other metabolites produced such as ethanol or carbon dioxide production along with the organic acids.

Moreover, different strains from the same LAB species that were used in the current study, are known to produce bacteriocins such as Plantaricin which is produced by *L. plantarum* (Gong et al. 2010; Kumar et al. 2016), Pediocin by *P. pentosaceus* (Khorshidian et al. 2021), Caseicin by *L. casei* (Rammelsberg et al. 1990), Rhamnocin by *L. rhamnosus* (Jeong and Moon 2015), Mesenterocin from *L. mesenteroides* (Daba et al. 1991) etc. It is also important to highlight that certain LAB produce bacteriocins which lose their antimicrobial activity in neutral or alkaline pH conditions (Peng et al. 2023). Moreover, Keersmaecker et al. (2006) observed a non-proteinaceous broad spectrum antimicrobial compound which is synthesized by a *L. rhamnosus* strain and active under lower pH as mediated by accumulation of lactic acid. Therefore, apart from specific fermentation metabolites produced, it is also possible that these LAB displayed different degrees of inhibition due to the production of other proteinaceous or non-proteinaceous antimicrobial compounds which are only active under lower pH. Nevertheless, this theory must be confirmed by further investigation.

In the co-culture, not a single *Salmonella* colony forming unit (CFU) was observed at 24 h while considerable numbers of *Salmonella* were present after 10 h incubation. Nevertheless, the pH of both time points was similar. Thus, we suggest that although the pH was similar at both time points (by production of organic acids) it might take some time for the organic acids to diffuse into pathogenic cells, and interfere with the metabolic functions of *Salmonella* to completely eradicate them from the co-culture. However, another consideration is that these LABs produce a strong antimicrobial metabolite which can eradicate *Salmonella*, later in their exponential growth which might be activated at a lower pH as previously observed by Keersmaecker et al. (2006).

Moreover, the co-aggregation ability of a probiotic with a pathogen, is a good indication of *in-vivo* inhibition of pathogen colonization in the GIT. If a probiotic is able to co-aggregate with a pathogen, it is an advantage for the probiotic to release the antimicrobial compounds at a close proximity to these pathogenic bacteria preventing their colonization in the gut (Tuo et al. 2013). Therefore, *L. mesenteroides*, from our results displays the most promise to combat *Salmonella* Typhimuriumcolonization in the GIT of broiler chickens.

Conclusion

Among the different strains of different genera belonging to lactic acid bacteria studied, *Leuconostoc mesenteroides* displayed the most significant overall anti-pathogenic properties against all the food borne pathogens used suggesting its potential for *in-vivo* applications to combat foodborne pathogens in broiler chickens.

Acknowledgements We acknowledge JHJ sp z o.o., Poland for providing LAB for our experiment. We also thank APC Microbiome, Ireland and *Salmonella* National Reference Laboratory, DAFM, Ireland for providing *Salmonella* Typhimurium and *Salmonella* Braenderup strains, respectively and Aarhus University, Denmark for providing *Campylobacter jejuni* strain used in the current experiment. We specially thank Helen Slattery from Teagasc Food Research Center, Ireland for performing the HPLC for organic acid characterization.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ramesha N. Wishna-Kadawarage. The first draft of the manuscript was written by Ramesha N. Wishna-Kadawarage. Funding acquisition, supervision, review and editing was by Rita M. Hickey and Maria Siwek. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This research was carried out under the funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 955374.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Authority EFS, European Centre for Disease Prevention and Control (2022) The European Union One Health 2021 Zoonoses Report. EFSA J 20(12):e07666. https://doi.org/10.2903/j.efsa.2022.7666

Balakrishna A (2013) In vitro evaluation of adhesion and aggregation abilities of four potential probiotic strains isolated from Guppy

(*Poecilia reticulata*). Braz Arch Biol Technol 56(5):793–800. https://doi.org/10.1590/S1516-89132013000500010
Bintsis T (2017) Foodborne pathogens. AIMS Microbiol 3(3):529–563. https://doi.org/10.3934/microbiol.2017.3.529
Burin RCK, Silva A, Nero LA (2014) Influence of lactic acid and acetic acid on *Salmonella* Spp. Growth and expression of acid tolerance-related genes. Food Res Int 64:726–732. https://doi.org/10.1016/j.foodres.2014.08.019
Campana R, van Hemert S, Baffone W (2017) Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. Gut Pathog 9:12. https://doi.org/10.1186/s13099-017-0162-4
Centers for Disease Control and Prevention (2022) Chicken and Food Poisoning. https://www.cdc.gov/foodsafety/chicken.html. Accessed 31 October 2022
Cizeikiene D, Juodeikiene G, Paskevicius A, Bartkiene E (2013) Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. Food Control 31(2):539–545. https://doi.org/10.1016/j.foodcont.2012.12.004
Daba H, Pandian S, Gosselin JF, Simard RE, Huang J, Lacroix C (1991) Detection and activity of a bacteriocin produced by *Leuconostoc mesenteroides*. Appl Environ Microbiol 57(12):3450–3455. https://doi.org/10.1128/aem.57.12.3450-3455.1991
De Giani A, Bovio F, Forcella M, Fusi P, Sello G, Di Gennaro P (2019) Identification of a bacteriocin-like compound from Lactobacillus plantarum with antimicrobial activity and effects on normal and cancerogenic human intestinal cells. AMB Express 9(1):88. https://doi.org/10.1186/s13568-019-0813-6
Gong HS, Meng XC, Wang H (2010) Mode of action of plantaricin MG, a bacteriocin active against *Salmonella typhimurium*. J Basic Microbiol 50(Suppl 1):S37–45. https://doi.org/10.1002/jobm.201000130
Hazards (BIOHAZ) E. P. on, Koutsoumanis B, Allende K, Alvarez-Ordóñez A et al (2020) A Update and review of control options for *Campylobacter* in broilers at primary production. EFSA J 18(4):e06090. https://doi.org/10.2903/j.efsa.2020.6090
Ibrahim SA, Ayivi RD, Zimmerman T, Siddiqui SA, Altemimi AB, Fidan H, Esatbeyoglu T, Bakhshayesh RV (2021) Lactic acid Bacteria as Antimicrobial agents: Food Safety and Microbial Food Spoilage Prevention. Foods 10(12):Article. https://doi.org/10.3390/foods10123131
Jeong YJ, Moon GS (2015) Antilisterial Bacteriocin from *Lactobacillus rhamnosus* CJNU 0519 presenting a narrow Antimicrobial Spectrum. Korean J Food Sci Anim Resource 35(1):137–142. https://doi.org/10.5851/kosfa.2015.35.1.137
JHJ Sp. z o.o (2021) Lavipan. http://jhj.pl/files/karty_prod/Ulotki_2021/jhj-lavipan-2021-pl_A4.pdf. Accessed 14 July 2023
Keeratikunakorn K, Kaewchomphunuch T, Kaeoket K, Ngamwongsatit N (2023) Antimicrobial activity of cell free supernatants from probiotics inhibits against pathogenic bacteria isolated from fresh boar semen. Sci Rep 13(1). https://doi.org/10.1038/s41598-023-33062-w. Article 1
Keersmaecker SCJD, Verhoeven TLA, Desair J, Marchal K, Vanderleyden J, Nagy I (2006) Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. FEMS Microbiol Lett 259(1):89–96. https://doi.org/10.1111/j.1574-6968.2006.00250.x
Khan RU, Naz S, Raziq F, Qudratullah Q, Khan NA, Laudadio V, Tufarelli V, Ragni M (2022) Prospects of organic acids as safe alternative to antibiotics in broiler chickens diet. Environ Sci Pollut Res 29(22):32594–32604. https://doi.org/10.1007/s11356-022-19241-8
Khorshidian N, Khanniri E, Mohammadi M, Mortazavian AM, Yousefi M (2021) Antibacterial activity of Pediocin and pediocin-producing Bacteria against *Listeria monocytogenes* in Meat products. Front Microbiol 12:709959. https://doi.org/10.3389/fmicb.2021.709959
Kim J, Kim YM, Lebaka VR, Wee YJ (2022) Lactic acid for Green Chemical Industry: recent advances in and future prospects for Production Technology, Recovery, and applications. Fermentation 8(11):Article11. https://doi.org/10.3390/fermentation8110609
Kumar V, Sheoran P, Gupta A, Yadav J, Tiwari SK (2016) Antibacterial property of bacteriocin produced by *Lactobacillus plantarum* LD4 isolated from a fermented food. Ann Microbiol 66(4). https://doi.org/10.1007/s13213-016-1230-6. Article 4
Kumariya R, Garsa AK, Rajput YS, Sood SK, Akhtar N, Patel S (2019) Bacteriocins: classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. Microb Pathog 128:171–177. https://doi.org/10.1016/j.micpath.2019.01.002
Ołdak A, Zielińska D, Łepecka A, Długosz E, Kołożyn-Krajewska D (2020) Lactobacillus plantarum strains isolated from Polish Regional cheeses exhibit anti-staphylococcal activity and selected Probiotic properties. Probiotics Antimicrob Proteins 12(3):1025–1038. https://doi.org/10.1007/s12602-019-09587-w
Peh E, Kittler S, Reich F, Kehrenberg C (2020) Antimicrobial activity of organic acids against *Campylobacter* Spp. And development of combinations—A synergistic effect? PLoS ONE 15(9):e0239312. https://doi.org/10.1371/journal.pone.0239312
Peng Z, Xu X, Fan P, Qiao B, Xie M, Huang T, Xiong T (2023) Identification and characterization of a novel pH and heat stable bacteriocin-like substance lactococcin036019 with food preserving potential. Food Control 148:109682. https://doi.org/10.1016/j.foodcont.2023.109682
Rammelsberg M, Müller E, Radler F (1990) Caseicin 80: purification and characterization of a new bacteriocin from *Lactobacillus casei*. Arch Microbiol 154(3):249–252. https://doi.org/10.1007/BF00248963
Smialek M, Burchardt S, Koncicki A (2018) The influence of probiotic supplementation in broiler chickens on population and carcass contamination with *Campylobacter* spp. - Field study. Res Vet Sci 118:312–316. https://doi.org/10.1016/j.rvsc.2018.03.009
Smialek M, Kaczorek E, Szczucińska E, Burchardt S, Kowalczyk J, Tykałowski B, Koncicki A (2019) Evaluation of *Lactobacillus* spp. And yeast based probiotic (Lavipan) supplementation for the reduction of *Salmonella enteritidis* after infection of broiler chickens. Pol J Vet Sci 22(1):5–10. https://doi.org/10.24425/pjvs.2018.125616
Tuo Y, Yu H, Ai L, Wu Z, Guo B, Chen W (2013) Aggregation and adhesion properties of 22 *Lactobacillus* strains. J Dairy Sci 96(7):4252–4257. https://doi.org/10.3168/jds.2013-6547
Vieco-Saiz N, Belguesmia Y, Raspoet R, Auclair E, Gancel F, Kempf I, Drider D (2019) Benefits and inputs from lactic acid Bacteria and their bacteriocins as Alternatives to Antibiotic Growth promoters during Food-Animal production. https://doi.org/10.3389/fmicb.2019.00057. Front Microbiol 10
Webb L, Ma L, Lu X (2022) Impact of lactic acid bacteria on the control of *Listeria monocytogenes* in ready-to-eat foods. Food Qual Saf 6:1–11. https://doi.org/10.1093/fqsafe/fyac045
Wyszyńska AK, Godlewska R (2021) Lactic acid Bacteria – A Promising Tool for Controlling Chicken *Campylobacter* infection. Front Microbiol 12:703441. https://doi.org/10.3389/fmicb.2021.703441

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Poultry Science®

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

Ramesha N. Wishna-Kadawarage ,[*,1] Katarzyna Połtowicz,[†] Agata Dankowiakowska,[‡] Rita M. Hickey,[§] and Maria Siwek [*]

[*]*Department of Animal Biotechnology and Genetics, Faculty of Animal Breeding and Biology, Bydgoszcz University of Science and Technology, Mazowiecka 28, Bydgoszcz 85-084, Poland;* [†]*Department of Poultry Breeding, National Research Institute of Animal Production, Krakowska 1, Balice 32-083, Poland;* [‡]*Department of Animal Physiology and Physiotherapy, Faculty of Animal Breeding and Biology, Bydgoszcz University of Science and Technology, Mazowiecka 28, Bydgoszcz 85-084, Poland; and* [§]*Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork P61 C996, Ireland*

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

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

2024 Poultry Science 103:103512
https://doi.org/10.1016/j.psj.2024.103512

INTRODUCTION

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

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

© 2024 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Received December 6, 2023.
Accepted January 25, 2024.

[1]Corresponding author: ramesha.wishna-kadawarage@pbs.edu.pl

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

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

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

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

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

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

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

MATERIALS AND METHODS

Egg Incubation and Experimental Design

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

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

Preparation of Injection for PB Group

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

Preparation of Injection for PPB Group

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

Hatching and Data Collection

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

Animal Rearing and Sample Collection

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

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

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

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

Slaughter Analysis

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

Meat Quality Analysis

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

Extraction of DNA

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

Extraction of RNA

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

Analysis of Relative Abundance of Bacteria

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

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

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

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

93.67051692

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

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

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

E universal: qPCR Efficiency of universal bacteria primers

Ct universal: Ct value of qPCR reaction for universal bacteria

E target: qPCR Efficiency of target bacteria primers

Ct target: Ct value of qPCR reaction for target bacteria

Analysis of Immune Related Gene Expression

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

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

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

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

94:19916375

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

Analysis of Cecal Histology

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

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

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

VW= villus width,
VH = villus height.

Statistical Analysis of Data

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

RESULTS AND DISCUSSION

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

Hatch Properties

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

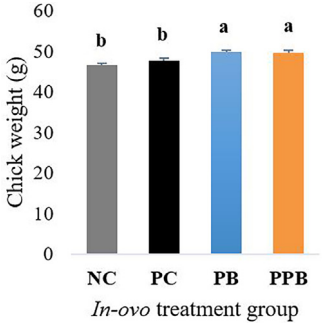

Figure 1. The weight of chicks at hatch across *in-ovo* treatment groups. Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending order). Abbreviations: NC: negative control group, PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic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

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

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

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

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

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

Body Weight and Feed Efficiency

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

Slaughter and Meat Quality Analysis

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

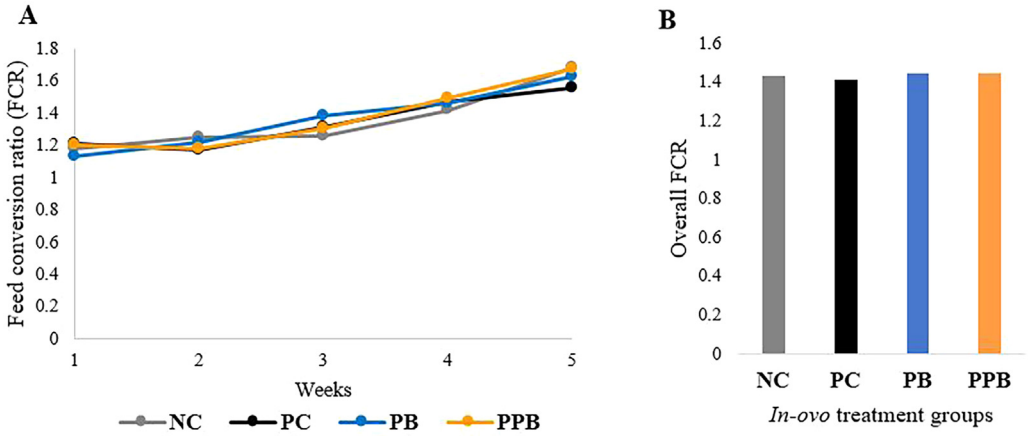

Figure 2. Feed conversion ratio (FCR) of the chickens of *in-ovo* treatment groups. (A) Weekly FCR. (B) Overall FCR. Abbreviations: NC: Negative control group, PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract)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

96:59300703;

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

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

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

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

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

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

Relative Abundance of Bacteria in Feces

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

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

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

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

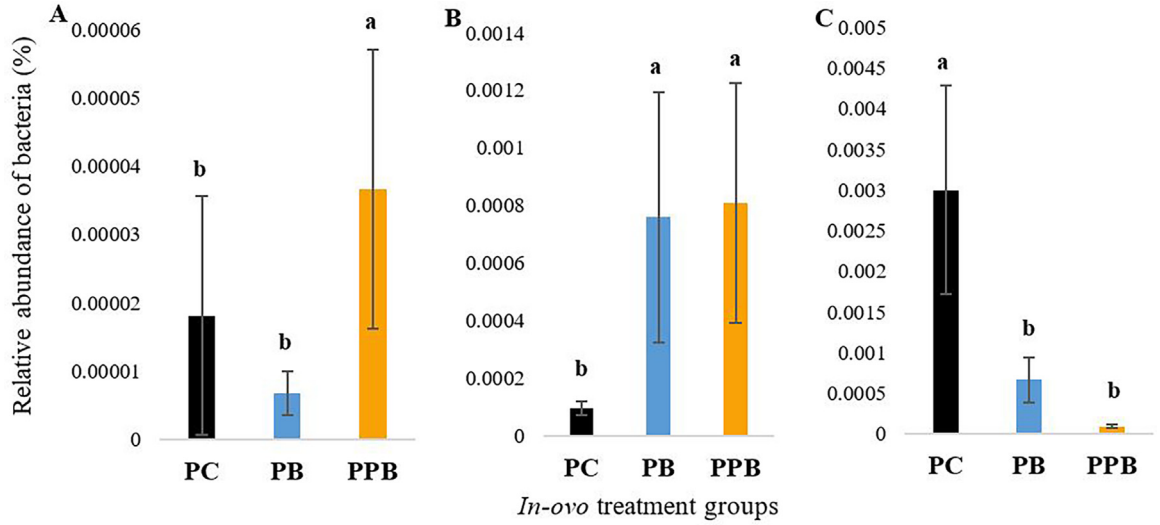

Figure 3. The relative abundance of bacterial communities in the feces of chickens of different *in-ovo* treated groups. (A) D 7 − *Bifidobacterium* sp. (B) D 7 − *Faecalibacterium* sp. (C) D 34 − *E. coli.* Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending order).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

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

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

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

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

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

https://doi.org/10.1016/j.psj.2024.103754

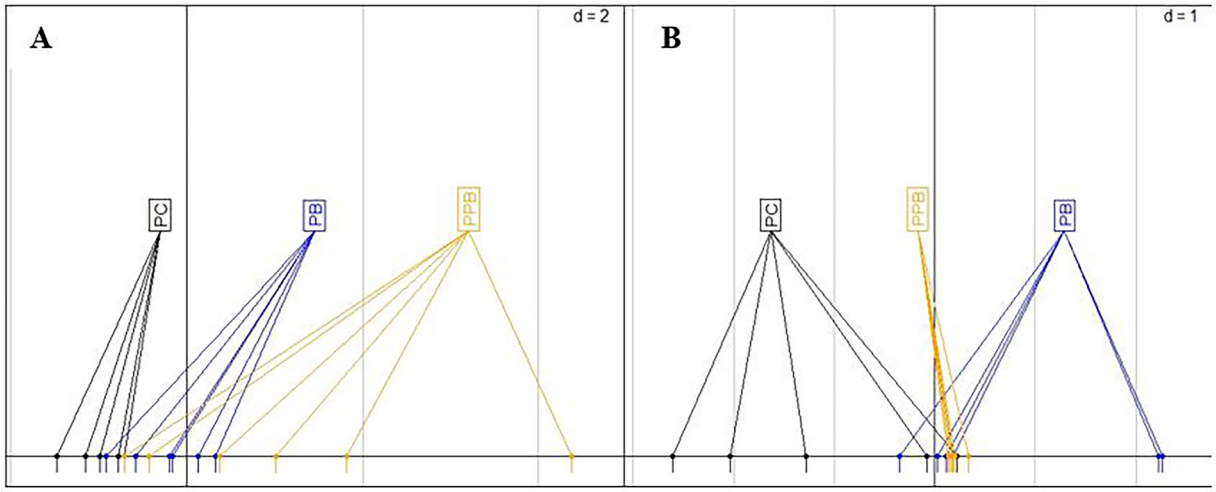

Figure 4. The between-class analysis plot for microbiological data in feces (A) D 7 (B) D 34. Abbreviations: PC: positive control, PB: probiotic (*Leuconostoc mesenteroides*) group, PPB: prophybiotic (*Leuconostoc mesenteroides* + garlic aqueous extract) group.

Relative Abundance of Bacteria in the Ceca

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

Expression of Immune Related Genes in Cecal Mucosa

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

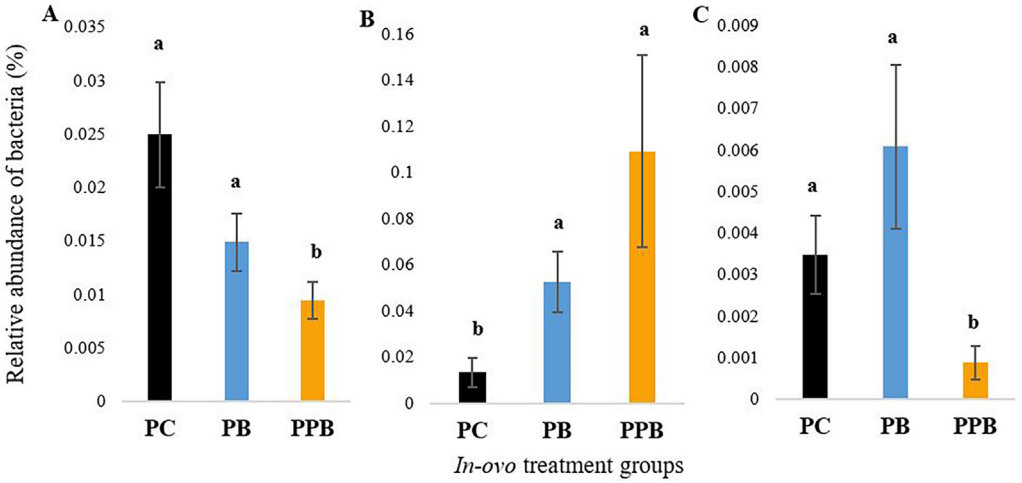

Figure 5. The relative abundance of bacterial communities in the luminal content of ceca of chickens of different *in-ovo* treated groups. (A) *Faecalibacterium sp.* (B) *Akkermansia sp.* (C) *Escherichia coli.* Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending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

10.3382/ps/pead359

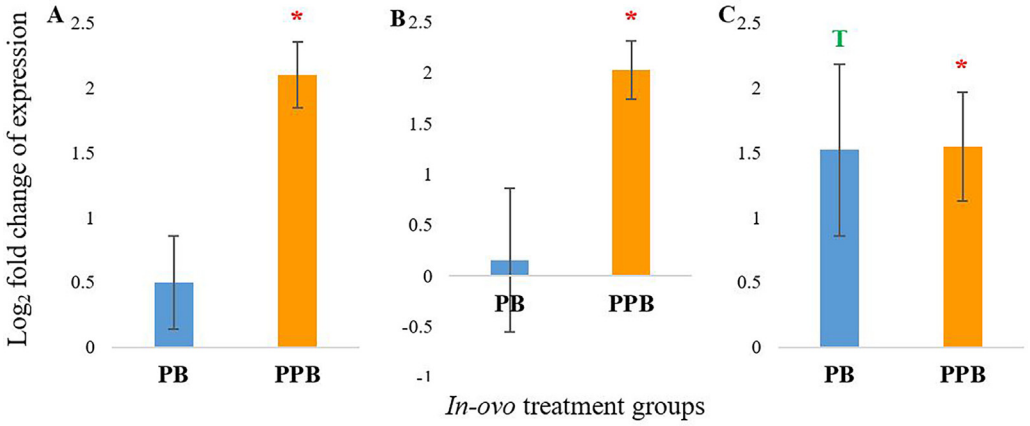

Figure 6. Immune-related gene expression in the cecal mucosa of chickens of different *in-ovo* treated groups. (A) *AVBD1* (B) *FFAR2* (C) *MUC6*. Error bars: ± SE. Red color asterick (*) indicates significant changes (*P*- value < 0.05) The letter T in green indicates there is a tendency (*P*-value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

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

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

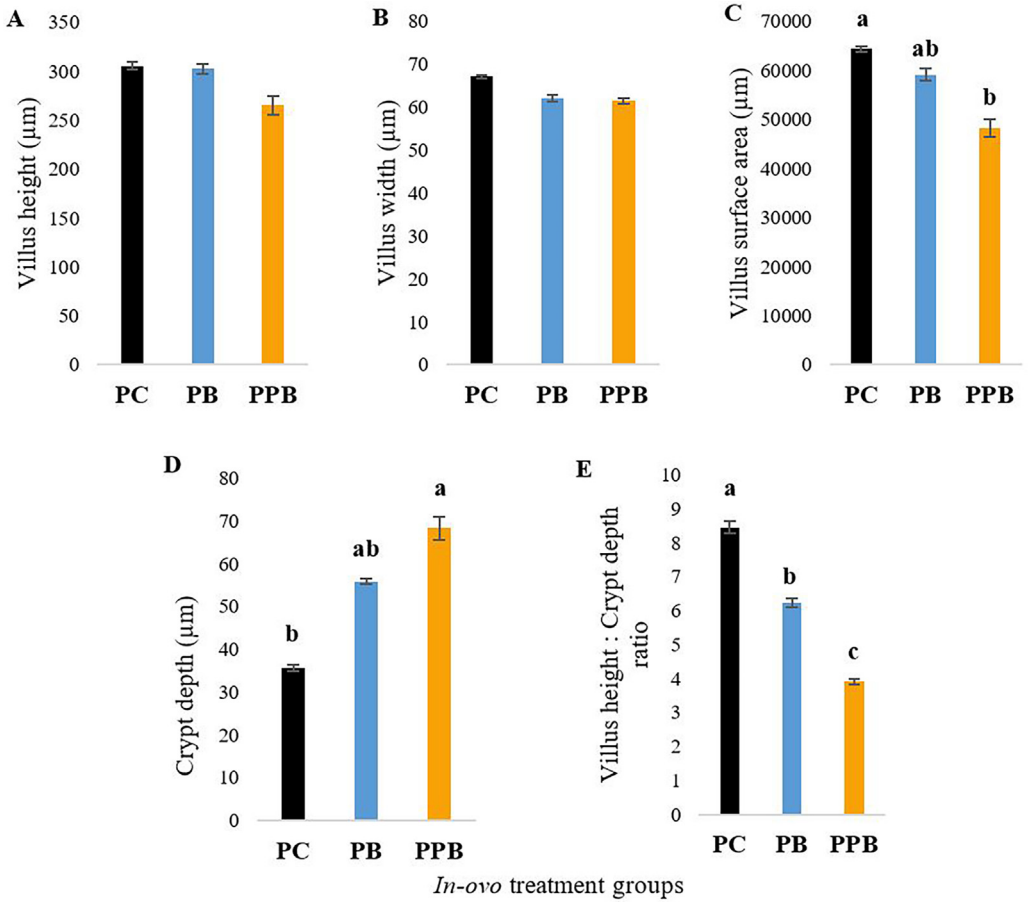

Figure 7. The analysis of histomorphological parameters of the cecal tissue of the *in-ovo* treated chickens. (A) Villus Height. (B) Villus Width. (C) Villus Surface Area. (D) Crypt depth. (E) Villus Height to Crypt Depth Ratio. Error bars: ± SE. Homogenous means have been indicated by similar letters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

100564272891

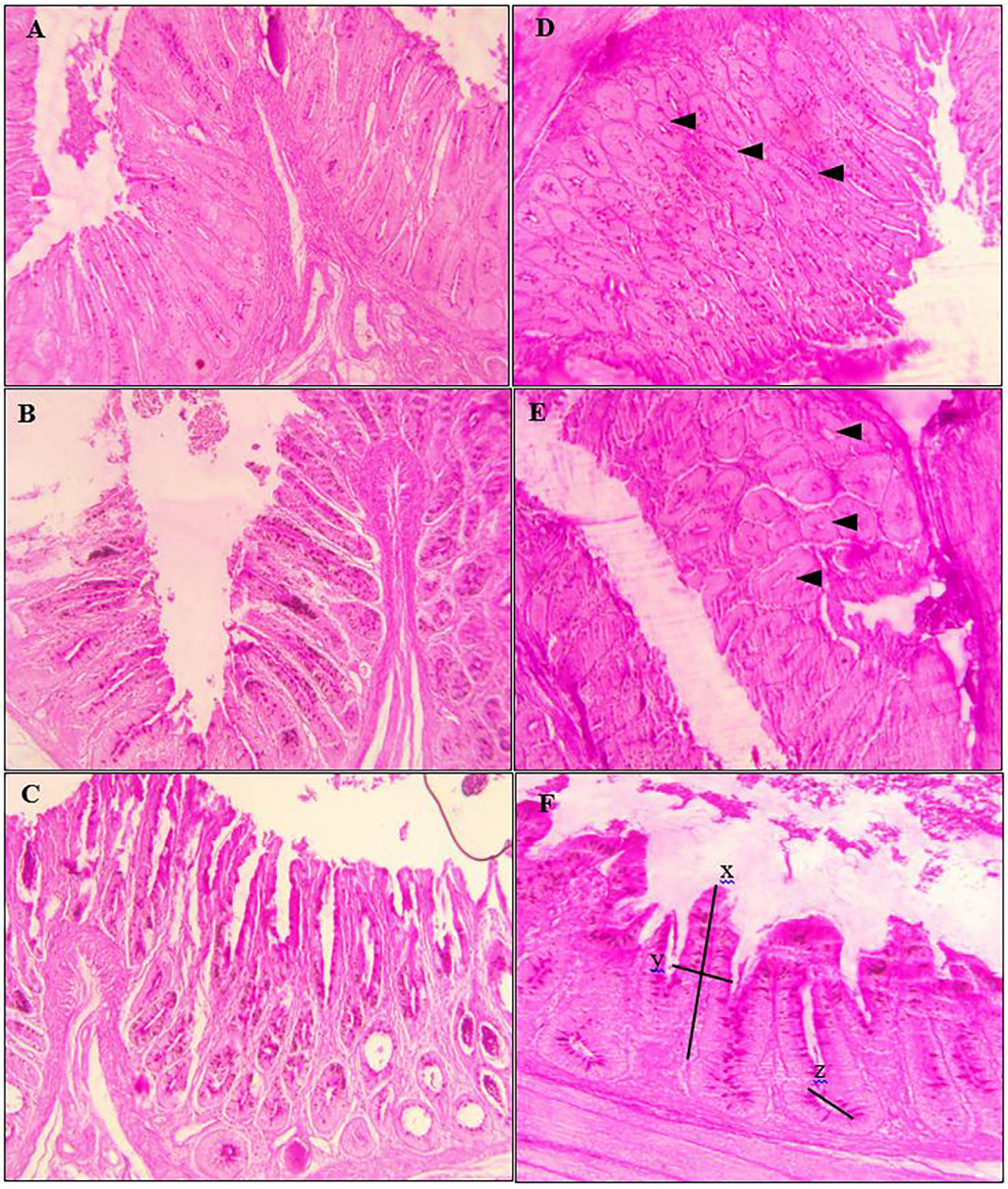

Figure 8. Histomorphological analysis of cecal tissue of *in-ovo* treated birds with Periodic acid−Schiff (PAS) staining (Magnification 100×). (A) Positive control (PC). (B) Probiotic (*Leuconostoc mesenteroides*) group (PB). (C) Prophybiotic (*Leuconostoc mesenteroides* + 0.5% (w/v) garlic aqueous extract)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

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

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

10.1093/ps/pab561

approach to mitigate pathogenic stress in broiler chickens.

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

Histology of Cecal Tissue

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

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

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

CONCLUSION

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

ACKNOWLEDGMENTS

This research was carried out under the funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 955374. This article has been financially supported by the Minister of Science under the program "Regional Initiative of Excellence" (RID/SP/0017/2024/01). We acknowledge JHJ sp z o.o., Poland for providing the probiotic for this experiment. We also thank Martin Jensen from Aarhus University, Denmark for providing garlic and relevant technical knowledge for preparing garlic aqueous extract in this study.

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

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

DISCLOSURES

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

REFERENCES

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Statement of the author of the doctoral dissertation

Mgr. Ramesha Nirmali Wishna Kadawarage

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. **Ramesha N. Wishna-Kadawarage**, Martin Jensen, Szymon Powałowski, Rita M. Hickey and Maria Siwek, *In-vitro* screening of compatible synbiotics and (introducing) "prophybiotics" as a tool to improve gut health, International Microbiology (Springer), 2024, 27, 645–657, https://doi.org/10.1007/s10123-023-00417-2, MNiSW points $_{2024}$: 40, Impact Factor $_{2023}$: 2.3

Tasks performed by the PhD student as part of the article:
a) Experiment design
b) Conducting the experiments
c) Data collection and analysis
d) Writing the first draft of the manuscript and revision

2. **Ramesha N. Wishna-Kadawarage**, Rita M. Hickey and Maria Siwek, *In-vitro* selection of lactic acid bacteria to combat *Salmonella enterica* and *Campylobacter jejuni* in broiler chickens, World Journal of Microbiology and Biotechnology (Springer), 2024, 40, 133, https://doi.org/10.1007/s11274-024-03946-8, MNiSW points $_{2024}$: 70, Impact Factor $_{2023}$: 4.0

Tasks performed by the PhD student as part of the article:
a) Experiment design
b) Conducting the experiments
c) Data collection and analysis
d) Writing the first draft of the manuscript and revision

105:11395235

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

3. **Ramesha N. Wishna-Kadawarage**, Katarzyna Połtowicz, Agata Dankowiakowska, Rita M. Hickey and Maria Siwek, Prophybiotics for *in-ovo* stimulation; validation of effects on gut health and production of broiler chickens, Poultry Science (Elsevier), 2024, 103, 4, https://doi.org/10.1016/j.psj.2024.103512, MNiSW points $_{2024}$: 140, Impact Factor $_{2023}$: 3.8

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

a) Experiment design

b) Conducting the *in-ovo* experiment

c) Sample collection and wet and dry lab/data analysis

d) Writing the first draft of the manuscript and revision

Bydgoszcz 11.07.2024
.....
Place, date

.....
Signature of the author of the doctoral dissertation

Rita Hickey
.....
Signature of the second supervisor
(Dr. Rita Hickey)

.....
Signature of the first supervisor

106:50656847

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

Co-author's Declaration

Prof. 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 articles mentioned below was as follows *:

1. Ramesha N. Wishna-Kadawarage, Martin Jensen, Szymon Powałowski, Rita M. Hickey and **Maria Siwek**, *In-vitro* screening of compatible synbiotics and (introducing) "prophybiotics" as a tool to improve gut health, International Microbiology (Springer), 2024, 27, 645–657, https://doi.org/10.1007/s10123-023-00417-2, MNiSW points $_{2024}$: 40, Impact Factor $_{2023}$: 2.3

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

2. Ramesha N. Wishna-Kadawarage, Rita M. Hickey and **Maria Siwek**, *In-vitro* selection of lactic acid bacteria to combat *Salmonella enterica* and *Campylobacter jejuni* in broiler chickens, World Journal of Microbiology and Biotechnology (Springer), 2024, 40, 133, https://doi.org/10.1007/s11274-024-03946-8, MNiSW points $_{2024}$: 70, Impact Factor $_{2023}$: 4.0

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

3. Ramesha N. Wishna-Kadawarage, Katarzyna Połtowicz, Agata Dankowiakowska, Rita M. Hickey and **Maria Siwek**, Prophybiotics for *in-ovo* stimulation; validation of effects on gut health and

production of broiler chickens, Poultry Science (Elsevier), 2024, 103, 4, https://doi.org/10.1016/j.psj.2024.103512, MNiSW points $_{2024}$: 140, Impact Factor $_{2023}$: 3.8

Tasks completed as part of the article:

a) Funding acquisition

b) Experiment design

c) Supervision

d) Review and editing of the manuscript

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

Bydgoszcz 02.07.2024
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 (eg the creator of the research hypothesis, the originator of the research, performance of specific research – eg 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.*

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

Co-author's Declaration

Dr. Rita M. Hickey

Teagasc Food Research Centre
Moorepark, Fermoy
Co. Cork, P61 C996
Ireland

DECLARATION

I declare that my authorial contribution to the scientific articles mentioned below was as follows *:

1. Ramesha N. Wishna-Kadawarage, Martin Jensen, Szymon Powałowski, **Rita M. Hickey** and Maria Siwek, *In-vitro* screening of compatible synbiotics and (introducing) "prophybiotics" as a tool to improve gut health, International Microbiology (Springer), 2024, 27, 645–657, https://doi.org/10.1007/s10123-023-00417-2, MNiSW points $_{2024}$: 40, Impact Factor $_{2023}$: 2.3

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

2. Ramesha N. Wishna-Kadawarage, **Rita M. Hickey** and Maria Siwek, *In-vitro* selection of lactic acid bacteria to combat *Salmonella enterica* and *Campylobacter jejuni* in broiler chickens, World Journal of Microbiology and Biotechnology (Springer), 2024, 40, 133, https://doi.org/10.1007/s11274-024-03946-8, MNiSW points $_{2024}$: 70, Impact Factor $_{2023}$: 4.0

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

3. Ramesha N. Wishna-Kadawarage, Katarzyna Połtowicz, Agata Dankowiakowska, **Rita M. Hickey** and Maria Siwek, Prophybiotics for *in-ovo* stimulation; validation of effects on gut health and production of broiler chickens, Poultry Science (Elsevier), 2024, 103, 4, https://doi.org/10.1016/j.psj.2024.103512, MNiSW points $_{2024}$: 140, Impact Factor $_{2023}$: 3.8

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

Tasks completed as part of the article:

a) Funding acquisition

b) Experiment design

c) Supervision

d) Review and editing of the manuscript

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

Teagasc, Ireland, 08/07/2024

....

Place, date Co-author's signature

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

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 authorial contribution to the scientific article mentioned below was as follows *:

1. Ramesha N. Wishna-Kadawarage, **Katarzyna Połtowicz**, Agata Dankowiakowska, Rita M. Hickey and Maria Siwek, Prophybiotics for *in-ovo* stimulation; validation of effects on gut health and production of broiler chickens, Poultry Science (Elsevier), 2024, 103, 4, https://doi.org/10.1016/j.psj.2024.103512, MNiSW points $_{2024}$: 140, Impact Factor $_{2023}$: 3.8

Tasks completed as part of the article:

a) Experiment design
b) Supervision of the *in-vivo* experiment and the meat quality analysis
c) Review and editing of the manuscript

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

Balice, 2.01.2024 — Place, date

Katarzyna Połtowicz — 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 (eg the creator of the research hypothesis, the originator of the research, performance of specific research – eg 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.*

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

Co-author's Declaration

Dr. Martin Jensen

Department of Food Science
Aarhus University
AgroFoodPark 48
8200, Århus N
Denmark

DECLARATION

I declare that my authorial contribution to the scientific article mentioned below was as follows [*]:

1. Ramesha N. Wishna-Kadawarage, **Martin Jensen**, Szymon Powałowski, Rita M. Hickey and Maria Siwek, *In-vitro* screening of compatible synbiotics and (introducing) “prophybiotics” as a tool to improve gut health, International Microbiology (Springer), 2024, 27, 645–657, https://doi.org/10.1007/s10123-023-00417-2, MNiSW points $_{2024}$: 40, Impact Factor $_{2023}$: 2.3

Tasks completed as part of the article:
a) Experiment design
d) Review and editing of the manuscript

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

Aarhus, 2nd July 2024

....

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 (eg the creator of the research hypothesis, the originator of the research, performance*

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

Co-author's Declaration

Dr. Agata Dankowiakowska

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

DECLARATION

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

1. Ramesha N. Wishna-Kadawarage, Katarzyna Połtowicz, **Agata Dankowiakowska**, Rita M. Hickey Maria Siwek, Prophybiotics for *in-ovo* stimulation; validation of effects on gut health and production of broiler chickens, Poultry Science (Elsevier), 2024, 103, 4, https://doi.org/10.1016/j.psj.2024.103512, MNiSW points $_{2024}$: 140, Impact Factor $_{2023}$: 3.8

Tasks completed as part of the article:

a) Experiment design
b) Supervision of the histology analysis
c) Review and editing of the manuscript

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

Bydgoszcz, 02.07.24
Place, date

Agata Dankowiakowska
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 (eg the creator of the research hypothesis, the originator of the research, performance of specific research – eg 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.

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

Co-author's Declaration

Dr. Szymon Powałowski

Univeristy of Humanities Król Stanisław Leszczyński
Królowej Jadwigi 10
64-100, Leszno
Poland

DECLARATION

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

1. Ramesha N. Wishna-Kadawarage, Martin Jensen, **Szymon Powałowski**, Rita M. Hickey and Maria Siwek, *In-vitro* screening of compatible synbiotics and (introducing) "prophybiotics" as a tool to improve gut health, International Microbiology (Springer), 2024, 27, 645–657, https://doi.org/10.1007/s10123-023-00417-2, MNiSW points $_{2024}$: 40, Impact Factor $_{2023}$: 2.3

Tasks completed as part of the article:

a) Experiment design

d) Review and editing of the manuscript

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

Leszno, 04.07.2024
Place, date

Szymon Powałowski
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 (eg the creator of the research hypothesis, the originator of the research, performance of specific research – eg 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.*