Interaction Between Early in Ovo Stimulation of the Gut Microbiota and Chicken Host – Splenic Changes in Gene Expression and Methylation

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Abstract

Epigenetic regulation of the gene expression results from interaction between the external environment and transcription of the genetic information encoded in DNA. Methylated CpG regions within the gene promoters lead to silencing of the gene expression. Factors contributing to epigenetic regulation include intestinal microbiota, which in chicken can be potently modified by *in ovo* stimulation. The main aim of this study was to determine global and specific methylation patterns for the spleen under the influence of host-microbiome interaction. Incubating eggs were *in ovo* stimulated on day 12 of incubation in two chicken genotypes: Ross broilers and Green-legged Partridgelike. The injected compounds included: probiotic – *Lactococcus lactis* subsp. *cremoris*, prebiotic – galactooligosaccharides, and synbiotic – combination of both. Chickens were sacrificed on day 42 post-hatching. Spleen was collected, RNA and DNA were isolated and intended to gene expression, gene methylation and global methylation analysis. We have proved that negative regulation of gene expression after administration of bioactive substances *in ovo* might have epigenetic character. Epigenetic changes depend on the genotype and the substance administered *in ovo*. Epigenetic nature of microbial reprogramming in poultry and extension of issues related to host-microbiome interaction is a new direction of this research.

Introduction

Epigenetic regulation of the gene expression is an interaction of the external environment with the genetic information. They are potentially heritable changes in the gene expression which does not involve alteration in DNA sequence. Changes in the gene expression may be triggered by factors such as: nutrition, health, climate or stress[1]. Epigenetic mechanisms responding to these environmental factors include expression of microRNA (miRNA) sequences, histone modification and DNA methylation. The methylation process might be influenced by many components present in the chicken diet such as probiotic bacteria[2] and also, the prebiotic fermentation product: the short-chain fatty acid, called butyrate[3,4]. These components mainly modulate the microbiota of the gastrointestinal tract (GIT), which affects the host organism by regulating its immune response, metabolism, digestive processes, or nutrient absorption[5]. Supplementation by bioactive substances can directly modulate composition of the host microbiota, which indirectly acts on the whole host organism[6]. Modulation effects depend on the time and mode of administration of the bioactive substance[7]. In chickens prebiotics, probiotics or synbiotics are routinely delivered in-feed and in-water directly after hatching[6]. Supplementation with bioactive substances is continued for another two weeks. During this period GIT is colonized by beneficial bacteria[8]. In poultry, an alternative route of bioactive substances administration is provided by *in ovo* technology. It is based on single dose of prebiotics, probiotics or synbiotics injected on the day 12 of embryonic development into the air chamber of the egg[9–12]. Such a route of delivery ensures contact of GIT with bioactive substances as early as possible. In the previous studies we proved efficiency of *in ovo* technology for reprogramming the chicken microbiome at an early stage of embryonic development by administering prebiotics (e.g. inulin, GOS, RFO) or synbiotics (e.g. inulin + *Lactococcus lactis* subsp. *lactis*, GOS + *Lactobacillus salivarius*, RFO + *Lactobacillus plantarum*)[10–12].

DNA methylation involves the addition of methyl residues to the cysteine, present within the CpG island, what blocks the enzymes accession (including transcriptases) thereby inhibiting the DNA transcription. DNA methylation is characterized by the stability of cytosine modifications within the CpG dinucleotides[13]. Remodeling of DNA methylation in genome can be global or locus-specific[14]. During embryogenesis, global demethylation of DNA occurs, and a *de novo* methylation profile appears at the blastocyst stage[15]. Therefore, the chicken environment, egg composition and conditions of fertilized eggs incubation might significantly influence an embryo DNA methylation. DNA requires donors of methyl groups and cofactors, originating from the outside environment (e.g. food). Studies have repeatedly confirmed DNA methylation as a conservative epigenetic mechanism in organism development[16,17]. However, little is still known about tissue-specific DNA methylation patterns.

It has been generally recognized that the overall pattern of the DNA methylation is conserved in many species[18,19]. The knowledge about these patterns and the epigenetic regulation of gene expression in poultry is still scarce. We hypothesize that in chickens stimulated with bioactive substances *in ovo* on the day 12 of embryonic development, an epigenetic mechanism of the gene expression was developed. Therefore, the main aim of this study was to determine global and specific methylation patterns for the spleen as a main immune organ under the influence of host-microbiome interaction. We carried out this analysis in a few steps: (1) meta-analysis of the whole transcriptome data of immune organs, to select candidate genes for DNA methylation analysis; (2) estimation of the global methylation level in spleen stimulated *in ovo*; (3) estimation of the expression and methylation level of candidate genes CpG islands' in spleen. The innovativeness of the study is expressed by acquiring new knowledge about the mechanisms of gene silencing, where the gene expression regulation resulted from the stimulation of chicken microbiome by bioactive substances injected *in ovo*.

Results

Meta-analysis based on published microarray data and gene selection

The meta-analysis of high-throughput transcriptome data (expression microarrays) allowed the identification of down – regulated genes in spleen. The gene expression was silenced after synbiotics and prebiotics administration *in ovo*. The comparison of down regulated genes is presented in Venn diagram (Fig. 1).

Analysis showed 108 down-regulated genes in spleen for five analyzed experimental groups. The selected gene sequences and their connection are presented in Figure 2. A connection was shown between 6 genes. These gene were intended for further analysis using the qMSP reaction.

Global methylation

The results of global methylation for spleen after *in ovo* stimulation with prebiotic, probiotic and synbiotic *in ovo* are presented in the Table 1. There are statistically significant differences between the analyzed

genotypes (GP and Ross) and between the probiotic and prebiotic/synbiotic injected groups in Ross genotype.

Table 1. The mean global (total) level of methylation of cytosine in DNA in the spleen in two genetic groups of chickens – Ross and Green-legged Partridgelike (GP) after *in ovo* stimulation of prebiotic, probiotic and synbiotic. Statistical analysis was performed using the GLM procedure and Duncan test.

Genetic group	Substance	Mean	SD	CV
Ross	C	11.79^{a}	6.41	54.33
	PRE	29.74^{b}	24.55	82.53
	PRO	6.82^{a}	1.18	17.35
	SYN	28.67^{b}	15.79	55.08
GP	C	10.56	4.32	40.88
	PRE	9.79	2.48	25.27
	PRO	8.05	2.05	25.44
	SYN	12.31	5.76	46.84

SD – standard deviation; CV – coefficient of variation

Relative gene expression

Gene expression analysis showed a statistically significant decrease in the expression of 11 selected genes after the administration of a prebiotic and a synbiotic in Ross. The administration of the probiotic increased gene expression in Ross. There was a significant increase in: *CYR61* expression after administration of the prebiotic; in *CXCR5*, *NFATC1, CYR61, IKAZ1, ANGPTL4* after administration of synbiotic, and an increase in *NR4A3* after administration of all substances in GP genotype. There was a significant decrease in the expression of the *CD72* gene after the administration of the probiotic. The results are presented in the Table 2.

Table 2. Changes in relative expression of the selected gene panel in spleen of Ross and GL injected *in ovo* with probiotic, prebiotic and synbiotic (mRNA abundance – LOG_2 Fold Change) * $P<0.05$

Genetic group	Ross			GP		
Substance	PRE	PRO	SYN	PRE	PRO	SYN
CD72	-4.19*	1.06	-5.0*	0.42	-0.38*	0.05
CXCR5	-4.23*	0.82	-4.74*	0.79	0.32	1.08*
NFATC1	-4.10*	0.55	-4.59*	-0.39	-0.41	1.50*
SYK	-2.95*	1.70	-3.19*	0.11	-0.58	-0.67
CYR61	-4.15*	0.76	-4.54*	1.38*	0.38	0.84*
NR4A3	-4.44*	0.99	-3.97*	1.64*	0.79*	1.44*
SERPING1	-5.27*	0.48	-5.90*	0.15	-1.13	0.30
TNFRSF14	-3.94*	1.25	-4.64*	0.25	-0.22	0.56
IKZF1	-4.14*	1.29	-5.14*	0.37	0.15	1.58*
KLHL6	-3.95*	0.71	-4.36*	-0.28	0.01	0.15
ANGPTL4	-3.06*	3.0	-3.19*	0.40	-0.59	0.90*

Gene – specific methylation (qMSP reaction)

DNA methylation analysis shows statistically significant changes in the level of methylation in the experimental groups relative to control. The differences were detected for 6 genes: *SYK, ANGPTL4, TNFRSF14, IKZF1, NR4A3* and *NFATC1*. A statistically significant increase in the methylation level in *SYK* gene equals: 2% in C group, 24% in SYN, 26% in PRE and 28% in PRO group in GP genotype. The methylation level of *ANGPTL4* was between 60 and 64% in Ross and 61% in C, 67% in PRO, 72.5% in PRE and 68% in SYN of GP. The methylation level of *TNFRSF14* gene, in the spleen of Ross increased from 69% (C) to 83% after PRE administration. On the contrary, in GL genotype methylation levels decreased from 81% in C group to 72% in PRO and 68% in PRE group. *IKZF1* methylation decreases in both genotypes after PRO administration by approximately 20%. However, after administration of the PRE in Ross, *IKZF1* methylation level increases from 50% to 87%. The administration of bioactive substances significantly increased the methylation of the *NR4A3* from 15% in the C group in the spleen of Ross, to 47% in PRO and SYN groups and 67% in PRE group. The similar picture is observed for the *NFATC* gene. Its methylation increased from 2% in C group to 39% in PRE group. The results are presented in the Figure 3.

Discussion

This research aims to verify the epigenetic character of spleen gene expression stimulated by the administration of bioactive substances (prebiotic, probiotic, synbiotic) during embryo development on day 12 of egg incubation in two different chicken genotypes.

Meta-analysis based on published microarray data and gene selection

The effects of the *in ovo* administration of bioactive substances on broiler chickens were already proved and published elsewhere[11]. A significant, long-term effects of *in ovo* delivery of bioactive substances were also determined at the molecular level, using transcriptomic approach[10]. Gene expression silencing was determined especially in genes related to the immune responses. Delivery of synbiotic GOS with *Lactobacillus salivarius in ovo* showed a negative modulation of gene expression in the spleen[12]. Out of 50 DEG, 32 genes showed strong inhibition of mRNA expression[12]. The phenomenon of negative modulation of gene expression indicates that stimulation of the microbial composition contributes to the development of food tolerance in rapidly growing broiler chickens. As a result, metabolic energy is used for growth and development, local immune response is muted instead of maintaining the lymphatic system in excitation[20]. Maintaining active immune system is energetically costly, what might adversely affect the body growth[21]. Intestinal epithelium is constantly exposed to large amount of foodborne and bacterial antigens[22]. Gut-associated lymphoid tissue (GALT) is a vital part of the immune system that protects the body from foreign antigens and pathogens, while also tolerates commensal food antigens. Peripheral part of immune system (spleen) is the main organ which generates an immune response. It plays a significant role in the organism protection from invading pathogens and antigens, maintaining the immune homeostasis and tissue regeneration processes [23]. GALT developed regulatory and anti-inflammatory mechanisms which eliminate or tolerate microbiota[24]. These mechanisms control host responses' and develop tolerance to pathogens, what leads to recognition of commensal bacteria and activation of transient and non-inflammatory immune response[25].

Hereby, we present the first and comprehensive study of gene expression and methylation levels after administration of individual components of the synbiotic, as well as the synbiotic itself, in two extremely different genotypes of chickens. Earlier studies on which the meta-analysis was based concerned only transcriptome changes after administration of prebiotic and synbiotic in chicken broilers[10,12].

Some differentially methylated genes have been relatively hypomethylated, suggesting that administration of bioactive substances may be associated with lower level or reduction of methylation, which consequently leads to changes in gene expression. However, the effect of methylation on gene expression also depends on many factors, such as the location of CpG, which does not allow a clear conclusion to be made regarding the increase or decrease of gene expression[26]. Little is known about the spleen DNA methylome and its potential cause-and-effect role in shaping the immune response. Due to the fact that methylation is tissue- dependent, it was necessary to select the tissue which plays a key role in the context of poultry production. It is unknown if transcriptional silencing of gene expression is associated with epigenetic mechanisms such as DNA methylation or histone acetylation . Identifying mechanisms that cause gene silencing would be key to understanding the molecular basis of environmental impact[27].

Literature provides the evidence that microbiome has a significant impact on the regulation of epigenetic mechanisms in mammals[28]. It has also been shown that there is a change in the microbiological profile in chicken intestines due to the *in ovo* administration of the GOS prebiotic[29] and the synbiotic consisting of GOS and lactic acid bacteria[11]. In this study, GOS was used as a prebiotic, lactic acid bacteria as a probiotic, and their combination in the form of a synbiotic, proving that their *in ovo* administration cause transcriptomic and epigenetic changes at the level of DNA methylation of genes in spleen. Despite the well-known intestinal microbiota and the growing knowledge of epigenetic regulation such as methylation, there are only few studies combining both issues[30,31].

Genotype-dependent methylation

Global methylation analysis showed differences between two distinct chicken genotypes stimulated *in ovo* at the early stage of embryo development. The analyzes were based on two genotypes of different origin and selection history - the broiler chicken line and the native breed of dual purpose hens. The experimental conditions were exactly the same for both genotypes. Broiler chicken - Ross 308 is a meat-type which was created as a result of the ongoing genetic intensive selection program. It is characterized by high resistance to diseases, an excellent pace of weight gain, excellent production parameters, namely the growth rate and feed efficiency in large-scale production. Breeding of chicken broilers usually take place in commercial hatcheries. The post – hatching process due to chicken's vaccination and transport cause so called hatching window, i.e. a gap in the access to food and water. At this time, the possibility of inoculation of the microbiota is reduced, which results in microbiota development disorders[32]. Green-legged Partridgelike (GP) is a dual purpose Polish native breed. It is characterized by high disease resistance and low environmental and food requirements[33]. The GP was included in the genetic resources conservation program in a state where no selection was carried out. This may result in differentiation in response to the stimulation of the intestinal microbiota directly, and indirectly of the immune system, by external factors. Earlier research shows that *in ovo* administration of a synbiotic affects the development of the immune organs: bursa of Fabricius (in broiler chickens) and spleen (in dual purpose chickens). Improvement of GP spleen development *by in ovo* synbiotics improves the sensitivity of the immune system to immunomodulating environmental factors[34]. These two genotypes (Ross and GP) are characterized by a diverse gene methylation level as a response to bioactive substances given during embryo development. It can be assumed that the difference is due to the genotype. GP is more resistant and more environmentally adapted, therefore environmental factors do not show such strong effects as in chicken broilers. GP native chickens and Ross broilers were the subject of the analysis in our previous studies. We have determined the morphological differences and immune responses to environmental antigens - lipoteichoic acid (LTA) and lipopolysaccharide (LPS) in broilers (Ross) and native chickens (GP) stimulated *in ovo* with bioactive compounds (prebiotic, probiotic, and synbiotic)[35]. *In ovo* stimulation enhanced colonization of the immune organs with lymphocytes in broilers to higher extent than in native chickens. It might be speculated that there was not much potential for further stimulation in GP. However, the significant changes in the immune system morphology expressed by the increased number of germinal centers (GC) in spleen was determined in native chickens (but not in broilers) [35]. The

number of GC in spleen indicates the activation of humoral immunity in animals by T-dependent antigens. Immune responses triggered in chickens stimulated *in ovo* are breed-dependent. The splenic (systemic) immune responses of broilers and native chickens challenged with LTA and LPS also showed distinct patterns. Genotype influenced gene expression signatures of all immune-related genes analyzed in spleen of broilers and native chickens ($P < 0.001$) (Slawinska - personal communication). The higher number of cytokines was up-regulated in broiler chickens in comparison to native chickens. We conclude that the GP has more potent immune system than Ross, assessed by higher proportion of immune cells in spleen. GP is also less sensitive to environmental changes such as external stimulation of the microbiota, which can be indirectly observed in gene methylation levels.

Substance-dependent methylation

Analysis of the global methylation in spleen showed statistically significant differences between the substances administered *in ovo* in Ross broiler chicken. The obtained results indicate that the response after administration of the probiotic is similar to the control. However, the prebiotic and synbiotic differ significantly from the probiotic, with no differences between the prebiotic and the synbiotic. It could be speculated that the administration of an exogenous dose of bacteria into the egg does not constitute such a strong environmental signal as the administration of a prebiotic or synbiotic. It can also be assumed that in the case of synbiotic, the prebiotic component plays a key role in modulation of methylation and expression profiles. Analysis of the expression of single genes confirmed statistically significant negative regulation of all analyzed genes after *in ovo* administration of the prebiotic and synbiotic in Ross. In most analyzed genes, negative regulation of gene expression can be related to methylation level of the gene. Our analysis showed that *in ovo* administration of various substances differentiated the level of gene methylation. The perinatal period is crucial in the reprogramming of the microbiota, enabling colonization of the gastrointestinal tract of the embryo with beneficial bacteria before hatching[36]. *In ovo* stimulation performed on the day 12 of egg incubation assumes the administration of bioactive substances during embryonic development and stimulation of the native intestinal microbiota of the embryo before hatching. The prebiotic can penetrate the subcutaneous membrane and penetrate the embryonic circulatory system; while the probiotic becomes available during hatching, when the membrane is broken[32]. GOS effect on the modulation of gene expression after *in ovo* administration to chicken broilers has been extensively described [10,29]. Relative analysis of the number of bacteria in the intestinal contents after GOS administration showed that its effect depends on the segment of the intestine. It mainly affects the number of *Bifidobacterium spp*. and *Lactobacillus spp*.[29].

In summary, negative regulation of the gene expression after administration of bioactive substances *in ovo* on day 12 of egg incubation of broiler chicken and native polish chicken may have epigenetic character. Epigenetic mechanisms depend on the genotype being analyzed and the substance administered *in ovo*. Epigenetic nature of this research is a new direction of microbial reprogramming in poultry and extension of issues related to host-microbiome interaction. This study indicates that there is potential in bioactive substances administered *in ovo* to target silencing gene expression in spleen that is behind DNA methylation.

Methods

Experimental outline

In the first stage of the study, (1) a meta-analysis was performed on the basis of whole-transcriptome microarray data generated in previous experiments[10,12] in order to select immune-related genes that were silenced. Bioactive compounds used in those experiments for *in ovo* delivery were two prebiotics (P1 – GOS; P2 – inulin) and synbiotics (S1 – *Lactococcus lactis* subsp. *lactis* + inulin; S2 – *Lactobacillus salivarius* + GOS, S3 – *Lactococcus lactis* subsp. *cremoris* + GOS *in ovo* to broiler chickens.

The present experiment follows the same route of the substance delivery and focuses on single synbiotic the synbiotic and its individual components: prebiotic (GOS) and probiotic (*Lactococcus lactis* subsp. *cremoris*). These three bioactive substances were *in ovo* administered into chicken embryos of two contrasting chicken genotypes: Ross 308 and Green-legged partridgelike.

Based on spleen isolated from *in ovo* stimulated chickens a following analysis were performed: (2) global methylation to verify the epigenetic nature of the changes, and then (3) gene expression analysis (to confirm expression regulation under the influence of various substances) and (4) methylation analysis of individual selected genes.

Meta-analysis of published microarray data for gene selection

Gene selection for methylation analysis was based on two sets of microarray data. These data sets contained broiler chicken transcripts generated from individuals which received prebiotic and synbiotic *in ovo* on day 12 of egg incubation[10,12]. Both projects carried out whole-genome microarray analyzes (Affymetrix, Santa Clara, US), based on genetic material isolated from spleen in experiment with *in ovo* injection of S1, S2 and S3. Meta-analysis aimed to select genes which were silenced at mRNA level in spleen. The analysis was carried out based on gene lists generated by Affymetrix Expression Console software. *In silico* selection of gene sequences was based on the following criteria: p-value ($p<0.05$) and fold change (down-regulation; $FC< -1.0$). Subsequently, selected gene groups were compared with each other based on the Venn diagrams. Connection of the selected genes was analyzed with STRING[37]. A final list was selected based on gene function and possibility of designing primers for the qMSP reaction.

Experimental setup and tissue collection

600 eggs of Ross 308 (Ross) broiler chicken and 600 eggs of Green-legged Partridgelike (GP) were incubated in standard conditions. On the day 12 of incubation, eggs were randomly distributed into experimental groups (150 eggs per group): (1) probiotic (PRO) – *Lactococcus lactis* subsp. *cremoris*, (2) prebiotic (PRE) – galactooligosaccharides (GOS) (3) synbiotic (SYN) – *Lactococcus lactis* subsp. *cremoris* with GOS. The set amount of bacteria was 10^5 bacteria CFU egg^{-1} and the amount of prebiotic was 3.5 mg egg^{-1}. The control group (C) was mock-injected with 0.2 mM physiological saline (0.9%). Eggs were injected into an air cell with 0.2 mL of aqueous solution of each substance. After hatching,

birds were housed in litter pens (4 replicates/group, 8 animals each). Six randomly selected individuals from each group (PRO, PRE, SYN and C) were sacrificed on the day 42 post-hatching and spleen was collected. The experiment was approved by the Local Ethics Committee for Animal Experiments (Bydgoszcz, Poland) (study approval reference number 16/2014). All methods were carried out in accordance with relevant guidelines and regulations.

RNA and DNA isolation

Tissues for RNA isolation were fixed in stabilizing buffer (fix RNA, EURx, Gdansk, Poland). RNA isolation was prepared by using TRI reagent (MRC, Cincinnati, USA) and commercial kit for RNA purification (Universal RNA Purification Kit, EURx, Gdansk, Poland). Spleen was homogenized with the TissueRuptor homogenizer (Qiagen GmbH, Hilden, Germany) in TRI reagent. Isolation of DNA from spleen was carried out by phenol – chloroform method[38] as described by Dunislawska *et al*. 2020[31]. RNA and DNA quality and quantity was checked by electrophoresis and NanoDrop2000 (Scientific Nanodrop Products, Wilmington, USA).

Global methylation analysis

Global DNA methylation analysis was prepared using commercial set for methylated DNA quantification (MDQ1, Imprint Methylated DNA Quantification Kit, Sigma-Aldrich) according to manufacturer's protocol and based on Gryzinska *et al*. 2013[39]. DNA isolated spleen (n=6) was diluted in binding solution to final concentration 150ng/µl. DNA was intended for estimation of methylated DNA level based on the ELISA principle on 96-well plates. Positive (methylated) and blank controls were analyzed together with DNA samples. The absorbance was measured at 450 nm. For each stimulated group, six samples, each derived from a different individual, were analyzed. The absorbance measurements were performed in two technical repeats, each using the same amount of DNA. Two measurements were averaged, and the mean value was used for the further analyses. Global DNA methylation levels are percentages relative to the methylated control and were calculated using the following formula:

$$\frac{A_{450}S - A_{450}B}{A_{450}MC - A_{450}B} \; x \; 100\%,$$

where $A_{450}S$ is the average absorbance of the sample, $A_{450}B$ is the average absorbance of the blank, $A_{450}MC$ is the average absorbance of the methylated control. Statistical analysis was carried out by using SAS statistical software.

Gene expression analysis – RT-qPCR

Gene expression analysis was performed by quantitative reverse transcription PCR (RT-qPCR). cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific/Fermentas, Vilnius, Lithuania), following the manufacturer's recommendations. The qPCR reaction mixture included Maxima SYBR Green qPCR Master Mix (Thermo Scientific/Fermentas, Vilnius, Lithuania), 1 µM of 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. Gene expression analysis was performed for selected genes in meta-analysis step. Sequences of primers were based on the literature or were designed by NCBI Primer BLAST tool[40] based on NCBI sequence. Primer sequences are shown in Table 3. Relative gene expression analysis was conducted separately for each experimental group by the ΔΔCt method[41] using *ACTB*[42] and *G6PDH*[43] as reference genes. Geometric means of cycle threshold (Ct) values of reference genes were used in the analysis[44]. Statistical analyses were performed by comparing the Ct value of each experimental group with that of the control group by Student's *t*-test ($P < 0.05$).

Table 3. Primer sequences used in the RT-qPCR reaction

Gene	Primers sequence	Amplicon size	NCBI no.
CD72 (chB1)	F: AGGAAGGTAGGGCAGCAATG R: CTGACCTGAGGTTCGCCAAA	134	395923
CXCR5	F: GCTCTGACTGTAGGGTGACG R: TGAAATGATGGGCAGTGGCT	145	419784
NFATC1	F: TCGAGTTCAAGCACAGCGAT R: GAAGGACCCCCTCGGAAGA	155	420815
SYK	F: AAGGGACAGCAATGGTTCCT R: AATTTAACAGACCTGCCAGAGG	142	427272
CYR61	F: ATCGCTCGTTCAGACGCATA R: TGTCTGGGCTCCGCTAAAAG	144	429089
NR4A3	F: GGCATCCCCGGAGTTTCTCTG R: TTTGACGAGGCCGCTCATT	237	420996
SERPING1	F: GTCCTCGTGCCACACTTACC R: TTGACCAATGCTTGCCCACC	111	423132
TNFRSF14	F: TGAGCACCATCAGGGGTATC R: AGGTACGGATGCTTCCCAAG	170	420403
IKZF1	F: GCGTGTGAAAGAGCGACTTC R: GAACACTCCGCACAACACCT	149	395974
KLHL6	F: ATGGTTTCTGCGTCAACTCC R: CATCCTGGCTGGGATGCAATA	120	424762
ANGPTL4	F: TCCTCGATTCGCGAGTTCTG R: CAGGGCACTGGGAGCTG	148	769087

Real-time quantitative methylation-specific polymerase chain reaction (qMSP)

DNA methylation is a specific chemical modification of a nucleic acid, involving the attachment of a methylene group to cytosine or adenine nucleotides. The isolated DNA was subjected to methylation analysis using the qMSP method as described by Dunislawska et al. 2020[31]. The QMSP method is a methyl specific quantitative PCR (qPCR) preceded by DNA conversion using bisulfite. Due to this

modification, unmethylated cytosine undergoes deamination and uracil is formed. In contrast, 5-methylcytosine (which is a methylation product) is resistant to this modification, making it possible to make it visible in the qPCR reaction with the help of primers specific for methylated and unmethylated DNA. The mechanism of methylation concerns only cytosines that are part of the cytidine-phosphate-guanosine (CpG) dinucleotide sequence. Therefore the primers for qMSP reactions were designed within CpG islands. Based on the selected gene list in meta-analysis, gene primers in two variants were designed: methylated and unmethylated (Table 4). The conversion was carried out using the EpiJet Bisulfite Conversion Kit (Thermo Fisher Scientific/Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. In the next stage, the qPCR reaction was performed for the selected genes, where for each gene two primer pairs were designed - specific for methylated and non-methylated DNA using the MethPrimer tool. Primers for qMSP were complementary to the gene promoter region and were designed based on criteria: island size >100, GC% >50.0; obs./exp >0.60. DNA oligonucleotides were synthesized by Sigma-Aldrich. The qPCR analysis was performed in LightCycler 480 (Roche Diagnostics, Risch-Rothreuz, Switzerland) thermal cycler. The reaction mixture contained the Maxima SYBR Green qPCR Master Mix intercalating dye (Thermo Fisher Scientific/Fermentas, Vilnius, Lithuania). The optimized melting temperature was 58°C. After amplification, a melting curve was generated for each product (n=6/group). This was due to a gradual increase in temperature up to 98°C with continuous measurement of fluorescence. The relative level of DNA methylation [%] was calculated based on the results of melting curves (read fluorescence level) for each individual according to the formula[45]: 100 x (M/M+u), where M - average fluorescence intensity of the methylated product, U - average fluorescence intensity of the unmethylated product. Statistical analysis was performed using the student T-test (n=6; $p<0.05$) - comparison to control within the genotype and substance between genotypes.

Table 4. Sequences of the primers designed to qMSP reaction by using MethPrimer tool. M – specific for methylated DNA; U – specific for unmethylated DNA.

Gene		Primers sequence	GC%	Amplicon size	NCBI no.
CD72	M	F: AACGGGTTATGTGTCGTTATTAGTC	60.00	107	395923
		R: AAACTAAACCCTACTACCTTCTCGC	72.00		
	U	F: TGGGTTATGTGTTGTTATTAGTTGT	64.00	103	
		R: ACTAAACCCTACTACCTTCTCACA	70.83		
CXCR5	M	F: AGAGGTTGGGATTTACGGTAATAAC	56.00	156	419784
		R: ACAACTTTCTACCTTTACAAACGCT	56.00		
	U	F: AGGTTGGGATTTATGGTAATAATGT	56.00	154	
		R: ACAACTTTCTACCTTTACAAACACT	56.00		
NFATC1	M	F: CGATTCGGAAATATTAATAAAGC	52.17	100	420815
		R: AAAAATAATATAAACCCTACCCGAC	60.00		
	U	F: TTTGTATTGATTTGGAAATATTAATAAAGT	46.67	109	
		R: ACAAAAATAATATAAACCCTACCCAAC	62.96		
SYK	M	F: TATTAGGCGTTTTCGGGAAC	70.00	115	427272
		R: AAATTAATACATTTACTCGCCGCT	54.17		
	U	F: GTTTATTAGGTGTTTTTGGGAATGA	68.00	120	
		R: CCAAATTAATACATTTACTCACCACT	57.69		
CYR61	M	F: TTTGGTTTTAGTGTTTAAAGACGT	58.33	150	429089
		R: TTATATTTACCTTCAAAAAAACGTA	44.00		
	U	F: TTTTGGTTTTAGTGTTTAAAGATGT	56.00	154	
		R: TATTTATATTTACCTTCAAAAAAACATA	42.86		
NR4A3	M	F: GGGAAAGGATAAAGTTTTTGTAGTC	52.00	179	420996
		R: AAACTCAAACGTAACCCTAAACGTA	56.00		
	U	F: GGGAAAGGATAAAGTTTTTGTAGTTG	53.85	179	
		R: AAACTCAAACATAACCCTAAACATA	56.00		
SERPING1	M	F: GGTAACGAGAGTTTGGATTTGTAAC	56.00	163	423132
		R: CCTAAATAAACCCTAAAAACTACGC	64.00		
	U	F: TGGTAATGAGAGTTTGGATTTGTAAT	53.85	163	
		R: CTAAATAAACCCTAAAAACTACACC	64.00		

TNFRSF14	M	F: GTTTTAGTTATTTTTGTTTTTACGTTCGT	65.52	298	420403
		R: CCGCTATCACTATACAACTTCTCG	62.50		
	U	F: AGTTATTTTTGTTTTTATGTTTGT	62.50	292	
		R: CACTATCACTATACAACTTCTCACC	64.00		
IKZF1	M	F: GTAGTAGTAATTGTTGGAGGAGGC	62.50	192	395974
		R: AAAAATAACTTTACGAAACAACGAA	64.00		
	U	F: GTAGTAGTAATTGTTGGAGGAGGTG	64.00	192	
		R: AAAAATAACTTTACAAAACAACAAA	64.00		
KLHL6	M	F: TTTTTTGGATAATGAGTGTTTAACG	52.00	100	424762
		R: AAACACCAAAAAAAATCCCGTA	63.64		
	U	F: TTTTTGGATAATGAGTGTTTAATGA	48.00	102	
		R: CTAAAACACCAAAAAAAATCCCATA	64.00		
ANGPTL4	M	F: TAATTTTAACGGGAAGTATTTTCGT	56.00	156	769087
		R: CAACTTTAAAACTCTACCTCCAACG	60.00		
	U	F: TAATTTTAATGGGAAGTATTTTTGT	56.00	154	
		R: ACTTTAAAACTCTACCTCCAACACA	60.00		

Declarations

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Author contributions

A.D. performed molecular analysis (RNA and DNA isolation, quantitative and qualitative evaluation, designed primers for the reactions, performed RT-qPCR and qMSP reactions) and processed the results. M.G. performed global methylation protocol. A.D. developed the concept of the manuscript and wrote the main manuscript text. M.S. revised the main text of the manuscript and approved the final version for publication. A.S. and A.D. prepared all the figures. A.S. and M.G. revised the manuscript. A.S. made available biological material for analysis from his project. A.D. and M.S. obtained research funding.

Competing interests

The author declare no competing interests.

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Figures

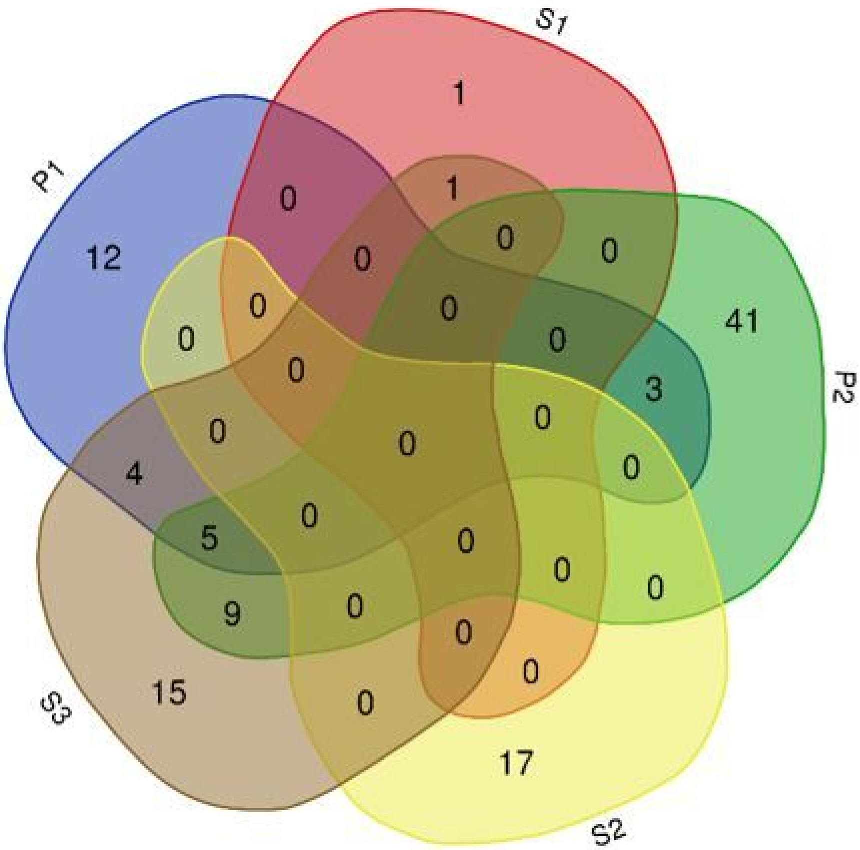

Figure 1

Venn diagram. The number of down-regulated genes in spleen detected in five experimental groups injected in ovo with: P1 – GOS; P2 – inulin; S1 – Lactococcus lactis subsp. lactis + inulin; S2 – Lactobacillus salivarius + GOS, S3 – Lactococcus lactis subsp. cremoris + GOS

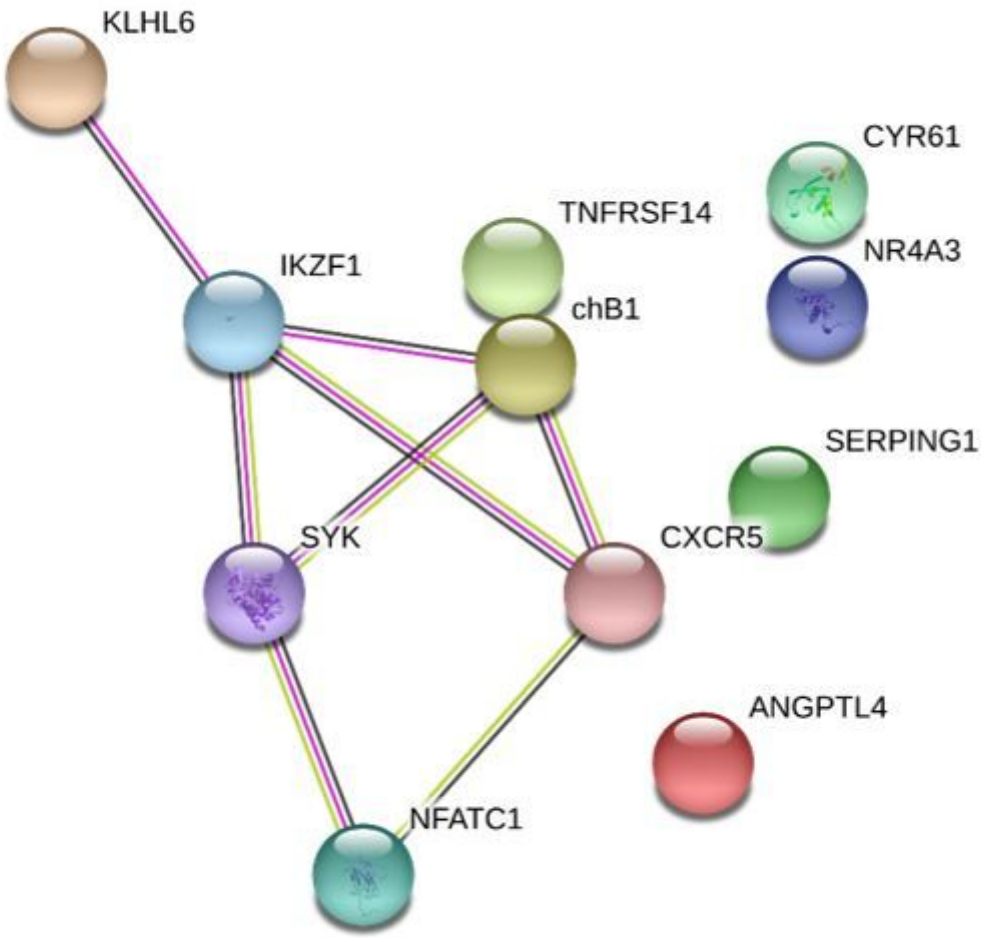

Figure 2

Analysis of the relationship between proteins encoded by down-regulated genes selected based on microarray data.

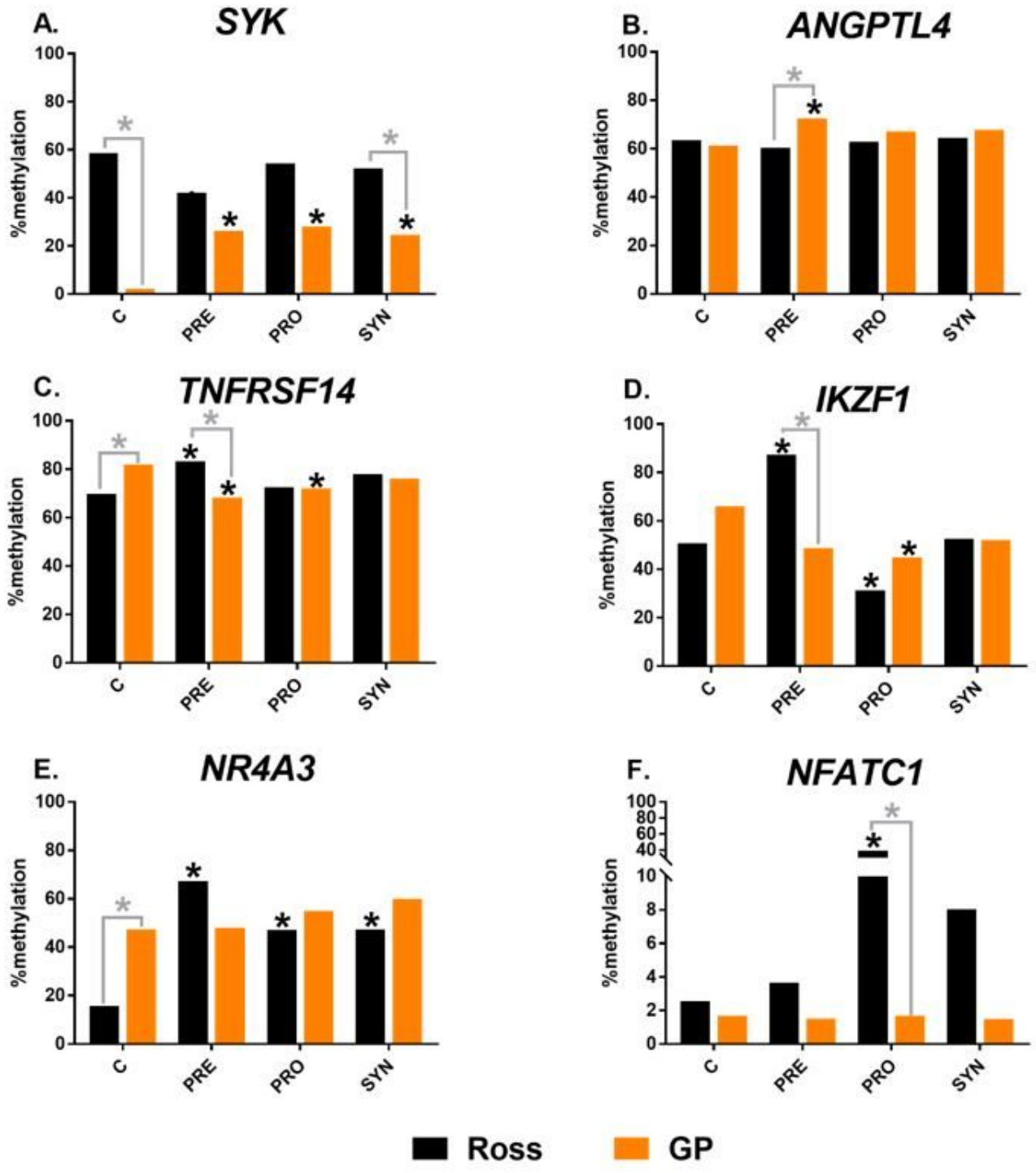

Figure 3

DNA methylation of the SYK, ANGPTL4, TNFRSF14, IKZF1, NR4A3, NFATC1 genes in spleen. X-axis – genetic groups: Ross and Green-legged Partridgelike (GP); groups: C – control, PRO – probiotic, PRE – prebiotic, SYN – synbiotic. Y-axis – percentage of methylation. * $p<0.05$ (n=6) - comparison to control within the genotype (black asterisk) and substance between genotypes (grey asterisk).