Journal of Animal and Feed Sciences, 32, 2, 2023, 152–163 https://doi.org/10.22358/jafs/156098/2023
The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna

Response in liver gene expression and DNA methylation to changes in the intestinal microbial profile after *in ovo* stimulation of chickens

A. Dunisławska[1,*], E. Pietrzak[1], A. Bełdowska[1], A. Sławińska[2] and M. Siwek[1]

[1] Bydgoszcz University of Science and Technology, Faculty of Animal Breeding and Biology, Department of Animal Biotechnology and Genetics, 85-796 Bydgoszcz, Poland
[2] Nicolaus Copernicus University in Torun, Faculty of Biological and Veterinary Sciences, Institute of Veterinary Medicine, Department of Basic and Preclinical Sciences, Gagarina 7, 87-100 Torun, Poland

KEY WORDS: early stimulation, gene silencing, microbiota, prebiotic, probiotic, symbiotic

Received: 31 July 2022
Revised: 27 October 2022
Accepted: 27 October 2022

* Corresponding author:
e-mail: aleksandra.dunislawska@pbs.edu.pl

ABSTRACT. The intestinal microbiota plays an important role in the signalling between the gut and the liver. Intestinal bacteria produce short-chain fatty acids and other signalling molecules that affect internal communication. Changes in the composition of the gut microbiota can lead to alternations in this communication, which may ultimately lead to modifications in gene expression driven by epigenetic mechanisms. This study aimed to determine the effect of early microbiome modifications by bioactive substances delivered *in ovo* on changes in the relative abundance of bacteria in the intestinal contents and the level of expression and methylation of hepatic genes. On day 12 of incubation, a probiotic, a prebiotic, and a synbiotic were injected into the eggs of broiler and Green-legged Partridge-like chickens. Samples were collected post-mortem on day 42. Relative bacterial abundance was analysed using qPCR, gene expression analysis by RT-qPCR, and gene methylation using the MS-qPCR method. It was shown that the relative abundance of the analysed bacteria changed in both genotypes. An increase was observed in the number of *Lactobacillus* and *Bifidobacterium*, and a decrease in the number of *Escherichia coli* in the Polish native breed. A significant increase was demonstrated in DNA methylation of the spleen associated tyrosine kinase gene after prebiotic administration in both groups, and the nuclear receptor subfamily 4 group A member 3 gene in broiler chickens after administration of the synbiotic. Changes in gene methylation correlated with alterations in gene expression. Early stimulation of the gut microbiota at the embryonic stage led to changes in the intestinal microbial profile in adults.

Introduction

The importance of the gut microbiota and its impact on intestinal function in poultry is well understood. However, there is a gap in knowledge regarding the interaction between the gut, its ecosystem and organs. It is an undeniable fact that the activity of intestinal bacteria extends far beyond the intestines and the associated lymphoid tissue (i.e. GALT). Interestingly, the hepatic metabolism related to the functioning of the gut is not yet well understood. The gut-liver axis is defined as the anatomical and functional relationship between the gastrointestinal tract and the liver (Albillos et al., 2020).

The gut microbiota does not directly affect host metabolism, but acts by producing metabolites and other signalling molecules (Koh and Bäckhed, 2020). From the point of view of poultry production, it is beneficial to support the intestines and their microbiota in order to achieve a multi-organ effect in the form of improved metabolism and immune status. In the first two weeks of life, *Lactobacillus* spp. and *Enterococcus* spp. are the dominant bacteria in all sections of the gastrointestinal tract (Aruwa et al., 2021), but as reported in the literature, *Escherichia coli* are also present in large numbers (Yadav and Jha, 2019). In the following weeks of life, the microbial profile of the gut is variable and unstable, which may be accompanied by mild inflammation (Berghof et al., 2013). This condition can lead to changes in the expression level of genes related to the immune system and basic metabolism, which affects the host body. The liver has both immune and metabolic functions (Hastings et al., 2020); therefore, it is crucial to characterise the abundance of major bacteria in the gut and mRNA expression profiles in the liver to understand the signalling at the level of the enterohepatic axis.

To support the intestinal microbiota an *in ovo* technology was developed to stimulate intestinal bacteria during embryonic development (day 12). It results in long-lasting effects in adult chickens. *In ovo* stimulation is a well-established method of introducing significant modulation of the gastrointestinal microbiota (Dunislawska et al., 2017; Siwek et al., 2018; Slawinska et al., 2019). *In ovo* administration of prebiotics can selectively modulate the entire gut microbiome ecosystem, resulting in the formation of a new metabolic environment (Siwek et al., 2018). The administration of probiotics, on the other hand, may result in the colonization of the intestines by beneficial bacterial strains and the elimination of pathogenic bacteria (Jha et al., 2020). In previous studies, we have proved that *in ovo* administration of synbiotics affects the regulation of immunological and metabolic gene expression, which depends on DNA methylation (Dunislawska et al., 2020). The results have clearly indicated that the negative regulation of metabolic gene expression in the liver by *in ovo* stimulation shows features of epigenetic control. This fact is largely supported by the available literature, as the process of DNA methylation has been shown to be modulated by environmental factors such as bioactive substances and their effects on the gut microbiota (Zhang, 2015; Ansari et al., 2020).

The objective of this study was to analyse changes in gene expression and methylation profiles in the liver under the influence of confirmed *in ovo* stimulation with bioactive substances of the intestinal microbiota in a line of broiler chickens and a Polish native breed.

Material and methods

Selection of gene panel for transcriptomic and epigenetic analysis

A panel of five genes was selected based on microarray data (Chicken Gene 1.1 ST Array Strip, Affymetrix, Santa Clara, CA, USA), as described by Dunislawska et al. (2020). This dataset contained broiler chicken transcripts generated from individuals that received *Lactobacillus* synbiotics *in ovo* on day 12. The main criteria for gene selection were strong downregulation of gene expression and statistical significance ($P < 0.05$). Two genes (spleen associated tyrosine kinase – *SYK*, kelch like family member 6 – *KLHL6*) were associated with the immune system, while three genes had metabolic functions (cysteine rich angiogenic inducer 61 – *CYR61*, nuclear receptor subfamily 4 group A member 3 – *NR4A3*, angiopoietin like 4 – *ANGPTL4*).

Experimental design

Six hundred eggs of ROSS 308 broiler chicken (ROSS broilers) and 600 eggs of Green-legged Partridge-like native chicken (GP) were incubated in a commercial hatchery using an automated incubator in standard conditions. On day 12 of incubation, the eggs were randomly distributed into experimental groups (150 eggs per group): (1) probiotic (PRO) – *Lactococcus lactis* subsp. *cremoris* (IBB PAS, Warsaw, Poland), (2) prebiotic (PRE) – galactooligosaccharides (GOS) (Bi^{2}tos, Clasado Biosciences Ltd., Reading, UK), (3) synbiotic (SYN) – *L. lactis* subsp. *cremoris* with GOS. The determined count of bacteria was 10^5 bacterial CFU/egg and the amount of prebiotic was 3.5 mg/egg (dose optimisation described in Bednarczyk et al., 2016 and Dunislawska et al., 2017). The control group (C) was mock-injected with 0.2 mM physiological saline (0.9%). Eggs were injected into the air chamber with 0.2 ml of an aqueous solution of each substance. After hatching, the birds were housed in litter pens (4 replicates/group, 8 animals each). Feed (prepared at the experimental station) and water were delivered manually and were available *ad libitum*.

Table 1. Chemical composition of feeds used for Ross broiler chickens (ROSS broilers) and Green-legged Partridge-like native chickens (GP)

Items	ROSS broilers				GP	
	Starter (D 1–10)	Grower I (D 11–21)	Grower II (D 22–33)	Finisher (D 34–42)	Starter (D 1–28)	Grower (D 19–42)
Metabolizable energy, MJ/kg	12.50	12.95	13.35	13.41	11.9	11.7
Crude protein, g/kg	220	200	190	184	200	185
Crude fiber, g/kg	28.00	30.0	31.0	32.0	34.0	35.0
Lysine, g/kg	13.8	12.5	11.3	10.5	11.0	10.0
Methionine + Cystine, g/kg	10.3	9.5	8.8	8.2	8.2	7.2
Threonine, g/kg	9.2	8.3	7.6	7.2	7.6	7.0
Tryptophan, g/kg	2.2	2.0	1.9	1.9	2.1	2.0

The feeding program was applied according to the requirements of the respective genotype. Table 1 presents the chemical composition of the feed for the ROSS broilers and Green-legged Partridge-like native chickens. Eight randomly selected individuals from each group (PRO, PRE, SYN, and C) were sacrificed on day 42 post-hatching, and the liver and intestinal contents (caecum) was collected. The experimental design is presented in Figure 1.

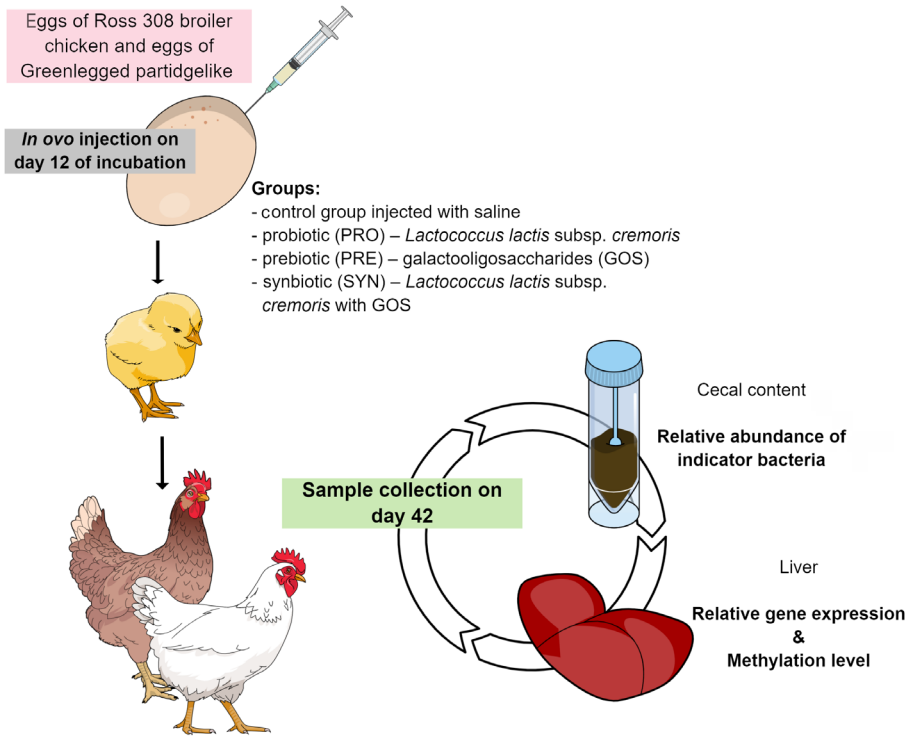

Figure 1. Experimental setup

Production data (feed consumption, percentage mortality, body weight) were collected during the rearing period. Statistical analysis of body weight was performed using a one-way ANOVA and Duncan's post-hoc test (SAS Enterprise Guide 8.2; SAS Institute Inc., Cary, NC, USA).

Relative abundance of indicator bacteria

The ceacal contents of chickens (n = 8/group) were collected into 5 ml Eppendorf tubes. Immediately after collection, samples were placed on dry ice, until transported to the laboratory and stored at −80 °C. Bacterial DNA was extracted from ceacal content samples (approximately 150 mg/sample) using the GeneMATRIX Stool DNA Purification Kit (EURx, Gdańsk, Poland) according to the manufacturer's instructions. The extracted DNA was subjected to qualitative (agarose gel electrophoresis) and quantitative (Scientific Nanodrop Products, Wilmington, DE, USA) assessments.

Relative quantification of the total number of *Bifidobacterium* spp., *Lactobacillus* spp., *E. coli,* and

Clostridium difficile was carried out by qPCR using a LightCycler 480 II (Roche-Diagnostics, Basel, Switzerland). The qPCR reaction mixture contained Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 1 μM each primer specific to 16S rRNA (synthesized by Sigma-Aldrich, Schnelldorf, Germany) and 20 ng of DNA. The primer sequences from the literature data are listed in Table 2.

Table 2. Primer sequences for the identification of bacteria

Bacteria	Forward sequence 5'→3'	Reverse sequence 5'→3'	Reference
Universal bacteria	ACTCCTACGGGAGGCAGCAGT	GTATTACCGCGGCTGCTGGCAC	Christensen et al., 2014
Bifidobacterium spp.	GCGTGCTTAACACATGCAAGTC	CACCCGTTTCCAGGAGCTATT	Christensen et al., 2014
Lactobacillus spp.	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	Christensen et al., 2014
Escherichia coli	CATGCCGCGTGTATGAAGAA	CGGGTAACGTCAATGAGCAAA	Huijsdens et al., 2002
Clostridium difficile	TTGAGCGATTTACTTCGGTAAAGA	TGTACTGGCTCACCTTTGATATTCA	Penders et al., 2005

Each qPCR reaction was conducted in two technical replicates. The thermal profile of the qPCR reaction was as follows: initial denaturation at 95 °C for 5 min, followed by 40 amplification cycles: denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and elongation at 72 °C for 45 s. Thermocycling consisted of measuring fluorescence at the end of each elongation step. After amplification, a melting curve was generated by gradually increasing the temperature to 98 °C and measuring the fluorescence of the melting amplicons. PCR efficiency (E) for each primer pair was calculated using the LightCycler 480 II software based on separate reactions of 5 dilutions (1×, 0.5×, 0.25×, 0.125×, and 0.0625×) of the bacterial DNA template. The relative abundance of the bacteria in the caecal content was calculated using the following formula:

$$(1+E_{universal})^{Cq\ universal}/(1+E_{target})^{Cq\ target},$$

where: $E_{universal}$ – efficiency of universal bacterial primers, $Cq_{univer}ar_{gl}$ – quantification cycle for the universal bacterial primer, E_{target} – efficiency for the bacterial target primers and $Cq_{t}arg_{et}$ – quantification cycle for the bacterial target primer (Christensen et al., 2014). Statistical analysis was performed using the t-test comparing the test group with the control group.

Gene expression in liver

RNA isolation from the liver fixed in stabilisation buffer (fix RNA, EURx, Gdansk, Poland) was carried out using TRI reagent (MRC, Cincinnati, OH, USA) and a commercial kit for RNA purification (Universal RNA Purification Kit, EURx, Gdansk, Poland). Liver (n = 6/each group) was homogenized with a TissueRuptor homogenizer (Qiagen GmbH, Hilden, Germany) in TRI reagent and purified on columns. RNA was subjected to qualitative (agarose gel electrophoresis) and quantitative (NanoDrop spectrophotometer, Thermo Fisher Scientific Nanodrop Products, Wilmington, NC, USA) assessments.

Gene expression analysis was performed by quantitative reverse transcription PCR (RT-qPCR). cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific/Fermentas, Vilnius, Lithuania), according to the manufacturer's recommendations. The qPCR reaction mixture included Maxima SYBR Green qPCR Master Mix (Thermo Scientific/Fermentas, Vilnius, Lithuania), 1 μM each primer, and diluted cDNA (140 ng). Thermal cycling was performed in a LightCycler II 480 (Roche Diagnostics, Basel, Switzerland). Each RT-qPCR reaction was conducted in two technical replicates and 24 biological repeats (n = 6/each group). Sequences of primers were based on our previous experiment (Dunislawska et al., 2020; Table 3). Relative gene expression analysis was conducted separately for each experimental group using the ΔΔCt method (Livak and Schmittgen, 2001) and actin beta (Sevane et al., 2014) and glyceraldehyde-3-phosphate dehydrogenase (De Boever et al., 2008) as reference

Table 3. Primer sequences for RT-qPCR reactions

Gene	(Forward/reverse) sequence 5'→3'	NCBI no./ Reference
KLHL6	F: ATGGTTTCTGCGTCAACTCC R: CATCCTGGCTGGGATGCAATA	424762
SYK	F: AAGGGACAGCAATGGTTCCT R: AATTTAACAGACCTGCCAGAGG	427272
ANGPTL4	F: TCCTCGATTCGCGAGTTCTG R: CAGGGCACTGGGAGCTG	769087
NR4A3	F: GGCATCCCCGGAGTTTCTCTG R: TTTGACGAGGCCGCTCATT	420996
CYR61	F: ATCGCTCGTTCAGACGCATA R: TGTCTGGGCTCCGCTAAAAG	429089
ACTB	F: CACAGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	Sevane et al., 2014
GAPDH	F: GGCACGCCATCACTATC R: CCTGCATCTGCCCATTT	De Boever et al., 2008

KLHL6 – kelch like family member 6, *SYK* – spleen-associated tyrosine kinase, *ANGPTL4* – angiopoietin like 4, *NR4A3* – nuclear receptor subfamily 4 group A member 3, *CYR61* – cysteine rich angiogenic inducer 61, *ACTB* – actin beta, *GAPDH* – glyceraldehyde-3-phosphate dehydrogenase

genes. The geometric mean cycle threshold (Ct) values of the reference genes were used in the analysis (Vandesompele et al., 2002).

Gene methylation in liver

DNA isolation from liver (n = 6/group) was carried out based on the phenol-chloroform protocol for RNA isolation (Maniatis et al., 1982). Tissue sections were transferred into a 2.0-ml Eppendorf tubes containing 500 µl of lysis buffer (1 M Tris-HCl, 0.5 M EDTA, pH 8) and proteinase K. Tissues were homogenized using a TissueRuptor homogenizer (Qiagen, GmbH, Hilden, Germany), and after homogenization, the samples were vortex and centrifuged (12 000 *g*, 10 min). The supernatants were transferred to new tubes. An equal volume of phenol-chloroform-isoamyl mixture was added to the supernatants, and then mixed and centrifuged (13 000 rpm, 10 min, room temperature). The aqueous phase was transferred to a new tube without disturbing the protein interphase and centrifuged again (13 000 rpm, 10 min, 4 °C). The precipitated DNA was washed with 70% ethanol and allowed to dry. The dry precipitate was dissolved in TE buffer overnight at room temperature. DNA was subjected to qualitative and quantitative assessment.

The isolated DNA was subjected to methylation analysis using quantitative real-time methylation-specific polymerase chain reaction (qMSP). Primers for qMSP reactions were designed within CpG islands and previously published in Dunislawska et al. (2020). The conversion was carried out using the EpiJet Bisulfite Conversion Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. In the next step, qPCR reactions were performed for the selected genesusing a LightCycler 480 (Roche Diagnostics, Basel, Switzerland) thermal cycler. The reaction mixture contained the Maxima SYBR Green qPCR Master Mix intercalating dye (Thermo Fisher Scientific, Waltham, MA, USA). The melting temperature was optimised at 58 °C. After amplification, a melting curve was generated for each product. The relative level of DNA methylation [%] was calculated based on the results of melting curves (fluorescence level readings) for each individual according to the following formula (Fackler et al., 2004):

$$\% \text{ of methylation} = 100 \times \left(\frac{M}{M + U} \right),$$

where: M – average fluorescence intensity of the methylated product, U – average fluorescence intensity of the unmethylated product. Statistical analysis was performed using Student's t-test (n = 6; $P < 0.05$).

Results

Production parameters

The analysis of feed consumption did not show any significant numerical changes between the groups stimulated with bioactive substances ($P > 0.05$) (Table 4). The administration of the prebiotic in the chicken broiler group resulted in a slight numerical increase in feed consumption per individual, while a decrease in feed consumption per kg of gain. A lower percentage of mortality in the probiotic group was demonstrated, while in the prebiotic group, it increased relative to control (numerically). In GP, synbiotic supplementation resulted in a higher feed intake (Table 4). A percentage decrease in mortality in the probiotic group, and an increase after administration of the prebiotic and synbiotic was shown.

Table 4. Consumption of compound feed and intake per 1 kg of chicken weight gain throughout the rearing period (mean ± SD) and percentage mortality

Group	Compound feed consumption by one individual, g	Compound feed consumption per 1 kg of weight gain, kg	Mortality, %
ROSS broilers			
C	4673.2 ± 118.1	1.50 ± 0.07	4.7
PRO	4832.7 ± 198.0	1.49 ± 0.04	3.1
PRE	5080.5 ± 131.7	1.55 ± 0.03	7.7
SYN	4949.7 ± 231.0	1.66 ± 0.05	4.6
GP			
C	940.7 ± 74.2	2.1 ± 0.1	6.3
PRO	937.5 ± 41.7	2.1 ± 0.2	0.0
PRE	1059.7 ± 131.7	2.3 ± 0.3	9.4
SYN	1106.0 ± 90.9	2.5 ± 0.3	9.4

ROSS broilers – Ross broiler chickens, GP – Green-legged Partridge-like native chickens; C – control, PRO – *Lactococcus lactis* subsp. cremoris, PRE – prebiotic galactooligosaccharides (GOS), SYN – synbiotic – *Lactococcus lactis* subsp. *cremoris* with GOS; data are presented as mean value ± SEM; $P > 0.05$

Egg stimulation with the probiotic increased body weight of ROSS broilers from week 1 to 5 in comparison to the control group (Table 5). No effect of probiotic and synbiotic stimulation on body weight was observed in ROSS broilers from week 1 to week 5. At week 6, an increase in body weight was recorded in the PRE and SYN groups. However, stimulation with the probiotic and prebiotic exerted no effect on body weight of GP chicken in comparison to the control group. Stimulation with the synbiotic of GP chicken eggs decreased body weight of these chickens in comparison to the control and other groups.

Table 5. Body weight of chickens during the rearing period (mean ± SD)

Group	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
ROSS broilers						
C	194.3^{b} ± 16.3	522.1^{b} ± 56.5	990.5^{bc} ± 93.1	1723.8^{bc} ± 120.3	2565.9^{bc} ± 165.8	3129.4^{b} ± 219.6
PRO	204.9^{a} ± 17.7	556.2^{a} ± 52.2	1049.7^{a} ± 95.2	1799.6^{a} ± 109.5	2702.6^{a} ± 163.8	3229.7^{ab} ± 320.7
PRE	195.6^{b} ± 23.0	544.1^{ab} ± 73.4	1019.0^{ab} ± 135.9	1782.9^{ab} ± 148.1	2621.4^{ab} ± 284.1	3277.1^{a} ± 325.2
SYN	198.7^{ab} ± 18.9	529.0^{b} ± 56.5	972.7^{c} ± 102.5	1712.4^{c} ± 128.1	2492.6^{c} ± 178.2	2978.5^{c} ± 243.7
GP						
C	83.2^{ab} ± 8.8	150.1^{a} ± 21.0	230.4^{a} ± 36.9	325.9^{a} ± 53.8	419.1 ± 72.0	446.2 ± 77.7
PRO	83.4^{ab} ± 8.8	154.7^{a} ± 14.4	239.5^{a} ± 19.6	331.3^{a} ± 29.3	437.4 ± 39.8	448.8 ± 47.4
PRE	85.2^{a} ± 8.0	150.2^{a} ± 24.6	238.5^{a} ± 31.2	314.0^{ab} ± 43.6	418.3 ± 79.2	465.3 ± 57.1
SYN	81.6^{b} ± 8.5	142.2^{b} ± 17.3	199.8^{b} ± 36.8	299.4^{b} ± 52.3	417.5 ± 54.1	419.2 ± 56.2

ROSS broilers – Ross broiler chickens, GP – Green-legged Partridge-like native chickens; C – control, PRO – *Lactococcus lactis* subsp. *cremoris*, PRE – prebiotic galactooligosaccharides (GOS), SYN – symbiotic, *Lactococcus lactis* subsp. *cremoris* with GOS; data are presented as mean value ± SEM; ^{abc} – means within a column with different superscripts are significantly different at $P < 0.05$

Relative abundance of indicator bacteria

The results indicated that the highest number of *Bifidobacterium* spp. was determined in the caecum, and GOS showed a bifidogenic effect in this segment (increase from 1.3% to 3.9%). In this experiment, lower levels of *Bifidobacterium* spp. were recorded in both ROSS broilers and GP chickens (mean for C – 0.005%). However, significant changes were noted for both genotypes in the SYN group. In ROSS broilers, there was a reduction (to 0.002%) in the relative abundance of *Bifidobacterium* spp. (Figure 2). In GP chickens, the injected synbiotic exerted a bifidogenic effect, increasing the amount of bacteria to 0.02% (Figure 3).

In ROSS broilers, SYN numerically increased the abundance of *Lactobacillus* spp. in the caecal contents from approx. 0.06% to 0.12% (Figure 2). In GP chickens, a statistically significantly higher abundance of *Lactobacillus* spp. bacteria was found in the group of animals given SYN compared to C (from about 0.015% to 0.06%) (Figure 3).

In ROSS broilers and GP chickens, the highest abundance of *E. coli* was observed in the C group (0.005% and 0.09%, respectively). The lowest abundance of *E. coli* in ROSS broilers was determined in animals stimulated *in ovo* with the GOS prebiotic (approx. 0.002%) (Figure 2). In GP chickens, the lowest *E. coli* titre was determined in the SYN group (approx. 0.01%) (Figure 3). Native GP birds showed a higher abundance of *E. coli* in each group compared to ROSS broilers.

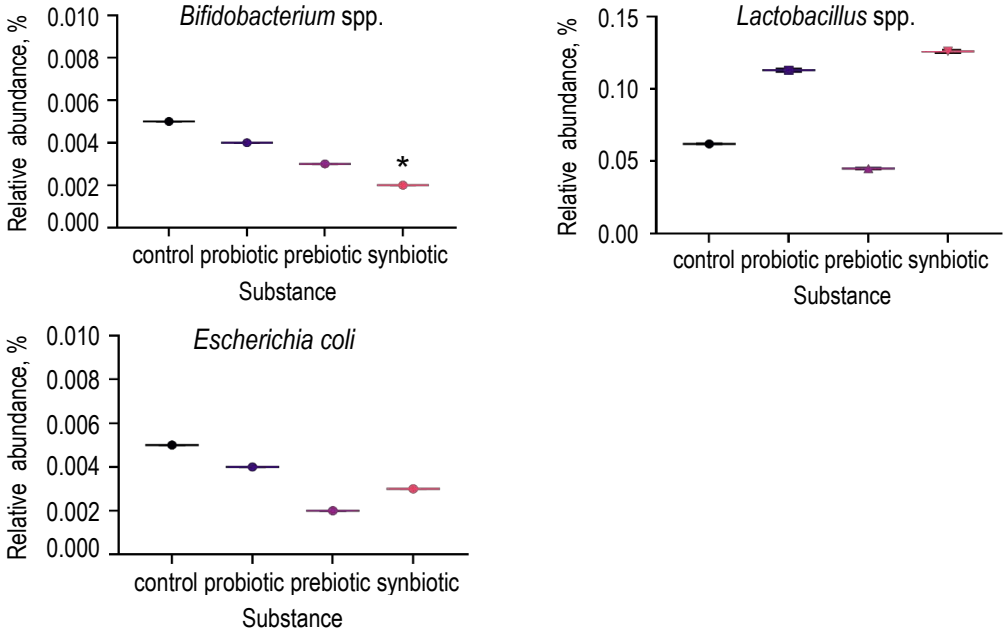

Figure 2. Relative abundance of bacteria in the caecal contents in ROSS 308 broiler chickens (n = 8/group) after *in ovo* stimulation with control (saline), probiotic (*Lactococcus lactis* subsp. *cremoris*), prebiotic galactooligosaccharides (GOS) and synbiotic (*L. lactis* subsp. *cremoris* with GOS); * – $P < 0.05$; *Clostridium difficile* not detected

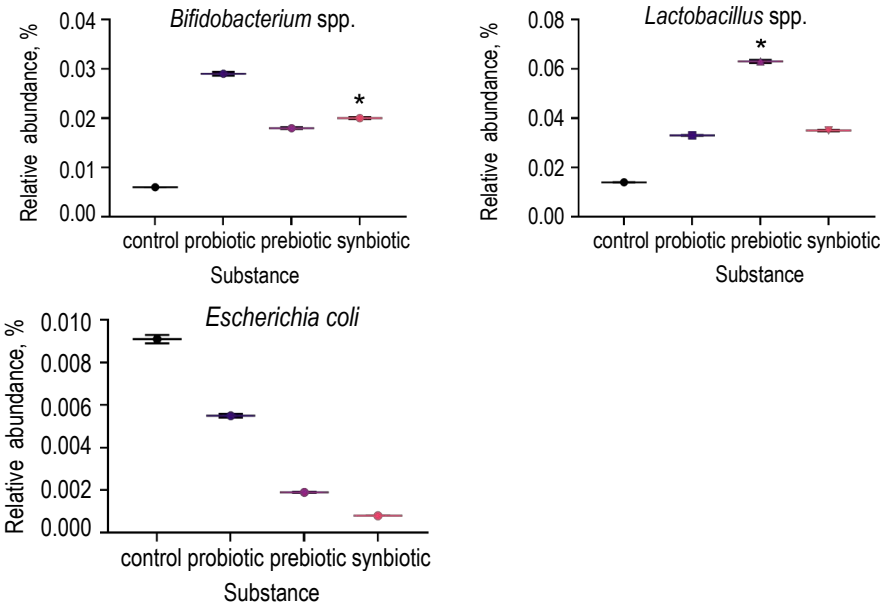

Figure 3. Relative abundance of bacteria in the caecal contents in native polish breed – Green-legged Partridge-like chickens (n = 8/group) after *in ovo* stimulation with control (saline), probiotic (*Lactococcus lactis* subsp. *cremoris*), prebiotic galactooligosaccharides (GOS) and synbiotic (*L. lactis* subsp. *cremoris* with GOS); * – $P < 0.05$; *Clostridium difficile* not detected.

Gene expression in liver

Gene analysis showed a significant decrease in the expression of immune-related genes, *KLHL6* and *SYK*, in ROSS chickens, and an increase in their expression in GP birds after SYN administration (Figure 4). An increase in *ANGPTL4* expression was also observed following SYN administration in GP chickens. In addition, the administration of all analysed substances

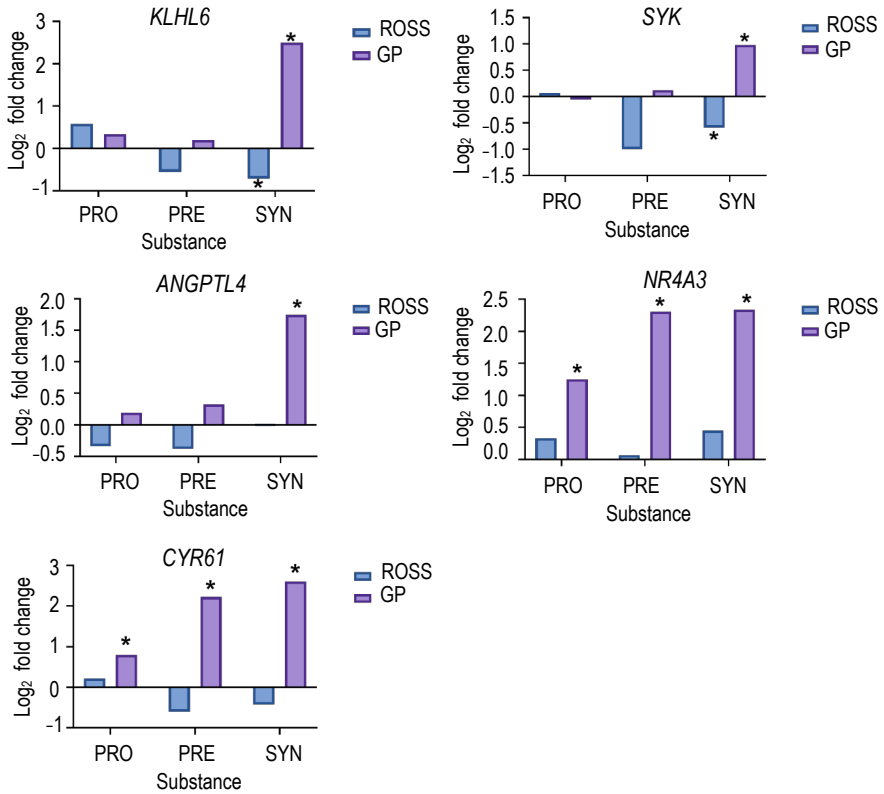

Figure 4. Expression of the *KLHL6*, *SYK*, *NR4A3*, *ANGPTL4* and *CYR61* genes in liver

X-axis – genetic groups: ROSS and Green-legged Partridge-like (GP); groups: PRO – probiotic, PRE – prebiotic, SYN – symbiotic; *KLHL6* – kelch like family member 6, *SYK* – spleen associated tyrosine kinase, *ANGPTL4* – angiopoietin like 4, *NR4A3* – nuclear receptor subfamily 4 group A member 3, *CYR61* – cysteine rich angiogenic inducer 61; Y-axis – percentage of methylation; * – $P < 0.05$ (n = 6)

significantly increased the expression of the *NR4A3* and *CYR61* genes in GP birds. In ROSS chickens, a decrease or no change in gene expression was observed after *in ovo* administration of bioactive substances.

Gene methylation in liver

There were statistically significant differences in methylation levels of the *KLH6, SYK, NR4A3,* and *ANGPTL4* genes after administration of bioactive substances. A statistically significant decrease was recorded in *KLHL6* methylation levels in the PRO group (50.9%) compared to control (59.1%). Significant methylation levels in the *SYK* gene were as follows: ROSS – 1% in C, 17.4% in PRE; GL – 9.3% in C, 2% in PRO, and 1.2% in SYN. A significant increase in *NR4A3* methylation was observed in ROSS chickens from the SYN group (51.1%) compared to control (5.5%). *ANGPTL4* methylation decreased in ROSS birds (80.1% in C, 61.1% in PRE), while it increased in GP chickens (69.5% in C, 81.1% in PRE). The results for all analysed genes are presented in Figure 5.

Discussion

The main aim of this study was to determine the impact of various bioactive substances, administered in ovo on day 12 of egg incubation (*in ovo* stimulation) into the air chamber, on changes in the relative abundance of indicator bacteria in the intestinal contents, and levels of hepatic gene expression and DNA methylation. These analyses were carried out to confirm the epigenetic nature of gene expression associated with early reprogramming of the microbiota.

Modification of the gut microbiota, as shown in the literature and in our previous studies, may be an important environmental signal for epigenetic regulation of gene expression. Changes in the microbiological profile of the intestines were confirmed by the analysis of indicator bacteria in the caecal contents. The caecum serves as the main fermentation chamber with the highest activity and density of anaerobic bacteria. Our previous experiments showed that the administration of a synbiotic at an early stage of embryonic development influenced

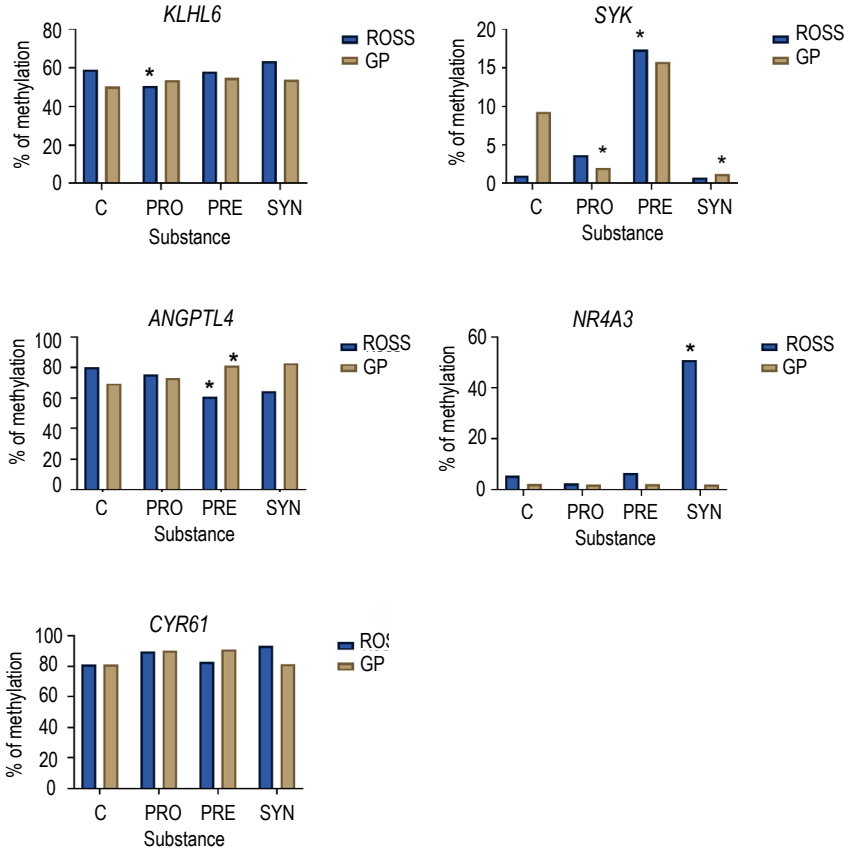

Figure 5. DNA methylation of the *KLHL6*, *SYK*, *NR3A3*, *ANGPTL4* and *CYR61* genes in liver

X-axis – genetic groups: ROSS and Green-legged Partridge-like (GP); groups: C – control, PRO – probiotic, PRE – prebiotic, *SYN* – symbiotic; *KLHL6* – kelch like family member 6, *SYK* – spleen associated tyrosine kinase, *ANGPTL4* – angiopoietin like 4, *NR4A3* – nuclear receptor subfamily 4 group A member 3, *CYR61* – cysteine rich angiogenic inducer 61; Y-axis – percentage of methylation; * – $P < 0.05$ (n = 6)

the growth of *Clostridium* bacteria, which in turn significantly affected intestinal health. Moreover, a lower number of *Lactobacillus* spp./*Enterococcus* spp. was also detected (Dunislawska et al., 2017). Our subsequent analyses proved that *in ovo* administration of the GOS prebiotic reduced the population of *Lactobacillus* spp. in all segments of the intestine (Slawinska et al., 2019). In the general interpretation of animal nutrition, lactic acid bacteria are considered beneficial to the host as they lower the pH by producing lactic and acetic acids. This change in pH is considered beneficial for the host organisms, allowing for the control of pathogenic bacteria.

Early colonization of the intestine is of great importance for poultry health and productivity. The chickens' microbiota develops rapidly in the first days after hatching; however, in a commercial broiler production, chickens hatch in a hatchery without contact with older birds. Delivering bioactive substances such as pro-, pre- or synbiotics *in ovo* is an effective method of early stimulation of the host microbiome, prior to direct contact of the newly hatched chicken with microorganisms from the external environment (Pedroso et al., 2016; Dunislawska et al., 2017; Slawinska et al., 2019). The microbial community in the intestines of 3-day-old chickens has been shown to be transient and replaced by stable microbiota later in life (Choi et al., 2015). In mature chickens, the caecum is the part of the gastrointestinal tract (GIT) with the highest concentration of microorganisms, and its impact on health and performance has been confirmed (Oakley and Kogut, 2016; Stanley et al., 2016). The caecal microbiota plays a key role in metabolising cellulose, starch and polysaccharides (Clench and Mathias, 1995). The main groups of bacteria that make up the caecal microbiota include *Firmicutes* and *Bacteroidates*, as well as *Actinobacteria* and *Proteobacteria* (Wei et al., 2013).

In our study, we analysed the caecal contents of 42-day-old commercial broiler chickens (ROSS) and a native polish chicken breed (Green-legged Partridge-like). In 42-day-old chicks (ROSS and Green-legged Partridge-like), no significant effect of *in ovo* probiotic and prebiotic administration on the relative abundance of *Bifidobacteria* spp. was observed; however, groups stimulated *in ovo* with the synbiotic (SYN) demonstrated a statistical difference (a decrease in the ROSS genotype, and an increase in the GP genotype) compared to the C group. *Bifidobacteria* spp. belong to the phylum *Actinobacteria*, which consists of non-spore-forming, non-motile, anaerobic Gram-positive bacteria. It is assumed that in the early stage of bird development, the population of *Bifidobacteriaceae* increases, which may be an important element of the maturation of the caecal microflora. Some bacteria, including *Bacteroides fragilis* have been shown to metabolise exopolysaccharides, i.e. carbohydrates produced by certain *Bifidobacterium* strains (Salazar et al., 2008).

Lactobacillus spp., belonging to the phylum *Firmicutes*, are responsible for the fermentation of carbohydrates. Their metabolites lower the pH and limit the growth of other bacterial species that may adversely affect host health (Crhanova et al., 2019). In the current study, *in ovo* stimulation had no statistically significant effect on the relative abundance of *Lactobacillus* spp. in the ROSS broiler genotype. A significant difference in the relative abundance of *Lactobacillus* spp. was determined in GP chickens stimulated *in ovo* with the prebiotic GOS. In broilers, 40 days of GOS supplementation increased the abundance of *Bifidobacteria* and *Lactobacilli,* as measured in animal faeces (Jung et al., 2008). Slawinska et al. (2019) demonstrated that the relative abundance of *Lactobacilllus* spp. was the lowest in the caecal contents compared to the other parts of the GIT, and the GOS-stimulated group had a lower *Lactobacillus* spp. content (0.02%) compared to control animals (0.4%). Here, in ROSS broilers, SYN numerically increased the abundance of *Lactobacillus* spp. in the caecal contents. Potentially, this increase could reduce the abundance of *Bifidobacterium* spp. in this group through competitive exclusion.

E. coli mainly inhabited the lower section of the GIT. In poultry, *E. coli* (phylum *Proteobacteria*) colonisation occurs in the first 24 h after hatching (Ballou et al., 2016). Some of *E. coli* strains are commensal, but others have acquired pathogenic capacity. *E. coli* can cause colibacillosis in poultry – a disease associated with perihepatitis, pericarditis, and airsacculitis (Johnson et al., 2008). Our study demonstrated a numerical (not statistically confirmed) decrease in *E. coli* abundance in all groups subjected to *in ovo* (PRO, PRE, and SYN) stimulation compared to the C group.

The current experiment also quantified the specific region of the 16S rRNA gene in *C. difficile*. We did not detect the presence of *C. difficile* in the tested caecal contents in the experimental animals (data not shown). *C. difficile* is a Gram-positive anaerobic, spore-forming bacteria belonging to the phylum *Firmicutes* of the family *Clostridiaceae* (Rupnik

et al., 2009). There is no evidence of significant pathogenicity of *C. difficile* in poultry. These bacteria may be present in the faeces of healthy chickens and their abundance may depend on age (Skraban et al., 2013), while in humans *C. difficile* is a pathogen causing severe diarrhoea. Due to the similarity of *C. difficile* ribotypes found in human and animal samples, there are some indications that animal carriers (including poultry) are one of the causes of *C. difficile* infections in humans (Keessen et al., 2011). Therefore, it is particularly important to control the occurrence of *C. difficile* in broilers as the main source of meat.

It is believed that the inhibition of the development of pathogenic gut microbiota may be mediated by the production of organic acids by resident lactic acid bacteria (LAB) in the gut (Gibson and Wang, 1994). In addition, oligosaccharides such as GOS selectively promote the growth of beneficial bacteria, limiting the excessive development of unfavourable strains (Shoaf et al., 2006). We found small differences in the relative abundance of indicator bacteria, including *Bifidobacterium* spp., *Lactobacillus* spp., *E. coli* and *C. difficile* between the control and the groups subjected to *in ovo* stimulation on day 12 of egg incubation (PRO, PRE, and SYN). These results applied to both ROSS broilers and GP chickens. We assumed that indicator bacteria selected for the study confirmed the relative stability of the caecal microbiota, which could result from its maturity in the analysed birds.

DNA methylation affects gene activity by providing and activating molecular mechanisms for biological and disease processes. DNA methylation contributes to the silencing of gene expression, which is due to the fact that it changes the chromatin structure into an inactive and condensed so-called heterochromatin. A significant portion of methylated nucleotides serve as a signal for chromatin-building proteins, ordering the initiation of chromatin condensation processes (Li et al., 2015). As reported in the literature and in our previous study, DNA methylation can be modulated by shaping the gut microbiota.

In our study, *in ovo* stimulation of GP with the synbiotic resulted in an increase in *SYK* gene expression with a simultaneous decrease in its methylation. SYK plays a role in transmitting adaptive immune signals. *SYK* is expressed in liver parenchymal cells (Torres-Hernandez et al., 2019). In ROSS chickens, there was an increase in methylation (hypermethylation) of the *NR4A3* gene with a simultaneous decrease in the expression of this gene in the group stimulated with the *Lactococcus*-based synbiotic. These results differ from the findings described in our previous report (Dunislawska et al., 2020), where the administration of the *Lactobacillus*-based synbiotic reduced *NR4A3* gene methylation (hypomethylation) in the liver of broiler chickens. In both studies, the level of methylation correlated with the expression of the *NR4A3* gene. This gene is an activator of transcription, binding to regulatory elements in the promoter region. It plays a role in the process of fatty acid utilisation, cell differentiation and apoptosis. It may also be responsible for increased food intake and weight gain. These cases demonstrate a strong correlation between silencing the expression of these genes and DNA methylation. After *in ovo* stimulation with the synbiotic, a significant increase was observed in the expression of the *ANGPTL4* gene in GP birds. The *ANGPTL4* gene is involved in processes related to the lipoprotein lipase activity, angiogenesis, and triglyceride homeostasis. This gene codes for a protein that regulates glucose homeostasis, insulin sensitivity and lipid metabolism. The liver usually produces *ANGPTL4* at a stable level (Dijk and Kersten, 2014). Administration of LAB can reduce the amount of adipose tissue during increased expression of the *ANGPTL4* gene. Interestingly, methylation of the *ANGPLT4* gene increased after synbiotic administration, indicating no correlation between methylation and expression in GP after synbiotic administration, and the lack of epigenetic gene silencing effect. This mechanism may be genotype-dependent.

Conclusions

The results demonstrated that alterations in the methylation of metabolic genes were correlated with changes in gene expression. It was shown that early stimulation of the intestinal microbiota in ovo with bioactive substances influenced the microbiological profile of the intestines in adults. Further high-throughput analyses are needed to address the issue of gut microbiota programming and its epigenetic effects. Based on these results, it can be concluded that the body responds with changes in gene expression and methylation levels in the liver to alterations in the profile of the gut microbiota. These analyses represent the next step in confirming the epigenetic nature of gene expression associated with *in ovo* programming of the gut microbiota.

Funding source

The authors would also like to thank Clasado Biosciences for providing the prebiotic for research. The study was financed by grant UMO-2017/25/N/NZ9/01822 funded by the National Science Centre (Poland). The manuscript was funded from a subsidy for the Bydgoszcz University of Science and Technology granted by the Ministry of Science and Higher Education in Poland (BN-0/2023).

Conflict of interest

The Authors declare that there is no conflict of interest.

References

Albillos A., de Gottardi A., Rescigno M., 2020. The gut-liver axis in liver disease: pathophysiological basis for therapy. J. Hepatol. 72, 558–577, https://doi.org/10.1016/j.jhep.2019.10.003

Ansari I., Raddatz G., Gutekunst J. et al., 2020. The microbiota programs DNA methylation to control intestinal homeostasis and inflammation. Nat. Microbiol. 5, 610–619, https://doi.org/10.1038/s41564-019-0659-3

Aruwa C.E., Pillay C., Nyaga M.M., Sabiu S., 2021. Poultry gut health - microbiome functions, environmental impacts, microbiome engineering and advancements in characterization technologies. J. Anim. Sci. Biotechnol. 12, 119, https://doi.org/10.1186/s40104-021-00640-9

Ballou A.L., Ali R.A., Mendoza M.A., Ellis J.C., Hassan H.M., Croom W.J., Koci M.D., 2016. Development of the chick microbiome: how early exposure influences future microbial diversity. Front. Vet. Sci. 3, 2, https://doi.org/10.3389/fvets.2016.00002

Bednarczyk M., Stadnicka K., Kozłowska I., Abiuso C., Tavaniello S., Dankowiakowska A., Sławińska A., Maiorano G., 2016. Influence of different prebiotics and mode of their administration on broiler chicken performance. Animal 10, 1271–1279, https://doi.org/10.1017/S1751731116000173

Berghof T.V.L., Parmentier H.K., Lammers A., 2013. Transgenerational epigenetic effects on innate immunity in broilers: an underestimated field to be explored? Poult. Sci. 92, 2904–2913, https://doi.org/10.3382/ps.2013-03177

De Boever S., Vangestel C., De Backer P., Croubels S., Sys S.U., 2008. Identification and validation of housekeeping genes as internal control for gene expression in an intravenous LPS inflammation model in chickens. Vet. Immunol. Immunopathol. 122, 312–317, https://doi.org/10.1016/j.vetimm.2007.12.002

Choi K.Y., Lee T.K., Sul W.J., 2015. Metagenomic analysis of chicken gut microbiota for improving metabolism and health of chickens - a review. Asian Australas. J. Anim. Sci. 28, 1217–1225, https://doi.org/10.5713/ajas.15.0026

Christensen E.G., Licht T.R., Leser T.D., Bahl M.I., 2014. Dietary xylo-oligosaccharide stimulates intestinal bifidobacteria and lactobacilli but has limited effect on intestinal integrity in rats. BMC Res. Notes 7, 660, https://doi.org/10.1186/1756-0500-7-660

Clench M.H., Mathias J.R., 1995. The avian cecum: a review. Wilson Bull. 107, 93–121

Crhanova M., Karasova D., Juricova H., Matiasovicova J., Jahodarova E., Kubasova T., Seidlerova Z., Cizek A., Rychlik I., 2019. Systematic culturomics shows that half of chicken caecal microbiota members can be grown in vitro except for two lineages of *clostridiales* and a single lineage of *bacteroidetes*. Microorganisms 7, 496, https://doi.org/10.3390/microorganisms7110496

Dijk W., Kersten S., 2014. Regulation of lipoprotein lipase by Angptl4. Trends Endocrinol. Metab. 25, 146–155, https://doi.org/10.1016/j.tem.2013.12.005

Dunislawska, A., Slawinska A., Siwek M., 2020. Hepatic DNA methylation in response to early stimulation of microbiota with Lactobacillus synbiotics in Broiler chickens. Genes 11, 579, https://doi.org/10.3390/genes11050579

Dunislawska A., Slawinska A., Stadnicka K., Bednarczyk M., Gulewicz P., Jozefiak D., Siwek M., 2017. Synbiotics for broiler chickens - *in vitro* design and evaluation of the influence on host and selected microbiota populations following *in ovo* delivery. PLoS One 12, e0168587, https://doi.org/10.1371/journal.pone.0168587

Fackler M.J., McVeigh M., Mehrotra J., Blum M.A., Lange J., Lapides A., Garrett E., Argani P., Sukamar S., 2004. Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. Cancer Res. 64, 4442–4452, https://doi.org/10.1158/0008-5472.CAN-03-3341

Gibson, G.R., Wang X., 1994. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J. Appl. Bacteriol. 77, 412–420, https://doi.org/10.1111/j.1365-2672.1994.tb03443.x

Hastings K.L., Green M.D., Gao B., Ganey P.E., Roth R.A., Burleson G.R., 2020. Beyond metabolism: role of the immune system in hepatic toxicity. Int. J. Toxicol. 39, 151–164, https://doi.org/10.1177/1091581819898399

Huijsdens X.W., Linskens R.K., Mak M., Meuwissen S.G.M., Vandenbroucke-Grauls C.M.J.E., Savelkoul P.H.M., 2002. Quantification of bacteria adherent to gastrointestinal mucosa by real-time PCR. J. Clin. Microbiol. 40, 4423–4427, https://doi.org/10.1128/JCM.40.12.4423-4427.2002

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

Johnson T.J., Wannemuehler Y., Doetkott C., Johnson S.J., Rosenberger S.C., Nolan L.K., 2008. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. J. Clin. Microbiol. 46, 3987–3996, https://doi.org/10.1128/JCM.00816-08

Jung S.J., Houde R., Baurhoo B., Zhao X., Lee B.H., 2008. Effects of galacto-oligosaccharides and a *Bifidobacteria lactis*-based probiotic strain on the growth performance and fecal microflora of broiler chickens. Poult. Sci. 87, 1694–1699, https://doi.org/10.3382/ps.2007-00489

Koh A., Bäckhed F., 2020. From association to causality: the role of the gut microbiota and its functional products on host metabolism. Mol. Cell 78, 584–596, https://doi.org/10.1016/j.molcel.2020.03.005

Keessen E.C., Gaastra W., Lipman L.J.A., 2011. *Clostridium difficile* infection in humans and animals, differences and similarities. Vet. Microbiol. 153, 205–217, https://doi.org/10.1016/j.vetmic.2011.03.020

Li C., Guo S., Zhang M., Gao J., Guo Y., 2015. DNA methylation and histone modification patterns during the late embryonic and early postnatal development of chickens. Poult. Sci. 94, 706–721, https://doi.org/10.3382/ps/pev016

Livak K.J., Schmittgen T.D., 2001. Analysis of relative gene expression data using Real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408, https://doi.org/10.1006/meth.2001.1262

Maniatis T., Fritsch E., Sambrook J., 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press. New York, NY (USA)

Oakley B.B., Kogut M.H., 2016. Spatial and temporal changes in the broiler chicken cecal and fecal microbiomes and correlations of bacterial taxa with cytokine gene expression. Front. Vet. Sci. 3, 11, https://doi.org/10.3389/fvets.2016.00011

Pedroso A.A., Batal A.B., Lee M.D., 2016. Effect of in ovo administration of an adult-derived microbiota on establishment of the intestinal microbiome in chickens. Am. J. Vet. Res. 77, 514–526, https://doi.org/10.2460/ajvr.77.5.514

Penders J., Vink C., Driessen C., London N., Thijs C., Stobberingh E.E., 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, https://doi.org/10.1016/j.femsle.2004.11.052

Rupnik M., Wilcox M.H., Gerding D.N., 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat. Rev. Microbiol. 7, 526–536, https://doi.org/10.1038/nrmicro2164

Salazar N., Gueimonde M., Hernández-Barranco A.M., Ruas-Madiedo P., de los Reyes-Gavilán C.G., 2008. Exopolysaccharides produced by intestinal *Bifidobacterium* strains act as fermentable substrates for human intestinal bacteria. Appl. Environ. Microbiol. 74, 4737–4745, https://doi.org/10.1128/AEM.00325-08

Sevane N., Bialade F., Velasco S., Rebolé A., Rodríguez M.L., Ortiz L.T., Cañón J., Dunner S., 2014. Dietary inulin supplementation modifies significantly the liver transcriptomic profile of broiler chickens. PLoS One 9, e98942, https://doi.org/10.1371/journal.pone.0098942

Shoaf K., Mulvey G.L., Armstrong G.D., Hutkins R.W., 2006. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. Infect. Immun. 74, 6920–6928, https://doi.org/10.1128/IAI.01030-06

Siwek M., Slawinska A., Stadnicka K., Bogucka J., Dunislawska A., Bednarczyk M., 2018. Prebiotics and synbiotics - *in ovo* delivery for improved lifespan condition in chicken. BMC Vet. Res. 14, 402, https://doi.org/10.1186/s12917-018-1738-z

Skraban J., Dzeroski S., Zenko B., Tusar L., Rupnik M., 2013. Changes of poultry faecal microbiota associated with *Clostridium difficile* colonisation. Vet. Microbiol. 165, 416–424, https://doi.org/10.1016/j.vetmic.2013.04.014

Slawinska A., Dunislawska A., Plowiec A., Radomska M., Lachmanska J., Siwek M., Tavaniello S., Maiorano G., 2019. Modulation of microbial communities and mucosal gene expression in chicken intestines after galactooligosaccharides delivery *in ovo*. PLoS One 14, e0212318, https://doi.org/10.1371/journal.pone.0212318

Stanley D., Hughes R.J., Geier M.S., Moore R.J., 2016. Bacteria within the gastrointestinal tract microbiota correlated with improved growth and feed conversion: challenges presented for the identification of performance enhancing probiotic bacteria. Front. Microbiol. 7, 187, https://doi.org/10.3389/fmicb.2016.00187

Torres-Hernandez A., Wang W., Nikiforov Y. et al., 2019. Targeting SYK signaling in myeloid cells protects against liver fibrosis and hepatocarcinogenesis. Oncogene 38, 4512–4526, https://doi.org/10.1038/s41388-019-0734-5

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, research0034.1, https://doi.org/10.1186/gb-2002-3-7-research0034

Wei S., Morrison M., Yu Z., 2013. Bacterial census of poultry intestinal microbiome. Poult. Sci. 92, 671–683, https://doi.org/10.3382/ps.2012-02822

Yadav S., Jha R., 2019. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. J. Anim. Sci. Biotechnol. 10, 2, https://doi.org/10.1186/s40104-018-0310-9

Zhang N., 2015. Epigenetic modulation of DNA methylation by nutrition and its mechanisms in animals. Anim. Nutr. 1, 144–151, https://doi.org/10.1016/j.aninu.2015.09.002