Method paper

Method: Protocol for *in-ovo* stimulation with selected pro-/prophy-biotics to mitigate *Campylobacter jejuni* in broiler chickens

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ABSTRACT

Broiler chickens are a natural reservoir for *Campylobacter* which is largely responsible for the highest reported zoonotic infection within the Europe, Campylobacteriosis. However, despite extensive scientific investment, the broiler industry is still in need of effective intervention strategies to control this pathogen in broiler production. *In-ovo* modulation has been studied extensively as a method to positively modulate the gastrointestinal microflora in broiler chickens. However, the efficacy of an *in-ovo* method against *Campylobacter* has not been studied to date to the best of our knowledge. Therefore, the current study was conducted to validate the efficacy of a protocol for *in-ovo* stimulation of ROS308 broiler chicken eggs with *Leuconostoc mesenteroides* B/00288 strain alone (probiotic) and in combination with garlic aqueous extract (prophybiotic) in reducing the abundance of *Campylobacter jejuni* in ceca. On 12th day of incubation, the selected doses of the probiotic and prophybiotic were injected into the air cell of the eggs as treatments. Two control groups (Negative control: without injections and positive control injected with physiological saline) were also included in the experiment. The impact of the protocol on hatch parameters (hatchability, chick length, chick weight and Pasgar score), BWs and feed conversion ratio was recorded. When the chickens were 21 days old, an infection challenge with *Campylobacter jejuni* was performed. A quantitative PCR method was used to quantify the *Campylobacter* relative abundance in faeces (one week postinfection) and in the cecal content (at the age of 35 days). The probiotic treatment significantly (*P*-value = 0.0020) reduced the *Campylobacter jejuni* numbers in the ceca while the prophybiotic treatment resulted in a statistical tendency (*P*-value = 0.0691) in reducing the abundance of *Campylobacter jejuni* in ceca. Both treatments had no adverse effects on the hatch or production parameters studied. In conclusion, *in-ovo* stimulation with the probiotic *Leuconostoc mesenteroides* demonstrated potential in reducing *Campylobacter jejuni* colonisation in the ceca of ROSS308 broiler chickens infected at 3 weeks of age.

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Implications

Broiler chickens carry *Campylobacter* in large quantities, the most widely reported zoonotic pathogen in Europe. Administration of probiotics into hatching eggs has proven beneficial in improving gut health but has not been studied with respect to controlling *Campylobacter* in poultry. Administration of the probiotic *Leuconostoc mesenteroides* into ROSS308 hatching eggs on day 12 of incubation displayed a significant reduction of *Campylobacter jejuni* abundance in the ceca at the end of the production period with no adverse impact on the production parameters. This protocol will be useful in future research aimed at eliminating *Campylobacter* from broiler production.

Specification table

Subject	Behaviour and Health Management
Specific subject area	Mitigating *Campylobacter jejuni* colonisation in broiler chicken gastrointestinal track via *in-ovo* stimulation method

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Type of data	Graphs and raw data
How data were acquired	Data on probiotic growth were obtained by measuring absorbance at optical density at 600 nm (OD600) using a Multiskan™ FC Microplate Photometer and a plate count method. The data on hatched chickens were recorded manually (Hatchability, chick weight, chick length and Pasgar score). Body weights and feed intake were recorded using electronic scales. *Campylobacter jejuni* relative abundance was quantified via a qPCR method using LightCycler 480 II (Roche Diagnostics).
Data format	Raw and analysed data are presented in Excel file format (.xlsx) Metadata are presented in Word file format (.docx)
Parameters for data collection	Probiotic growth data were collected in OD600 values and plate counts. The hatchability, length, weight and Pasgar score of the chicks hatched from the experimental groups were recorded. The BWs (individual) and feed disappearance (per group) were recorded weekly. Relative abundance of *Campylobacter jejuni* in faecal samples (one week postinfection) and the cecal content (at the end of production: 5 weeks) were determined via qPCR.
Description of data collection	To prepare the probiotic inoculum at the accurate dose with maximum viable cells, two parameters were determined, the time at which inoculum is prepared and the OD600 at which the bacterial suspension should be diluted. After performing the *in-ovo* stimulation, the hatch parameters were recorded. During the rearing period, feed intake and BW of chickens were recorded per experimental group on a weekly basis. The relative abundance of *Campylobacter jejuni* in faecal samples (collected 1 week postinfection) and cecal contents (collected at the age of 5 weeks) was measured to validate the infection protocol and the efficacy of the *in-ovo* protocol in reducing the *Campylobacter jejuni* colonisation in broilers.
Data source location	Bydgoszcz University of Science and Technology, Bydgoszcz, Poland.
Data accessibility	Data and supplementary materials used for this paper can be obtained from the following repository; Wishna Kadawarage, R. N., & Siwek, M. (2024). Method paper_Campylobacter challenge [Data set]. Zenodo. https://doi.org/10.5281/zenodo.12782959
Related research article	None.

Introduction

Campylobacter is a bacteria which causes gastrointestinal illnesses in humans and other animal species. However, chickens can carry *Campylobacter* in large numbers within their gut asymptomatically (Looft et al., 2019). According to the latest reports, *Campylobacter* is the most frequently reported zoonotic pathogen in Europe and contaminated broiler chicken meat has been identified as the most common source of human Campylobacteriosis (European Food Safety Authority, 2014). In fact, the European Food Safety Authority estimated that a $3\log_{10}$ reduction of *Campylobacter* content in chicken ceca will reduce human *Campylobacter* cases attributed to broiler meat by 58% (European Food Safety Authority, 2020). For many years, *Campylobacter* was identified as a commensal bacteria as part of the chicken gut microbiome as it did not cause symptomatic infection in the majority of cases (Hakeem and Lu, 2021). However, some scientists claim that *Campylobacter* is not merely a commensal, but rather a gut pathogen causing chronic inflammation in the gut of chickens (Humphrey et al., 2014; Awad et al., 2018). Therefore, controlling *Campylobacter* in broiler chickens is imperative with respect to food safety as well as in addressing animal welfare concerns.

Campylobacter spreads horizontally in poultry flocks, and the poultry production environment is generally enriched with multiple sources of this bacteria (Hakeem and Lu, 2021). It has also been reported that *Campylobacter* has multiple survival strategies, and its unique colonisation factors aid in successful growth in both the chicken gut (Hermans et al., 2011) and the external environment (Hakeem and Lu, 2021). Therefore, many researchers have studied the efficacy of different intervention strategies to control *Campylobacter* in broiler chickens. In a recent review by Taha-Abdelaziz et al. (2023), the authors claim that despite the many strategies that have been studied to control *Campylobacter* in broilers, such as immunisation with vaccines, feed and water supplemented with bioactive supplements, faecal microbial transplants, strict biosecurity measures and postslaughter contamination control measures, neither of them have shown sufficient efficiency in completely eliminating *Campylobacter* from broilers. Existing studies show the beneficial effects of *in-ovo* administration of probiotics in reducing *Salmonella* (de Oliveira et al., 2014), *Eimeria* (Pender et al., 2016) and pathogenic *E. coli* (Cuperus et al., 2016; Oliveira et al., 2024) in broilers. However, to our knowledge, the potential of *in-ovo* treatments in *Campylobacter* control has not been studied, to date. If proven effective, an *in-ovo* method would be an ideal strategy when compared to other intervention strategies mentioned above, as this is a one-time, precise dose application at the hatchery and would incur no additional costs to the farmers during the production period. Therefore, we hypothesised that *in-ovo* injection with carefully chosen bioactive substances may reduce the *Campylobacter* content in the ceca of broiler chickens. Accordingly, the current study was conducted to investigate the effect of *in-ovo* stimulation with the *Leuconostoc mesenterodies* B/00288 strain alone (as a probiotic) and in combination with garlic aqueous extract (as a prophybiotic) on the *Campylobacter* quantity in the ceca of adult ROSS308 broiler chickens.

The selected probiotic strain has been used in a multistrain probiotic supplement which has previously reduced the quantity of *Campylobacter* in broiler chicken gut (Smialek et al., 2018) and has displayed *in-vitro* anti-*Campylobacter* effects (Wishna-Kadawarage et al., 2024a). Previously, our research group coined the term prophybiotics for the use of probiotics in combination with a phytobiotic as a tool to improve the gut health and identified the combination of garlic aqueous extract (0.5%) (phytobiotic)

and *Leuconostoc mesenteroides* (probiotic) as a compatible prophy-biotic pair (Wishna-Kadawarage et al., 2023). We further, reported the beneficial effects of *in-ovo* stimulation with these two treat-ments, probiotic and prophybiotics on the cecal microbiome and gene expression in immune-related organs under experimental conditions (Wishna-Kadawarage et al., 2024b; c). This paper pre-sents a detailed methodology of the protocol together with the val-idation of its efficacy in reducing the abundance of *Campylobacter* in the ceca of broiler chickens. Possible mechanisms involved in this reduction will be presented in a follow−up article describing the physiological and genomic responses of these chickens.

Materials and methods

Egg incubation, experimental design and injection

For this study, ROSS 308 broiler chicken eggs (n = 200; maternal flock age = 30 weeks) were obtained from Drobex-Agro, Mako-wiska, Poland and the incubation of the eggs was performed under standard conditions (Temperature: 37.5 ℃ and Relative Humidity: 55%, egg turning once in every 1.5 h) using a Midi series I incubator (Fest Incubators, Poland). On the 12th day of incubation, egg can-dling was performed using a lighted torch to remove the unfer-tilised eggs and dead embryos. The eggs were subsequently randomly distributed into four experimental groups namely; neg-ative control (**NC**), positive control (**PC**), probiotic (**PB**) and Prophy-biotic (**PPB**). The eggs of the NC group did not receive any injection whereas the PC eggs received an injection (0.2 ml/egg) of sterile 0.9%NaCl physiological saline solution (Natrium Chloratum 0.9% Fresenius KabiPac, Fresenius Kabi, Poland). The eggs of the PB group received the same volume of probiotic *Leuconostoc mesen-teroides* B/00288 suspension in 0.9% NaCl physiological saline solu-tion which corresponds to a dose of 10^6 colony−forming units (**CFU**)/egg. The injection for the PPB group consisted of two compo-nents, the same probiotic suspension (10^6 CFU/egg) and garlic aqueous extract (0.5% (g/ml) in final injection mixture) in 2:1 ratio.

All injections were performed at the site of the air cell. The eggs (the blunt end) were first, disinfected with 70% ethanol and can-dled to locate the air cell. A 20G needle was then used to create a hole in the eggshell at the site of air cell. The respective injections (0.2 ml/egg) were made manually through these holes in the eggs without damaging the inner shell membranes using 1−ml syringes with 26G needles. Finally, the eggs were sealed with non-toxic glue (Elmer's school glue, Elmer's products Inc., USA) and returned to the incubator. On 18th day of the incubation, the eggs were trans-ferred into a hatcher (Midi series I hatcher, Fest Incubators, Poland) and were maintained stationary at 37.5 ℃ with 65% relative humidity until the chicks were hatched (on day 21).

Preparation of garlic aqueous extract for prophybiotic treatment

The garlic cultivar used for this protocol is Thermodrome which was organically grown in the 2021 season at Aarhus University, Department of Food Science at Research Centre at Årslev, Funen, Denmark. Initially, the fresh garlic bulbs were chopped (into 3–5 mm slices) and air-dried (2 days at 40 ℃ and 5 days at 50 ℃). Then, the air-dried garlic was ground into fine pieces which were then further sieved using a 1 mm sieve to obtain fine garlic pow-der. This powder was stored at −20 ℃ until usage. In our previous study, no growth inhibition of the selected probiotic (*Leuconostoc mesenteroides*) was observed when supplemented with 0.5% (g/ml) aqueous extract prepared using this garlic powder (Wishna-Kadawarage et al., 2023). Therefore, 0.5% was selected as the dose of the antimicrobial phytobiotic component of the PPB injection used in the current study. However, for the PPB treatment, we pre-

pared the probiotic suspension and garlic aqueous extract sepa-rately and mixed in the proportion, 2:1. Therefore, in order to have 0.5% concentration of garlic in the final injection mixture, we prepared a 1.5% garlic aqueous extract as follows.

First, the required volume of the injection was calculated based on the number of eggs (0.2 ml × no. of eggs). Then, the required amount of garlic aqueous extract was calculated according to the proportion (1/3) of the garlic component in the PPB injection as follows;

$$\text{Volume of garlic aqueous extract (ml)} = \frac{\text{Total volume of injection (ml)}}{3}$$

Next, the amount of garlic powder necessary to prepare a 1.5% garlic aqueous extract was calculated using the following equation;

$$\text{Garlic (g)} = \left(\frac{1.5}{100}\text{g/ml}\right) \times \text{Volume of garlic aqueous extract (ml)}$$

Then, the required amount of garlic powder was measured using a fine balance and added to sterile distilled water in the calculated volume of the garlic extract. The mixture was then mixed rigorously using a vortex for approximately 30 s. The mixture was then contin-uously shaken for 8 min at 550 rpm speed. The suspension was allowed rest for 2 min resulting in sedimentation of the powder. Finally, the suspension was centrifuged at 10 000 rpm for 5 min. The supernatant was filter sterilised using a 0.2 μm syringe filter (WHA69012502, Merck, Poland) and used for the PPB injection mixture.

Determination of the parameters for probiotic inoculum preparation

The two most important parameters to determine prior to preparing the probiotic inoculum for *in-ovo* applications are, the time at which inoculum is prepared (late exponential phase in the growth) and the optical density at 600 nm (**OD600**) corre-sponding to the correct dose/bacterial density of the probiotic sus-pension. As the growth of a bacteria is strain−specific, a preliminary experiment was conducted in order to generate a growth curve of the selected strain to determine these two param-eters. Accordingly, *Leuconostoc mesenteroides* B/00288 strain was grown in MRS broth media (BD Difco 288130, Fisher Scientific, Dublin, Ireland) at 37 ℃ for 24 h under aerobic conditions. The bac-terial density of the culture was determined at 0, 5, 15, 18, 20 and 24 h intervals using both OD600 measurements and plate count (CFU/ml) methods in triplicate.

Briefly, for OD600 measurements, at each time point, a 250 μl sample of the culture was obtained after mixing well. These culture samples were placed in wells of sterile TPP tissue culture test plates (92096, TPP, Switzerland), and the OD600 absorbance was recorded using a Multiskan™ FC Microplate Photometer (Thermo Scientific, Poland). For the plate count method, a 100 μl sample from the culture was obtained at each time point which was then serially diluted (100 folds) with sterile Ringer's solution (Merck 1.15525, Germany). From each dilution, a 100 μl was spread plated on MRS agar (1.10660, Merck, Germany) in triplicates. The plates were incubated for 48 h, and the number of colonies was counted to calculate the CFU/ml concentration in the original culture. The growth curves for the probiotic bacteria using both CFU/ml and OD600 were plotted. The hour at which the growth of the probiotic bacteria reached the stationary phase (late exponential phase) was selected as the number of hours for which the probiotic culture will be incubated to prepare the inoculum for the *in-ovo* injection.

As the two treatment groups (PB and PPB) had different vol-umes of the bacterial suspension in the final injection mixture

(PB: total volume and PPB: 2/3 of the total volume), to deliver the same dose of probiotic bacteria from both treatments, two separate suspensions of the probiotic with different doses were used. Accordingly, the required dose for the PB treatment was 5×10^6 CFU/ml (to deliver 10^6 CFU from 0.2 ml of the suspension/egg) and that for the PPB treatment was 7.5×10^6 CFU/ml (to deliver 10^6 CFU from 2/3 of the injection volume (0.133 ml)/egg). In order to determine the corresponding OD600 values for these two concentrations, the OD600 values Vs CFU/ml results from the preliminary experiment were plotted and a regression equation was employed. This equation was used to calculate the corresponding OD600 values used to prepare the respective probiotic suspensions for the two *in-ovo* treatments.

Preparation of probiotic inoculum for pro- and prophy-biotic treatments

Based on the above experiment, 15 h was selected as the time for preparing the probiotic inoculum for the treatments and therefore, *Leuconostoc mesenteroides* was grown for 15 h as described in the preliminary experiment. To harvest the bacterial cells, the culture was centrifuged at 4 200 rpm and 4 ℃ for 20 min. The bacterial pellet was washed twice using sterile 0.9% NaCl physiological saline and resuspended in two suspensions adjusting the OD600 to the corresponding values determined by the preliminary experiment. The suspension for PB treatment was entirely used for the injections whereas the suspension for PPB group was mixed with the garlic aqueous solution in 2:1 ratio for the injections.

Rearing and data collection

Upon completion of the hatching, the hatchability for each treatment was calculated using the following equation;

$$\text{Hatchability } (\%) = \frac{\text{No. of chicks hatched}}{\text{No. of eggs put to the hatcher}} \times 100$$

The quality of the chicks hatched from each group was assessed using the following parameters: Pasgar score, chick weight and chick length recorded from 20 randomly selected chicks per group. A measuring tape fixed in position along the flat surface of a table was used to measure the chick length. As previously described by Ipek and Sozcu (2015), the chick was placed stretched and face down along the measuring tape and the length from the tip of the break to the end of the middle toe of the right leg was recorded for each bird. The weight of chicks was measured using an electronic balance when they were completely dry after hatching. Each chicken was then assessed for their quality via Pasgar scoring method as described in the Lohmann hatchery guide (Lohmann Breeders, 2024). Out of the chicks hatched from each group, 32 randomly selected birds were raised in separate floor pens (area: 8 m^2) with *ad libitum* feeding and watering. Individual BWs of 16 birds (the same birds in each week) in each group and feed intake (feed disappearance) per group were measured weekly and feed conversion ratio (**FCR**) was calculated (overall and weekly) per group using the following equation;

$$\text{FCR} = \frac{\text{Total amount of feed consumed (kg)}}{\text{Total weight gained (kg)}}$$

Campylobacter jejuni infection

On 19th day of rearing, the birds belonging to each group were randomly divided into two subgroups, infected and non-infected (n = 16/group) and were housed separately. The non-infected groups were abbreviated using the same abbreviations for *in-ovo*

groups namely, NC, PC, PB and PPB, and the infected groups of the *in-ovo* groups were abbreviated as **NC_C, PC_C, PB_C** and **PPB_C**, respectively. On the 21st day, the birds allocated to pathogen infection (infected subgroup) from each *in-ovo* experimental group were infected with 6×10^8 CFU of *Campylobacter jejuni* DVI-SC 181 (origin: broiler) using the oral gavage method. Briefly, the individual birds were restrained carefully to open up their beak and then 3 ml of the *Campylobacter jejuni* suspension was inserted into the open beak using a syringe. This procedure was quickly performed to avoid additional stress to the birds.

Preparation of Campylobacter jejuni inoculum

All the handling and culturing steps of *Campylobacter jejuni* were performed under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2). The frozen (-80 ℃) *Campylobacter jejuni* stock was thawed at room temperature. Then, 100 µl of the stock was streaked using a sterile inoculation loop on Mueller-Hinton Agar (CM0337B, Oxoid, United Kingdom) plates supplemented with *Campylobacter* selective supplement (Skirrow SR0069E, Oxoid, United Kingdom) according to the manufacturer's recommendations. The plates were incubated at 42 ℃ for 48 h after which, 2–3 well- −grown colonies from these plates were picked up using a sterile inoculation loop and inoculated into 10 ml of Mueller-Hinton broth (BD 275730, Fisher Scientific, Ireland) supplemented with the *Campylobacter* selective supplement (Skirrow SR0069E) according to the manufacturer's recommendations. The inoculated broth was incubated at 42 ℃ for 24 h, and 0.5 ml of this culture was then, used to re-inoculate fresh Mueller-Hinton Broth (15 ml). After incubating this new culture for 24 h at 42 ℃, a bacterial cell pellet was obtained by centrifuging the culture tube at 5 000 rpm for 10 min at 4 ℃. The cell pellet was re-suspended in sterile 0.9% NaCl physiological saline solution adjusting the OD600 to correspond to approximately 2×10^8 CFU/ml in the cell suspension. From this suspension, 3 ml/bird was used to infect the birds delivering a dose of 6×10^8 CFU/bird.

Sample collection

On the 28th day (one week postinfection), eight faecal samples/group were collected into sterile 5 mL micro-centrifuge tubes and stored immediately at -80 ℃ until use. On 34th day, eight birds from PC, PC_C, PB, PB_C, PPB and PPB_C groups (except NC and NC_C) were euthanised by CO_2 inhalation using the UNO Euthanasia Unit (Uno Bio Science Solutions, Netherlands). Carcasses were then opened and luminal content of the ceca was collected for quantification of the relative abundance of *Campylobacter jejuni,* in order to validate the effect of the *in-ovo* protocols on the *Campylobacter jejuni* colonisation. Briefly, the luminal content of ceca was carefully transferred to sterile 5 mL micro-centrifuge tubes and placed immediately on dry ice. The samples were transported on dry ice and stored at -80 ℃ until use. The experimental design for the validation of the efficacy of *in-ovo* stimulation protocols in controlling *Campylobacter jejuni* in the ceca of broiler chickens is illustrated in Fig. 1.

Extraction of DNA

To quantify the relative abundance of *Campylobacter jejuni* in faeces and the cecal content using quantitative PCR (qPCR) method, total DNA was extracted from the samples using the GeneMATRIX Stool DNA Purification Kit (E3575, EURx, Poland) by optimising the manufacturer's protocol (the sample amount and times for incubating and shaking during the cell lysis). The quality and quantity of the DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Poland), and gel electrophoresis (2%
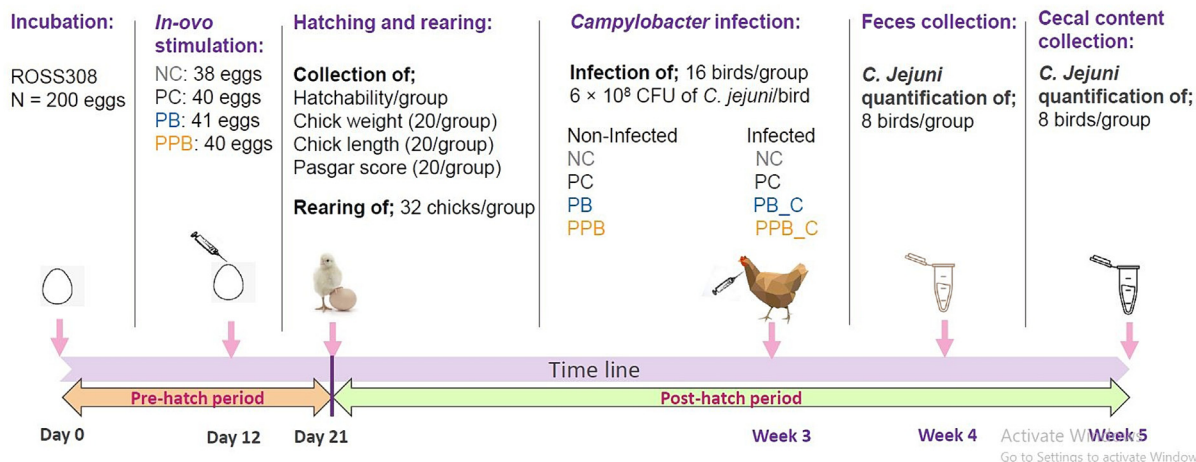
Fig. 1. Graphical illustration of the experimental design to validate the efficacy of *in-ovo* stimulation protocols in controlling *Campylobacter* in the ceca of broiler chickens. NC: Negative control, PC: Positive control, PB: Probiotic, PPB: Prophybiotic, NC_C: Negative control - infected, PC_C: Positive control - infected, PB_C: Probiotic - infected, PPB_C: Prophybiotic - infected.

agarose) was used to confirm the integrity of the DNA. Extracted DNA samples were stored at −80 ℃ until use.

Analysis of relative abundance of bacteria

Campylobacter jejuni abundance was quantified relative to the quantity of total bacteria (using universal primers) in each sample. The universal primers used in the current study are ACTCCTACGG-GAGGCAGCAGT (forward primer) and GTATTACCGCGGCTGCTGG-CAC (reverse primer) (Tannock et al., 1999) whereas the *Campylobacter jejuni* specific primer sequences were CTGAATTTGA-TACCTTAAGTGCAGC (forward primer) and AGGCACGCCTAAACC-TATAGCT (reverse primer) (Nogva et al., 2000). The qPCR reaction mixture (total volume: 12.5 µl) contained 6.25 µl of SG qPCR Master Mix (2x) (0401, EURx, Poland), forward and reverse primers (Universal primers: 1 µM and *C. jejuni* specific primers: 0.1uM) (Sigma-Aldrich, Germany) and 20 ng of DNA. The qPCR for the samples (in two technical replicates) was performed in 96 well plates (4TI-0955, AZENTA, Poland) using the LightCycler 480 II (Roche-Diagnostics, Switzerland).

The protocol for qPCR for both primers included an initial denaturation step of 5 min at 95 ℃ followed by 40 cycles of amplification (denaturation at 95 ℃ for 10 s, an annealing for 15 s and elongation at 72 ℃ for 30 s). For the universal primers, the annealing temperature was 58 ℃ whereas for the *C. jejuni* specific primers, the annealing temperature used was 60 ℃. To determine the PCR efficiency of each primer set, a standard curve for each set of primers was performed with five $2 \times$ dilutions (1x, 0.5x, 0.25x, 0.125x and 0.0625x) of the pooled DNA sample (by pooling the DNA from all samples from all treatment groups). The PCR efficiency was then, determined using the LightCycler 480 II software (Roche-Diagnostics, Switzerland). The following formula (Slawinska et al., 2019) was used to calculate the relative abundance of *Campylobacter jejuni* in the luminal content of ceca and faecal samples.

$$\text{Relative abundance } (\%) = \frac{\text{Efficiency Universal}^{\text{Ct Universal}}}{\text{Efficiency } C.jejuni^{\text{Ct } C.jejuni}}$$

Efficiency universal: qPCR Efficiency of universal bacteria primers
    Ct universal: Ct value of qPCR reaction for total bacteria.
    Efficiency *C. jejuni*: qPCR Efficiency of *C. jejuni* specific primers.
    Ct *C. jejuni*: Ct value of qPCR reaction for *C. jejuni*.

Validation of the in-ovo treatment protocol and data analysis

Validation of the *in-ovo* treatment protocols in the current study involved two steps. The first step was to validate if the *in-ovo* treatment is adversely affecting the production parameters. In this regard, the hatchability, chick quality, BW and FCR of the *in-ovo*−treated birds were compared to those of the non-treated (NC) and mock-treated (PC) birds. The second step was to validate the efficacy of the *in-ovo* treatments in mitigating *Campylobacter jejuni* colonisation in the broilers. In this regard, the relative abundance of *Campylobacter jejuni* in faeces 1 week postinfection (day 28) and in the luminal content of ceca at the end of production period (day 34) was compared between mock-treated (PC) birds and the treated birds (PB and PPB). The outliers (values which are greater than the 3rd quartile + (1.5 × interquartile range) and below 1st quartile + (1.5 × interquartile range)) were removed from the data before statistical analysis. The comparisons between the groups were performed by one-way ANOVA procedure followed by Tukey's HSD mean comparison. Kruskal Wallis test followed by Dunn's test was performed when the assumptions of ANOVA were not met. The comparison between the BWs of infected and non-infected birds of the same *in-ovo* treatment group was performed using two−sample *t*-test (Wilcoxon Rank-Sum test, when assumptions were not met). A statistical significance (P-value < 0.05) or tendency (P-value < 0.1) was identified using the obtained *P*-values. All the statistical procedures were conducted using R software (version 4.3.1).

Results

Parameters for probiotic inoculum preparation

The growth curves of *Leuconostoc mesenteroides* B/00288 resulting from both the OD600 method and the plate count method were similar (Fig. 2). According to the growth curve, the probiotic strain seemed to have reached optimum growth by 15 h of incubation and remained in the stationary phase until 24 h. Therefore, in order to obtain the maximum number of cells at the highest metabolically active stage, we selected 15 h of incubation as the time point to prepare the inoculum for the *in-ovo* application.

A regression line was obtained by plotting the corresponding OD600 and CFU/ml values of each replicate at each time point until 15 h (Fig. 3). The regression equation was used to calculate the
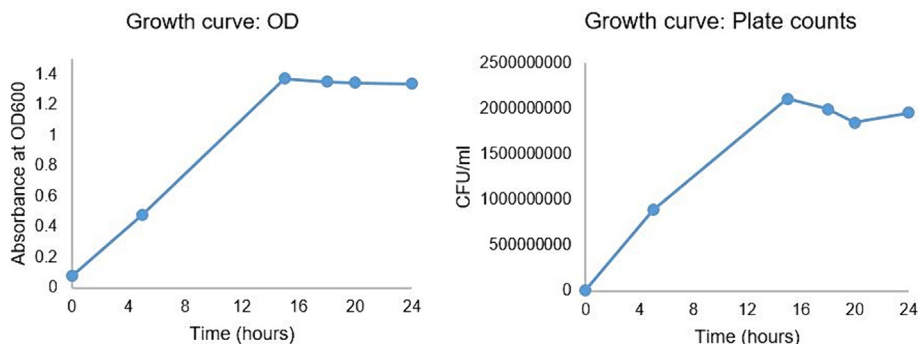
Fig. 2. Growth curves of *Leuconostoc mesenteroides* B/00288 obtained via Optical Density at 600 nm (OD600) method and plate count method. CFUs: Colony Forming Units.

OD600 value which corresponds to the dose required for each treatment as follows.

$$\text{Regression equation}: \ y\,(\text{OD600})$$
$$= (6 \times 10^{-10} \times (\text{dose in CFU/ml}))$$
$$+ 0.0249$$

$$\text{Probiotic (PB) treatment}: \text{required dose} = 5 \times 10^6 \ \text{CFU/ml}$$

$$y\,(\text{OD600}_{PB}) = \left(6 \times 10^{-10} \times \left(5 \times 10^6 \ \text{CFU/ml}\right)\right) + 0.0249$$
$$= 0.0279$$

$$\text{Probiotic (PB) treatment}: \text{required dose} = 5 \times 10^6 \ \text{CFU/ml}$$

$$y\,(\text{OD600}_{PB}) = (6 \times 10^{-10} \times (7.5 \times 10^6 \ \text{CFU/ml})) + 0.0249$$
$$= 0.0294$$

Accordingly, inoculums for respective treatments were prepared by adjusting the OD600 to the OD600 values calculated above. The coefficient of determinant of the regression equation was 0.9732 indicating a good precision (97%) in the dose of the inoculums we prepared for our *in-ovo* treatments.

Validation of the quality of in-ovo protocol/treatments

The quality of the *in-ovo* protocol and the treatments was evaluated by comparing the hatchability, hatch quality, BWs and FCR of the *in-ovo* treated birds to the non-treated and mock−treated birds
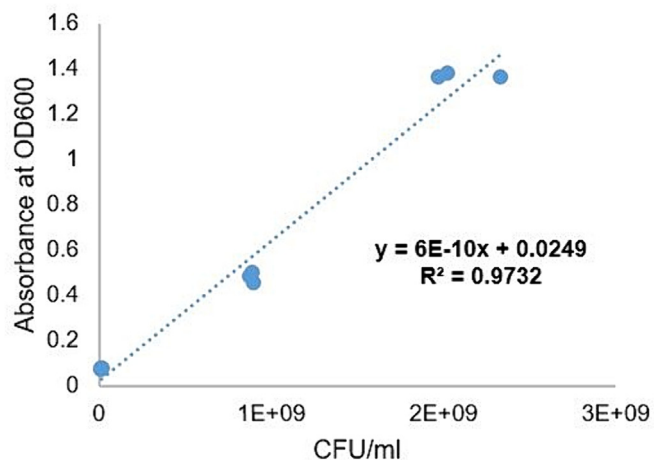

Fig. 3. Regression line to determine the corresponding *Optical Density at 600 nm (OD600)* values for probiotic inoculum preparation. CFUs: Colony Forming Units.

to determine if the treatments are impairing these parameters. The hatchability of the groups, NC, PC, PB and PPB was 97.4, 97.5, 97.6 and 100%, respectively. After removing the outliers (Chick weight: 1 from NC and 1 from PB and Chick length: 1 from PB and 2 from PPB), the chick length and chick quality (Pasgar score) did not significantly differ between groups (P-value > 0.1) whereas the PPB treatment group resulted in the highest chick weight (tendency; P-value = 0.0877) (Fig. 4). These results indicated that neither the *in-ovo* injection protocol nor the treatments adversely affected the hatch parameters of these chickens. After removing the outliers from the weekly BWs (NC: 1 from week 4, PB: 2 from weeks 3, 4 and 5 and PPB: 2 from week 2), it was observed that, during the 1st 2 weeks (P-values $7.966e^{-05}$ and 0.03323, respectively), the PC and PB groups had the highest BWs while the NC group displayed the lowest BWs. From the 3rd week onwards, no statistical differences in the weekly BWs were observed among the *in-ovo* groups (P-values > 0.1) (Fig. 5). The weekly FCR did not show any clear indication of one group being more efficient than another. On the contrary, the overall FCR of the *in-ovo* injected groups (PC, PB and PPB) was slightly higher than that of the NC group (Fig. 5).

When the infected and non-infected sub-groups of each *in-ovo* experimental group were compared separately, no significant differences in the BWs were observed (P-values > 0.1) except for the PB group (P-value = 0.01455). In PB treatment group, the infected birds displayed higher BW compared to the non-infected sub-group (Fig. 6). Considering the above results, we suggest that the *in-ovo* injection protocol and the treatments used in this study do not adversely affect the production parameters of the ROS308 broiler chickens.

Validation of the efficacy of in-ovo treatments in mitigating Campylobacter jejuni colonisation

To validate the infection protocol, quantification of *Campylobacter jejuni* in the faeces 1 week postinfection was performed via qPCR method. The results were negative for the samples from the non-infected groups while the samples of all infected groups were positive indicating the success of the infection protocol. The relative abundance of *Campylobacter jejuni* in the faeces, however, was statistically similar (P-value > 0.1) among the *in-ovo* treatment groups reflecting a similar level of colonisation and shedding of *Campylobacter jejuni* among the groups, 1 week postinfection (Fig. 7A). However, after removing the outliers (PC: 1 and PB: 1), the PB−treated chickens displayed a significantly lower relative abundance of *Campylobacter jejuni* in the ceca at the end of the production period (2 weeks postinfection/35 days of age) compared to those in the PC group (P-value = 0.0020). The PPB group displayed a statistical tendency (P-value = 0.0630) to have a lower *Campylobacter jejuni* abundance in ceca when compared to the PC group (Fig. 7B).
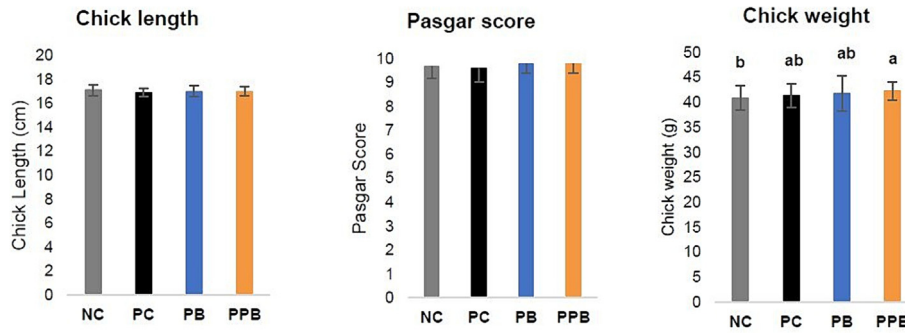
Fig. 4. The length of the broiler day old chicks of different experimental groups. Error bars: ± SD. Homogenous means have been indicated by similar letters (in descending order) (*P* < 0.05). NC: Negative control, PC: Positive Control, PB: Probiotic and PPB: Prophybiotic.
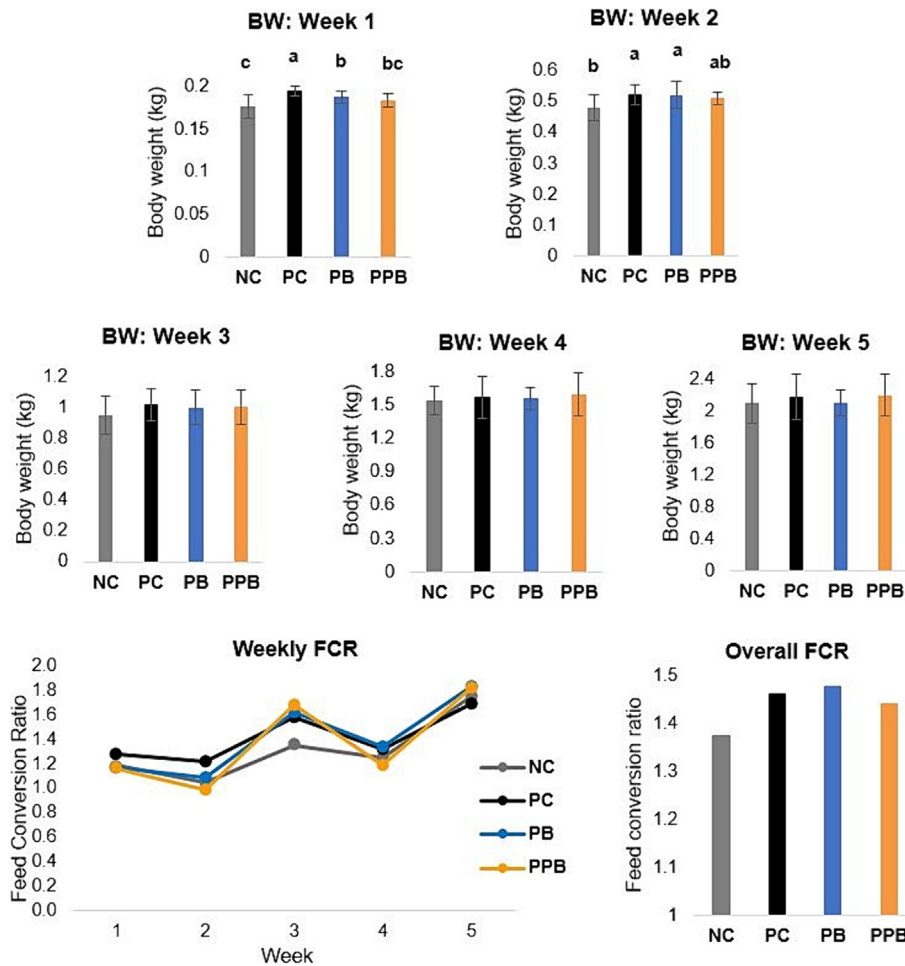

Fig. 5. BW and Feed Conversion Ratio (FCR) of the broiler chickens in the *in-ovo* experimental groups. Error bars: ± SD. Homogenous means have been indicated by similar letters (in descending order) (*P* < 0.05). NC: Negative control, PC: Positive Control, PB: Probiotic and PPB: Prophybiotic.

Author's point of view

- The main outcome of the study is an optimised protocol to perform *in-ovo* stimulation with a selected probiotic (*Leuconostoc mesenteroides* B/00288) or a prophybiotic (*Leuconostoc mesenteroides* B/00288 + garlic aqueous extract) to mitigate *Campylobacter jejuni* colonisation in ROSS308 broiler chickens without compromising the production parameters. The procedure or treatments did not adversely affect the hatch parameters (hatchability and chick weight, length and quality) and

BWs of the chickens whereas a significant reduction of (by probiotic treatment) and a statistical tendency in reducing (by prophybiotic treatment) *Campylobacter jejuni* colonisation was observed.
- This method is a one-time application applied at the hatchery level, and the reduction of *Campylobacter jejuni* colonisation was observed without continuous application of any supplement. Therefore, the authors believe that this method could be useful for the broiler industry by providing a low−cost yet promising solution to control *Campylobacter jejuni* contamina-
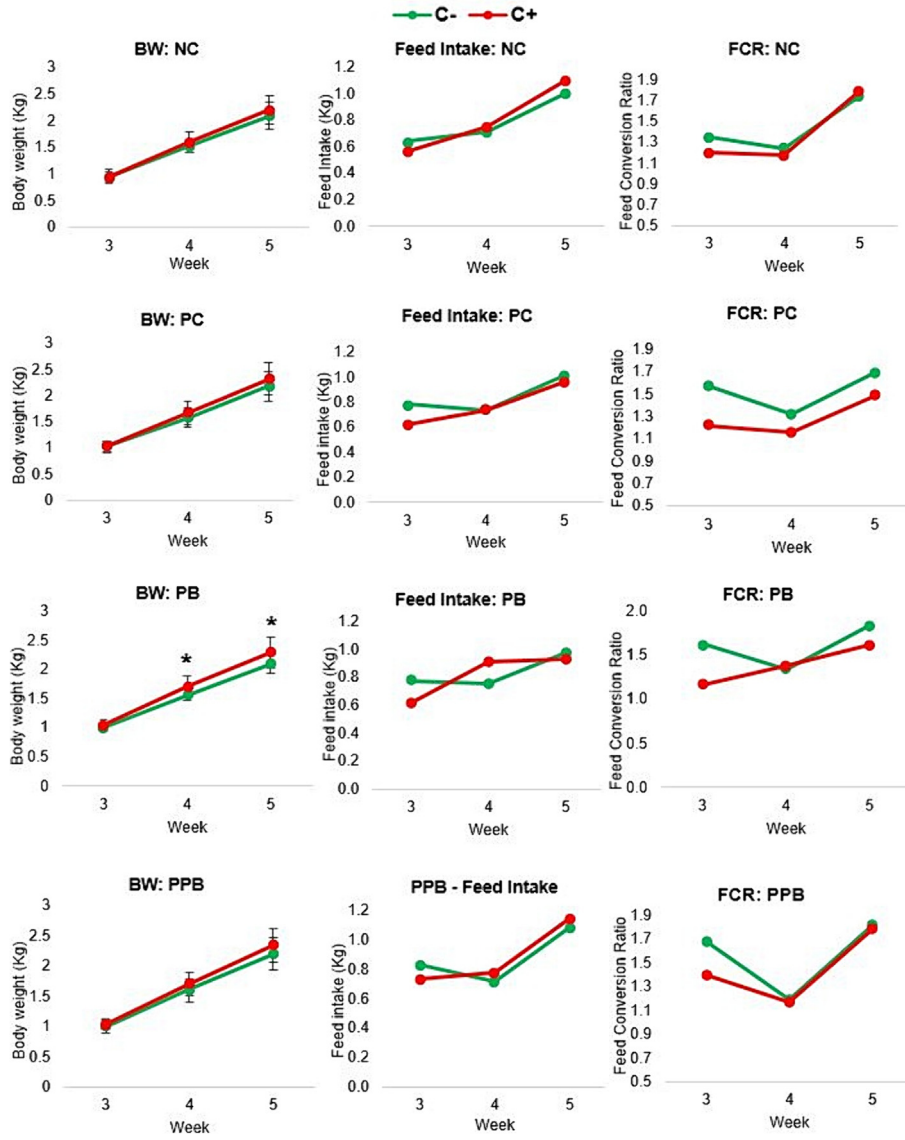
Fig. 6. BW, feed intake and FCR of the non-infected and infected broiler chickens in the *in-ovo* experimental groups. Error bars: ± SD. Significant differences indicated an asterix (*) (*P* < 0.05). C−: Non-infected C+: Infected, NC: Negative control, PC: Positive Control, PB: Probiotic and PPB: Prophybiotic.
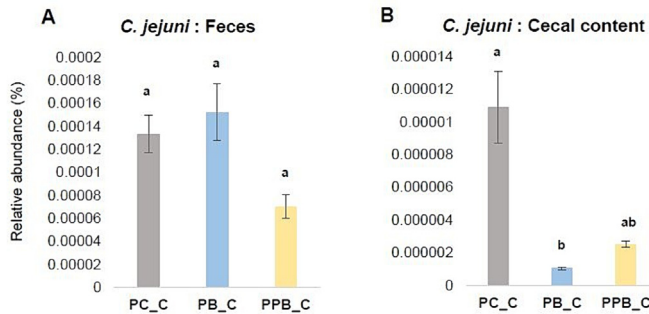

Fig. 7. *Campylobacter jejuni* relative abundance in A: Faeces on day 28 (one week postinfection) and B: Cecal content on day 34 (2 weeks postinfection) of the broiler chickens. Error bars: ± SE. Homogenous means have been indicated by similar letters (in descending order) (*P* < 0.05). PC_C: Positive control_infected, PB_C: Probiotic_infected, PPB_C: Prophybiotic_infected.

tion in broiler products. However, the constraint is the day of *in-ovo* injection. The usual egg handling days at the hatchery are day 7 and day 18. Therefore, day 12 would require an additional egg−handling step. However, our methodology will provide guidelines to other researchers working on *in-ovo* stimulation strategies, on how to determine optimal parameters in inoculum preparation for *in-ovo* application.

- There are some limitations in the current study. Only one strain of *Campylobacter jejuni* was used in the current study, and the colonisation potential and mechanisms differ in different strains. Moreover, the colonisation of *Campylobacter jejuni* also depends on factors such as the broiler strain and age at which infection is taking place which makes the control of *Campylobacter* in chickens a more complicated task. Therefore, further trials on infection with different strains/ combination of strains on different broiler chicken lines at different time periods would be necessary to further generalise the impact of this protocol.

- Other limitations related to the treatments are, that the different strains of the same probiotic (*Leuconostoc mesenteroides*) may display different growth performances, antimicrobial potential as well as compatibility with the phytobiotic component used in the prophybiotic (PPB) injection. Similarly, variations in the composition of antimicrobial substances (such as

allicin) and oligosaccharides may be expected in the garlic cultivar used in preparing the phytobiotic component of the PPB treatment. Therefore, the current protocol (the parameters and doses) might be unique with respect to the particular bioactive substances used and thus, optimisation is necessary when changes to the bioactive substances are made. However, this method paper will facilitate this optimisation process for future studies. Moreover, our protocol will encourage future researchers to employ more *in-ovo* approaches to mitigating *Campylobacter* infection in broiler chickens.

- The datasets generated by this study can be used by other researchers to compare the production parameters or *Campylobacter* abundance data of other studies. Our data can be used as a reference to compare with data from other chicken lines, chickens infected with different *Campylobacter* strains/ at different time points and those who are treated with different probiotics via different treatment methods (*in-ovo,* in feed or in water).

Conclusion

In conclusion, this study indicates the potential of mitigating *Campylobacter jejuni* colonisation in ROS308 broiler chickens via administering *Leuconostoc mesenteroides* B/00288 probiotic strain into hatching eggs without compromising the production parameters. This detailed method paper will provide a framework for *in-ovo* stimulation protocols and encourage more research on the use of novel bioactives in *in-ovo* stimulation to control *Campylobacter* colonisation in broiler chickens.

Peer Review Summary

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Ethics approval

Animal handling methodologies in this study were approved by the Local Ethical Committee (No 72/2023. 06.09.2023) and were in accordance with the animal welfare legislation of the European Union (directive 86/609/EEC).

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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All authors contributed to the Conceptualisation and methodology. Investigation, formal analysis, visualisation and writing- first draft were performed by RNWK. Funding acquisition, supervision, writing- review and editing were performed by RMH and MS. All authors read and approved the final manuscript.

Declaration of interest

None.

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