Mucosal and Systemic Immune Responses Modulated by in Ovo-Delivered Bioactive Compounds in Distinct Chicken Genotypes

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Research

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Abstract

Background

Mucosal and systemic immune responses are different strategies to cope with environmental stimuli. *In ovo* delivery of prebiotic, probiotic, or synbiotic into the avian embryo allows for indigenous microbiota stimulation. Intestinal microbiota in animals is responsible for immune system maturation. The genetic component is critical in host-microbiota crosstalk and immune system development. The goal of this study was to compare mucosal and systemic immune responses in two distinct chicken genotypes stimulated *in ovo*.

Results

The experiment was constructed in full-factorial design and was aimed to study the effects of two chicken genotypes (chicken broilers or native chickens), four *in ovo*-delivered compounds (GOS/galactooligosaccharides/prebiotic, *Lactococcus lactis* subsp. *cremoris*/probiotic, or GOS+*L.lactis*/synbiotic, vs. physiological saline) and three different stimuli (LPS or LTA vs. physiological saline) on a panel of cytokine genes (*IL-1B*, *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-12p40*, and *IL-17*) expressed in caecal tonsils and spleen. We tested significance of the main effects and their interactions. Genotype had the most significant influence on all gene expression signatures in both tissues ($P < 0.001$ for all genes, except $P < 0.05$ for *IL-10* in spleen). Immune challenge was the second most significant main effect, and influenced *IL-1β*, *IL-6 IL-10* ($P < 0.001$), and *IL-17* genes ($P < 0.05$) in caecal tonsils, and all genes in spleen ($P < 0.001$), except *IL-4* ($P > 0.05$). *In ovo stimulation* influenced *IL-2*, *IL-4*, and *IL-10* in caecal tonsils ($P < 0.05$), as well as *IL-2* and *IL-12p40* in spleen ($P < 0.05$).

Conclusions

The mucosal and systemic immune responses of chicken broilers and native chickens showed distinct patterns. Genotype influenced gene expression signatures of all immune-related genes, but chicken broilers developed stronger immune responses than native chickens. LPS triggered both mucosal (caecal tonsils) and systemic (spleen) immune responses in chicken broilers, but only systemic (spleen) in native chickens. *In ovo* stimulation with bioactive compounds (especially prebiotic) modulated innate immune responses to LPS. GOS delivered *in ovo* induced the most pronounced responses to LPS, which validated its further application as a potent immunomodulator for *in ovo* applications.

Background

Mucosal and systemic immune responses are different strategies to cope with pathogens. Mucosal immune responses are generated locally by the mucosa-associated lymphoid tissue (MALT). Mucosa lines body cavities and passages, and provides a barrier function between the microbiota inhabiting the mucosal surfaces and the milieu of the body. MALT comprises lymphoid cells and organs scattered along the mucosa. Mucosal immune responses are generated locally against pathogens while tolerating

commensal microbiota encountered at the mucosa (1). Pathogens that are not neutralized by the mucosal immune responses enter the bloodstream and are transported to other lymphoid organs, such as lymph nodes or spleen. These organs filter lymph and blood, and generate the systemic immune responses against recognized antigens. Systemic immune responses include innate and adaptive mechanisms, such as inflammation, phagocytosis, cell-mediated and humoral immune responses (2). Transient immune responses can be triggered by Toll-like Receptor (TLR) ligands, such as lipoteichoic acid (LTA) and lipopolysaccharide (LPS). LTA is a component of a cell wall of Gram-positive bacteria, whereas LPS is an endotoxin produced by Gram-negative bacteria. These compounds are recognized by TLR1/2 (LTA) and TLR4 (LPS) (3). Upon antigen recognition of the TLR ligand by the respective TLR, the immune responses are generated. The onset of the mucosal and systemic immune responses is manifested by the cytokine secretion, including cytokines involved in inflammation (*IL-1β*, and *IL-12*), Th1/Th2 polarization (*IL-6*, *IL-10* and *IL-12*), Th1 (*IL-2*), Th2 (*IL-4*), and Th17 (*IL17*)-mediated immune responses (4).

Both, mucosal and systemic immune responses are modulated by the microbiota and its metabolites inhabiting various niches of the mucosa (5). Mucosal immune responses in the MALT are typically associated with maintaining healthy microbiota composition and barrier function of the gut. The microbiota composition and immune responses of the host rely on environmental stimuli, biosecurity level, housing, litter, feed access, climate, and geographical location (6). Development of microbiota is correlated with maturation of the immune system and starts already in the perinatal period. An avian model allows for a direct stimulation of the microbiota prior to hatching, by injecting bioactive compounds *in ovo* (into the egg). *In ovo* delivered bioactive compounds (prebiotics, probiotics and synergistic combination of both, i.e., synbiotics) stimulate intestinal microbiota, which primes the avian immune system during perinatal period (7). In this manner, an early stimulation of the intestinal microbiota has a potent effect on the immune responses further in life (8-11), including responses to stress (12, 13).

The intensive selection of high-performing poultry resulted in development of distinct genetic types with superior growth and feed efficiency (i.e., fast-growing meat-type poultry known as chicken broilers) or egg laying potential (i.e., egg-type chickens). Aside from commercially selected chicken broilers and layers, there are dual-purpose breeds, which are slow-growing, native chickens used for meat and eggs production. Along with distinct anatomy and physiology of broilers and layers, they differ in type and level of the immune responses. In fast-growing broiler chickens, the immune responses are based on IgM antibodies, which represent short-term humoral immune responses. In contrast, layers mount higher titers of IgG antibodies, representing long-term adaptive immune responses, combined with strong cellular immune responses (14). The immune organs of the native chickens analyzed in this study (i.e., caecal tonsils and spleen) were populated with significantly higher proportion of B and T cells compared to broilers, which indicates higher immunocompetence of their immune system (15).

The poultry genetics determines the immune responses to intestinal microbiota modulation. For example, a disrupted intestinal homeostasis induced by administration of dextran sulfate sodium (DSS) early in

life (days 11-18 post-hatching) was less severe in broilers than in layers, which was manifested by lower mortality and less damaged intestinal morphology (16). Chicken broilers treated DSS, in contrast to layers, did not activate potent mucosal immune responses to inflammatory challenge. Their local humoral responses were based on increased IgM vs. IgY titers against LPS. On the other hand, improved intestinal homeostasis by *in ovo* delivered prebiotics, probiotics, and synbiotics resulted in clear differences in immune system morphology between chicken broilers and native GP chickens (10, 15). Spleen index, which is correlated with the immune responses, was higher in native chickens than in broilers, and it was even boosted in native chickens by *in ovo*-delivered synbiotic (10). We determined that synbiotic delivered *in ovo* enhanced development of germinal centers (GC) in spleen of native chickens (but not chicken broilers) (15). On the other hand, bioactive compounds (prebiotic, probiotic, and synbiotic) delivered *in ovo* increased the number of adaptive immune cells (T and B lymphocytes) in caecal tonsil and spleen of chicken broilers (but not native chickens) (15).

Based on the above, we hypothesize that distinct chicken genotypes will respond in a different manner to *in ovo* stimulation of intestinal microbiota with bioactive compounds followed by immune challenge with TLR ligands (LTA and LPS). The goals of this paper were to (1) determine the local and systemic immune responses in chickens to pro-inflammatory antigens, and (2) estimate the effects of host genetics and *in ovo* stimulation with prebiotic, probiotic, or synbiotic on the strength of the immune responses mounted upon the challenge.

Methods

Experimental design

The two trials have been conducted based on the full-factorial design, using chicken genotypes (chicken broiler and native chicken), four *in ovo*- injected compounds (prebiotic, probiotic, synbiotic, *vs.* physiological saline), and three types of immune challenge (LPS, LTA, *vs.* physiological saline) as factors. Figure 1 presents experimental design described in this study. The treatments were followed by harvesting caecal tonsils and spleen to study local and systemic immune-related gene expression.

Animal procedures

The animals used in the trials were: broiler chicken (Ross 308, Aviagen) for Trial 1 and native chicken (GP, Green-legged Partridgelike) for Trial 2. Both trials started with egg incubation (600 eggs/genotype) followed by *in ovo* injection of respective bioactive compound on day 12 of incubation. The bioactive compounds for *in ovo* injection included: prebiotic (GOS, galactooligosaccharides, 3.5 mg/egg), probiotic (*Lactococcus lactis* subsp. *cremoris*, 10^5 CFU/egg), or synbiotic (GOS, 3.5 mg/egg + *L. lactis*, 10^5 CFU/egg). Control eggs were mock-injected with sterile physiological saline. The injection volume for all eggs was 0.2 ml and the injection site was air cell. After *in ovo* injection, the hole was sealed and the incubation continued. For more details on *in ovo* procedures, refer to Slawinska, Dunislawska (17).

Table 1. Chemical composition of commercial feeds used for chicken broilers and native chickens

Items	Chicken broilers				Native chickens	
	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)
ME_N [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

After hatching, the chicks were housed in litter pens (4 replicates/group, 8 animals each) for 42 days. Feeding and environmental conditions were adjusted to age and genotype of the birds. Table 1 gives an overview of the diets applied. On the slaughter day, chickens were injected intraperitoneally with lipoteichoic acid (LTA, Sigma Aldrich, cat.# L2515, 0.5 mg/kg body weight), lipopolysaccharide (LPS, Sigma-Aldrich, cat.# L2880, 0.5 mg/kg body weight), or mock-injected with physiological saline. Animals were sacrificed two hours post-injection and gene expression of the major immune mediators was performed in caecal tonsils and spleen. Tissue samples (n=8) were collected two hours after immune challenge. Samples of spleen and caecal tonsils were dissected and preserved in 3 ml fixRNA (EURx, Gdansk, Poland).

RNA isolation and RT-qPCR

Total RNA was isolated from caecal tonsils and spleen samples. The tissues were first homogenized in 1ml TRI Reagent (MRC, Cincinnati, OH, US) using a rotor-stator homogenizer (TissueRuptor, Qiagen, GmbH, Hilden, Germany). The lysate was purified using Universal RNA Purification Kit (EURx, Gdansk, Poland). The concentration and purity of the eluted RNA was measured with NanoDrop (Thermo Scientific/NanoDrop Technologies, Wilmington, NC, USA). The total RNA was assessed for integrity by agarose gel electrophoresis.

Gene expression analysis was done using 2-step RT-qPCR. The amount of 5 µg of total RNA was reversely transcribed with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Vilnius, Lithuania). Obtained cDNA was diluted to working concentration of 70 ng/µl and stored at -20°C. RT-qPCR reactions were run using Maxima SYBR Green qPCR Master Mix (2x) (Thermo Scientific, Vilnius, Lithuania). RT-qPCR reaction mix included: 1x Maxima SYBR Green qPCR Master Mix, 1 µM of each oligonucleotide primer and 2 µl of diluted cDNA. The reaction volume was adjusted to 10 µl with

nuclease-free water. Each RT-qPCR reaction was performed in two technical replicates. Sequences of oligonucleotide primers used to amplify the immune-related genes are listed in Table 2.

Table 2. Immune-related genes and primers for RT-qPCR analysis

Gene symbol	Gene ID	Primer sequence (5'→3')	Reference
Target genes			
IL-1β	395196	F: GGAGGTTTTTGAGCCCGTC R: TCGAAGATGTCGAAGGACTG	(18)
IL-2	373958	F: GCTTATGGAGCATCTCTATCATCA R: GGTGCACTCCTGGGTCTC	(13)
IL-4	416330	F: GCTCTCAGTGCCGCTGATG R: GGAAACCTCTCCCTGGATGTC	(8)
IL-6	395337	F: AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT	(19)
IL-10	428264	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG	(20)
IL-12p40	404671	F: TTGCCGAAGAGCACCAGCCG R: CGGTGTGCTCCAGGTCTTGGG	(21)
IL-17	395111	F: GGGATTACAGGATCGATGAGGA R: GAGTTCACGCACCTGGAATG	(13)
Reference genes			
ACTB	396526	F: CACAGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	(22)
UB	396190	F: GGGATGCAGATCTTCGTGAAA R: CTTGCCAGCAAAGATCAACCTT	(22)

Thermal cycling was performed in LightCycler II 480 (Roche Diagnostics, Basel, Switzerland), using 384-well plate format. Thermal profile was as follows: initial denaturation (95°C for 20 min), followed by 40 cycles of amplification (15 s at 95°C), annealing (20 s at 58°C), and elongation (20 s at 72°C). Fluorescence acquisition was done after each elongation step. At the end of RT-qPCR run, a melting curve was generated, by gradual increase in temperature up to 98°C with continuous fluorescence measurement.

Statistical analysis

The experiment was performed according to three-way factorial design, in which genotype (broiler *vs.* native chicken), *in ovo* delivered bioactive compound (prebiotic *vs.* probiotic *vs.* synbiotic *vs.* control), and immune challenge (LTA *vs.* LPS *vs.* control) were considered independent variables and the dCt of each target gene was a dependent variable. Statistical analysis was performed independently for each tissue (caecal tonsils and spleen), using a three-way ANOVA model with interactions. The main factors and their interactions were considered significant at $P < 0.05$, $P < 0.01$, or $P < 0.001$. The statistical analysis was done using SAS Enterprise Guide 9.4 (SAS Institute, Cary, NC, USA).

Relative gene expression analysis was performed based on Ct values from RT-qPCR data. The delta delta Ct (ddCt) algorithm was used to calculate Fold Change (FC) of the gene expression in experimental groups in comparison to their respective controls (23). The normalization of the mRNA expression of the target genes was done with two reference genes (*ACTB* and *UB*). To calculate delta Ct (dCt), the mean Ct of the two reference genes were subtracted from Ct of each target gene ($dCt = Ct_{target} - Ct_{reference}$). Calibration of the relative gene expression was done for each genotype/tissue independently. A calibrator was C group (mock-injected *in ovo* and mock-immunized). The dCt value of the calibrator was subtracted from dCt of the experimental groups ($ddCt = dCt_{experimental} - dCt_{calibrator}$). FC was calculated with the following formula: $FC = 2^{-ddCt}$. The ddCt calculations were done in MS Excel and the FC values were visualized using GraphPad Prism 7 (GraphPad, La Jolla, CA, USA).

Results

Main effects

In this study, we tested significance of the main effects (i.e., genotype, *in ovo stimulation* and immune challenge), and their interactions on the immune-related gene expression in caecal tonsils and spleen, which represent mucosal and systemic immune responses (Table 3). The main effect with the most significant influence on all gene expression signatures in both tissues was genotype ($P < 0.001$ for all genes, except $P < 0.05$ for *IL-10* in spleen). The second most significant main effect was immune challenge, which influenced *IL-1β*, *IL-6 IL-10* ($P < 0.001$), and *IL-17* genes ($P < 0.05$) in caecal tonsils, and all genes in spleen ($P < 0.001$), except *IL-4* ($P > 0.05$). Results of ANOVA showed that *in ovo stimulation* influenced *IL-2*, *IL-4*, and *IL-10* in caecal tonsils ($P < 0.05$) as well as *IL-2* and *IL-12p40* in spleen ($P < 0.05$).

Table 3. Effects of genotype, in ovo stimulation, immune challenge, and their interaction on gene expression signatures in caecal tonsil and spleen in chicken

Gene	Genotype (G) [1]	Immune (I) [2]	In Ovo (O) [3]	(G) x (O) [4]	(G) x (I) [5]	(O) x (I) [6]	(G) x (O) x (I) [7]
Caecal tonsils							
IL-1β	< 0.001	< 0.001	ns	ns	< 0.01	ns	ns
IL-2	< 0.001	ns	<0.05	ns	ns	ns	ns
IL-4	<.0001	ns	<0.05	ns	ns	=0.05	=0.05
IL-6	<.0001	<.0001	ns	ns	< 0.01	ns	ns
IL-10	< 0.001	< 0.001	<0.05	ns	< 0.001	ns	ns
IL-12p40	< 0.001	ns	ns	ns	ns	ns	<0.05
IL-17	< 0.001	<0.05	=0.05	ns	ns	ns	<0.05
Spleen							
IL-1β	< 0.01	< 0.001	ns	<0.05	<0.01	ns	ns
IL-2	< 0.001	< 0.001	<0.01	ns	< 0.001	ns	ns
IL-4	< 0.001	ns	ns	ns	ns	ns	ns
IL-6	< 0.01	< 0.001	=0.05	< 0.001	ns	ns	ns
IL-10	>0.05	< 0.001	ns	ns	ns	ns	ns
IL-12p40	< 0.001	< 0.001	<0.05	ns	< 0.001	ns	<0.05
IL-17	< 0.01	< 0.001	ns	ns	ns	ns	ns

Effects: [1] Genotype (G): Ross broiler chicken or Green-legged Partridgelike; [2] Immune (I) challenge with lipopolysaccharide (LPS) or lipoteichoic acid (LTA) by intraperitoneal injection on day 42 post-hatching; [3] *In ovo* (O) stimulation with prebiotic (GOS - galactooligosaccharides), probiotic (*Lactococcus lactis* subsp. *cremoris*) or synbiotic (GOS + *L. lactis*) *vs.* physiological saline (C) on day 12 of egg incubation; [4] interaction between genotype and *in ovo stimulation*; [5] interaction between genotype and immune challenge; [6] interaction between *in ovo stimulation* and immune challenge; [7] interaction between genotype, *in ovo stimulation*, and immune challenge; Gene expression analysis was done with RT-qPCR. The significance of effects that were calculated with three-way ANOVA. Significance levels: $p < 0.05$, $p < 0.01$ or $p < 0.001$ (significant), and $p > 0.05$ (non-significant, NS).

Figures present mucosal (Figure 2) and systemic (Figure 3) immune responses to *in ovo* stimulation and immune challenge determined in caecal tonsils (Figure 2) and spleen (Figure 3) of chicken broilers and native chickens. Presented data indicate that the most potent systemic immune response was triggered by LPS in the spleen (Figure 3), in comparison to data obtained for mucosal immune responses in caecal

tonsils (Figure 2). The genes with the highest relative expression in spleen in response to LPS were: *IL1-β* (FC up to 60 in PRE, broilers), *IL-6* (FC up to 900 in PRE, broilers), *IL-10* (FC up to 32 in PRE, broilers) and *IL-17* (FC up to 120 in PRE, broilers).

Interactions

Mucosal immune responses in caecal tonsils

We determined a number of significant interactions between the main effects in the gene expression signatures in caecal tonsils. We found two-way interactions between genotype and immune challenge for: *IL-1β*, *IL-6* ($P < 0.01$), and *IL-10* ($P < 0.001$). Based on the gene expression patterns (Figure 2), *IL-1β*, *IL-6*, and *IL-10* genes were differentially expressed between chicken broilers and native chickens immunized with LPS. In broilers, caecal expression of pro-inflammatory genes, *IL-1β* (FC up to 4.4 in SYN/LPS) and *IL-6* (FC up to 12.66 in SYN/LPS) was higher than in native chickens (FC of *IL-1β* up to 2.18 in C/LPS and FC of *IL-6* up to 2.48 in SYN/LPS). Gene expression pattern of anti-inflammatory cytokine *IL-10* was also differentially expressed in chickens immunized with LTA and LPS. In broilers, *IL-10* gene expression was up-regulated, especially after LPS challenge (FC up to 4.69 in C/LPS). In native chickens, *IL-10* was down-regulated (FC down to -1.50 in PRE/LPS).

In caecal tonsils, we found three-way interactions (between all main effects) for *IL-12p40* and *IL-17* ($P < 0.05$). Among the different mRNA gene expression profiles, the most striking results were obtained for native chickens (PRE/LPS). *In ovo* control (C) native chickens challenged with LPS expressed up-regulated gene expression signatures of both genes (FC *IL-12p40* = 1.56 in C/LPS and FC *IL-17* = 2.05 in C/LPS), while *in ovo* delivery of prebiotic dampened expression of both genes (FC *IL-12p40* = -1.56 in PRE/LPS and FC *IL-17* = -1.93 in PRE/LPS) in caecal tonsils of native chickens.

Systemic immune responses in spleen

In spleen, we found two-way interactions between genotype and immune challenge for *IL-1β* ($P < 0.01$), *IL-2*, and *IL-12p40* ($P < 0.001$). In all those genes, the differences in the gene expression profile between broiler and native chickens were found in C (mock-challenged) and LTA-challenged groups. In C group, the gene expression profile of *IL-1β*, *IL-2*, and *IL-12p40* indicated low up-regulation in broilers and low down-regulation in native chickens. LTA was the same, except from *IL-1β*, which was moderately up-regulated in both broilers (FC up to 2.9 in SYN/LTA) and native chickens (FC up to 3.56 in PRO/LTA). LPS up-regulated *IL-1β* (FC up to 61 in PRE/LPS/broilers and 28 in C/LPS/native chickens) and *IL-2* (FC up to 3.83 in PRO/LPS/broilers and 4.09 in SYN/LPS/native chickens) in both genotypes, but down-regulated *IL-12p40* (FC down to -4.38 in PRE/LPS/broilers and -2.68 in C/LPS/native chickens). In summary, native chickens did not activate immune responses when not challenged. Upon LTA challenge, they activated only pro-inflammatory responses (*IL-1β*), but not Th1 responses (*IL-2* and *IL-12p40*). LPS activated pro-inflammatory (*IL-1β*) and Th1 responses (*IL-2*) responses in both genotypes. In spleen, we found two-way interactions between genotype and *in ovo* stimulation for *IL-1β* ($P < 0.05$) and *IL-6* ($P < 0.001$). Finally, we

found one three-way interaction (between all main effects) for systemic immune responses in mRNA expression of *IL-12p40* in spleen ($P < 0.05$).

Discussion

Genotype

Broilers and native chickens analyzed in this study represent distinct genotypes, that are characterized by different set of traits. Broilers are four-way crosses between heavily selected genetic lines. The extreme selection pressure implemented in contemporary broiler modulated not only growth and feed efficiency, but also mechanisms of the immune responses. Cheema, Qureshi (24) determined that broilers from 1957 had greater relative weight of bursa of Fabricius, caecal tonsils and spleen, and mounted higher humoral immune responses against SRBC in comparison to broiler from 2001. On the other hand, contemporary broiler scored higher in tests evaluating cellular and inflammatory immune responses (24). Genetics underlying such a shift in the immune responses due to selection towards growth rate has been attributed to variation accumulated in the genes *TLR3* and *PLIN3* (25). Native chickens analyzed in contrast to heavily selected broiler was Green-legged Partridgelike (GP). GP is a Polish heritage chicken line, unselected since 1960s and currently kept in conservative flocks (26). GP is a light-weight chicken, traditionally kept as a dual-purpose backyard chicken. It's an excellent model for immunological studies (8, 27) due to conserved genetic status (28), as well as their resilience and sturdiness (26).

Immune challenge

The intraperitoneal challenge with LTA and LPS antigens was performed to evaluate the level of the mucosal and systemic immune responses in caecal tonsils (Figure 2) and spleen (Figure 3) of the chickens stimulated *in ovo*. The intraperitoneal immunization is often referred to as mucosal vaccination, because it aims to stimulate local mucosal immune responses (i.e., secretory IgA production at the mucosal site). Mucosal vaccination has been used to develop local immunity against *Salmonella typhimurium* in chickens (29). In the current study, the chickens were challenged intraperitoneally with nonpathogenic antigens, which are purified microbe-associated molecular patterns (MAMPs) isolated from *Staphylococcus aureus* (LTA) and *Escherichia coli* (LPS).

Exposure to LPS is associated with the condition called endotoxemia, which results in overexpression of inflammatory mediators, leading to a septic shock and increased mortality (30). During intraperitoneal injection, the tested compound is deposited in the body cavity, from where it diffuses to the surrounding tissues as well as to the circulatory system (31). Since spleen filters the blood and samples the antigens from the bloodstream, it is the primary organ to mount immune responses and neutralize the antigens. On the other hand, caecal tonsils belong to GALT, which is also susceptible to endotoxemia-mediated injury (30). GALT is primed by the orally administered antigens rather than the peritoneal ones (32). Innate immune responses mounted in the GALT are either stimulatory (i.e., against oral pathogens) or tolerogenic (i.e., unresponsive to commensal or beneficial bacteria). Oral tolerance is developed in the GALT by early contact with intestinal microbiota and helps maintaining intestinal homeostasis. The

cellular mechanism by which harmful bacteria are differentiated from beneficial ones is mediated by GALT-associated dendritic cells (CD103+ DCs), which cooperate with other cells in the gut (33). In this manner, acquired oral tolerance primes caecal tonsils to exert lower level of inflammatory responses.

LTA challenge exerted less pronounced effects in both tissues compared to LPS, which is a strong inflammatory stimuli. LTA is an exopolysaccharide sourced from cell walls of Gram-positive bacteria, including many commensal species, including *Lactobacillus*. Even though LTA induces acute inflammatory responses in the gut by activating TLR2, its major function is to enhance gut integrity via stimulating tight junctions in the intestinal epithelia (34). *In vivo* studies clearly indicate that mice treated orally with TLR2 ligand, Pam3CSK4 (synthetic LTA), were less susceptible to mucosal injury by increased integrity of intestinal epithelium (34). Recognition of LTA in the gut is mediated by DCs (e.g., CD103+ DCs subtype, mentioned earlier), and results in production of inflammatory and regulatory cytokines (35). For this reason, LTA activated gene expression signatures in caecal tonsils, but exerted no immunostimulation in the spleen.

In ovo stimulation

Effects of *in ovo* stimulation on immune-related gene expression signatures in chickens is of particular interest due to an early effect of intestinal microbiota development on immune system maturation in the neonatal chicks. According to the review of Taha-Abdelaziz, Hodgins (36), the beneficial effects of early dietary interventions on the immune system in chickens are expressed in three areas: development of lymphoid organs, gastrointestinal microbiome, and immune competence. Our previous and the current research indicates that *in ovo*-delivered prebiotics, probiotics, and synbiotics affect all three aspects of poultry immunology. Regarding the first aspect, which is lymphoid organs development, *in ovo* stimulation significantly influenced colonization of caecal tonsils and spleen with Bu-1$^+$, CD4$^+$, and CD8$^+$ cells in the chickens analyzed in this paper (15). The influence depended on the bioactive compound, chicken genotype, and age. In broilers, *in ovo* stimulation increased CD4$^+$ cells in caecal tonsils (Day 7), CD4$^+$ and CD8$^+$ cells in caecal tonsils and spleen (Day 21), as well as Bu-1$^+$ cells in caecal tonsils and all type of lymphoid cells in spleen (Day 42). In native chickens, synbiotic increased CD4$^+$ and CD8$^+$ cells in spleen (but not in caecal tonsils) (Days 2, 21, and 42). Regarding the second aspect of early dietary interventions, i.e., gastrointestinal microbiome, to date we demonstrated that *in ovo* delivery of GOS prebiotic significantly increased counts of lactobacilli and bifidobacteria in feces of newly hatched chicks (37). These effects were long-lasting, and remained significant also on day of slaughter (11). *Lactobacillus*-based synbiotics delivered *in ovo* on day 12 of egg incubation increased *Lactobacillus* spp. and *Enterococcus* spp. in ileum of the Cobb broiler chickens (18). The current study reports that *in ovo* stimulation modulates immune competence (the third aspect of early dietary interventions) in broiler and native chickens.

The results presented in this paper strongly support our earlier findings, that *in ovo* stimulation with GOS prebiotic modulates splenic gene expression of *IL-4* in fast-growing chicken broilers (12), and both, *IL-2* and *IL-4*, in slow-growing chickens (13). We have also found that raffinose family oligosaccharides

(RFOs) and RFO-based synbiotics, modulate *IL-4* and *IL-12p40* gene expression in caecal tonsils and spleen of chicken broilers (38) and native chickens (GP) (8). In this study, we have complemented the earlier data with additional treatments, i.e., immune challenge with LTA or LPS antigens. Results of the interactions between genotype, *in ovo stimulation*, and immune challenge in caecal tonsils and spleen are discussed below (section 3.4).

Interactions

Mucosal immune responses in caecal tonsils

Caecal tonsils are the largest aggregates of lymphoid tissue present in the chicken GALT. As such, they represent a major site of mucosal immune responses. *IL-1β* and *IL-6* are involved in acute inflammatory responses. Both cytokines are activated by microbes, including enteric infection with *Eimeria* or *Salmonella* (39). Increased gene expression of *IL-1β* and *IL-6* in broiler chickens immunized with LPS reflects acute inflammatory responses mounted by GALT. Haghighi, Abdul-Careem (40) reported that the *IL-6* gene expression was increased in caecal tonsils of broiler chickens infected with *Salmonella*, but the inflammatory effects of infection were mitigated by probiotics. In the current study, broiler chickens immunized with LPS (also present in *Salmonella*) increased *IL-1β* and *IL-6* gene expression in caecal tonsils, but it was not influenced by *in ovo*-delivered bioactive compounds. *IL-10* is anti-inflammatory cytokine and its expression counterbalances pro-inflammatory activity of *IL-1β* and *IL-6* (41). Regulation of inflammation by the negative feedback is the supposed mechanism of *IL-10* up-regulation in individuals expressing high abundance of pro-inflammatory mediators (*IL-1β* and *IL-6*).

The regulatory effects of bioactive compounds delivered *in ovo* were more pronounced in native chickens rather than in broiler chickens. Such results are not surprising, since in our early research on *in ovo* stimulation we found that the prebiotics and synbiotics applied on day 12 of egg incubation, down-regulate immune related gene expression signatures in caecal tonsils of native chickens (GP) (8) and chicken broilers (18, 38). However, this is the first time that we report effects of *in ovo* stimulation (day 12 of egg incubation) together with the immune challenge in two distinct chicken genotypes. Regulatory effect of GOS prebiotic (PRE) on *IL-12p40* and *IL-17* gene expression in GALT is considered beneficial, since it helps reducing LPS-induced inflammation in the intestines. Both cytokines are expressed by CD4+ cells (T lymphocytes) in response to inflammatory agents. The role of *IL-12p40* cytokine is to drive pro-inflammatory Th1-type responses, while pleiotropic cytokine *IL-17* (also known as *IL-17A*) stimulates pro-inflammatory Th17-type responses (42). Th17-type immune responses has been investigated recently due to their involvement in autoimmune diseases (43). Increased number of Th17-type cells as well as *IL-17* cytokine eliminates therapeutic effects of oral tolerance in mice (44). Intestinal level of *IL-17* varies depending on microbiota composition. For example, *Lactobacillus fermentum* IM12 suppressed LPS-activated *IL-17* level in mice (45). On the other hand, dietary GOS increased *IL-17* in caecum of *Campylobacter*-infected broiler chickens, but did not ameliorate the infection (46). We suppose, that decreased activity of *IL-17* in prebiotic-supplemented and LPS-challenged native chickens might be a

good biomarker of anti-inflammatory effects of prebiotics and probiotics delivered *in ovo*. But it requires more insight to determine the specific effects on immune responses.

Systemic immune responses in spleen

The pronounced difference between broilers and native chickens in inflammatory immune responses to *in ovo* stimulation were found in C group (mock-challenged). In chicken broilers, splenic gene expression of *IL-1β* and *IL-6* was up-regulated, while in native chickens – it was down-regulated. In other words, chicken broilers mounted higher pro-inflammatory responses to *in ovo* stimulation than native chickens. As mentioned earlier, potent activation of the cellular and inflammatory immune responses is typical for the lymphoid system of chicken broilers (24). Broilers have been heavily selected for growth traits and feed efficiency (47). Such strong selection pressure affected immune system of the broiler chickens. Acquired immune responses takes weeks to develop functional antibodies against pathogens. Since, a typical lifespan of fast-growing broilers is only 35-42 days, strong innate immune responses are more likely to fight infection (48). Increased mRNA expression levels of pro-inflammatory cytokines are under genetic control. Thus, the chicken broilers analyzed in this study were more reactive to *in ovo* microbiota stimulation and activated stronger signatures of systemic, inflammatory immune responses than native chickens.

IL-12p40 encodes a subunit p40, which is shared between *IL-12* and *IL-23* cytokines in both avian and mammalian organisms (49). In spleen, *IL-12p40* is produced by activated antigen presenting cells, such as macrophages or dendritic cells. It drives natural killer (NK) cells to secrete IFN-□ and directs immune responses towards Th1-type cell-mediated immunity (50). The most pronounced interaction in the systemic immune responses was down-regulation of the *IL-12p40* mRNA abundance in the spleen of PRE/LPS/broilers (FC ~ -4.4). In our earlier studies we associated splenic mRNA abundance of *IL-12p40* with responses to thermal stress in chicken broilers (12) and native chickens (13). Responses mounted by the immune system after acute or chronic exposition to heat stress resemble responses to LPS injected intraperitoneally into the body cavity. Both of those immune responses are aimed to neutralize circulating LPS molecules. In heat stress studies, the LPS molecules were sourced from intestinal bacteria, and in this study they were directly injected. In previously mentioned heat stress studies and in the current study, we found that GOS delivered *in ovo* successfully dampened LPS-stimulated *IL-12p40* mRNA abundance. GOS has been known for its immunomodulatory properties in animal (51) and human models. In chickens, it promotes beneficial intestinal microbiota and alleviates the effects of the foodborne pathogens like *Salmonella* (52). In broiler chickens, GOS delivered *in ovo* has confirmed bifidogenic effects and beneficial effects on mucosal gene expression, enhancing gut barrier function (11).

Conclusions

The mucosal and systemic immune responses of chicken broilers and native chickens showed distinct patterns. Genotype influenced gene expression signatures of all immune-related genes, but chicken broilers developed stronger immune responses than native chickens. LPS triggered both mucosal (caecal

tonsils) and systemic (spleen) immune responses in chicken broilers, but only systemic (spleen) in native chickens. *In ovo* stimulation with bioactive compounds (especially prebiotic) modulated innate immune responses to LPS. GOS delivered *in ovo* induced the most pronounced responses to LPS, which validated its further application as a potent immunomodulator for *in ovo* applications, especially in chicken broilers.

Abbreviations

ACTB – beta actin

ddCt – delta delta Cycle threshold

DSS - dextran sulfate sodium

G - genotype

GALT – gut-associated lymphoid tissue

GC – germinal center

GP – green-legged partridgelike

GOS - galactooligosaccharides

I – immune challenge

IgG – Immunoglobulin G

IgM – Immunoglobulin M

IgY – Immunoglobulin Y

IL-1β – interleukin 1 beta

IL-2 – interleukin 2

IL-4 – interleukin 4

IL-6 – interleukin 6

IL-10 – interleukin 10

IL-12p40 – interleukin 12 subunit p40

IL-17 – interleukin 17

L. lactis – *Lactococcus lactis* subsp. *cremoris*

LPS - lipopolysaccharide

LTA – teichoic acid

MALT – mucosa-associated lymphoid tissue

O – *in ovo* stimulation

RT-qPCR – Reverse transcription-quantitative Polymerase Chain Reaction

TLR – Toll-like receptor

UB – ubiquitin beta

Declarations

Ethics approval and consent to participate

The animal study complied with Directive 2010/63/EU. The protocols were approved by the Local Ethics Committee for Animal Experiments (Bydgoszcz, Poland) (study approval reference number 16/2014).

Consent for publication

Not applicable

Availability of data and material

The datasets used in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AS and MS conceived the study and designed the work, AD performed the data acquisition, AK and EŁ performed the animal study. AS was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Figures

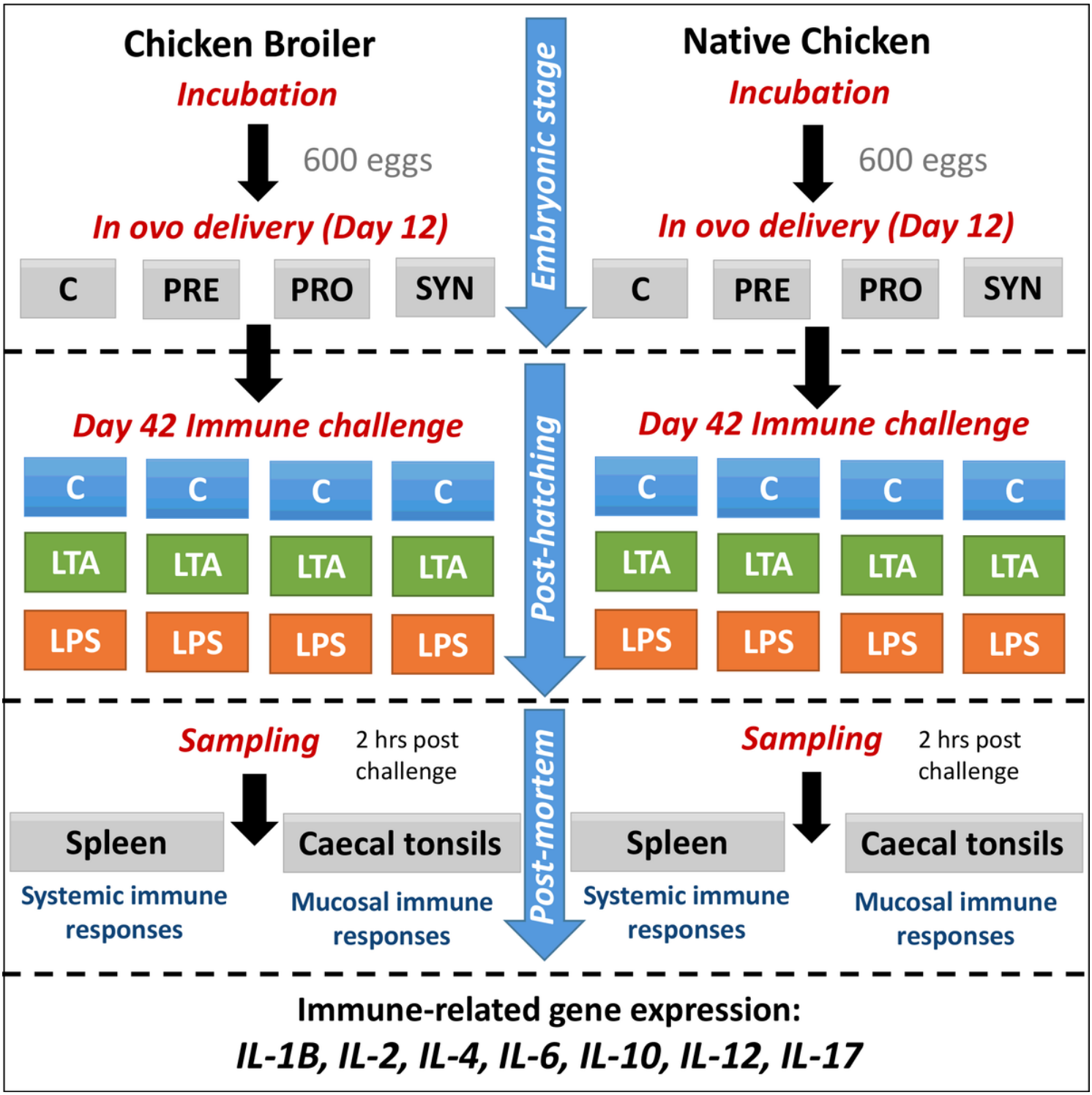

Figure 1

Experimental design of the study on effects of in ovo stimulation with bioactive compounds and immune challenge in broilers and native chickens. Experiment planned according to 2x4x3 factorial design. Independent variables included: two distinct genotypes (chicken broiler, native chicken), four in ovo stimulations (three experimental and mock control), and three in ovo challenge groups (LTA, LPS and mock control). Incubated eggs (n=600 per each genotype) were stimulated in ovo on day 12 of incubation with three bioactive compounds: prebiotic (GOS - galactooligosaccharides), probiotic (Lactococcus lactis

subsp. cremoris) and synbiotic (GOS + L. lactis). Hatched chicks were reared in the group pens. On day 42 post-hatching immune challenge was applied (intraperitoneal injection of LTA – lipoteichoic acis and LPS – lipopolysaccharides). Samples of caecal tonsils and spleen were harvested 2 hours post-challenge for relative gene expression analysis of cytokine genes.

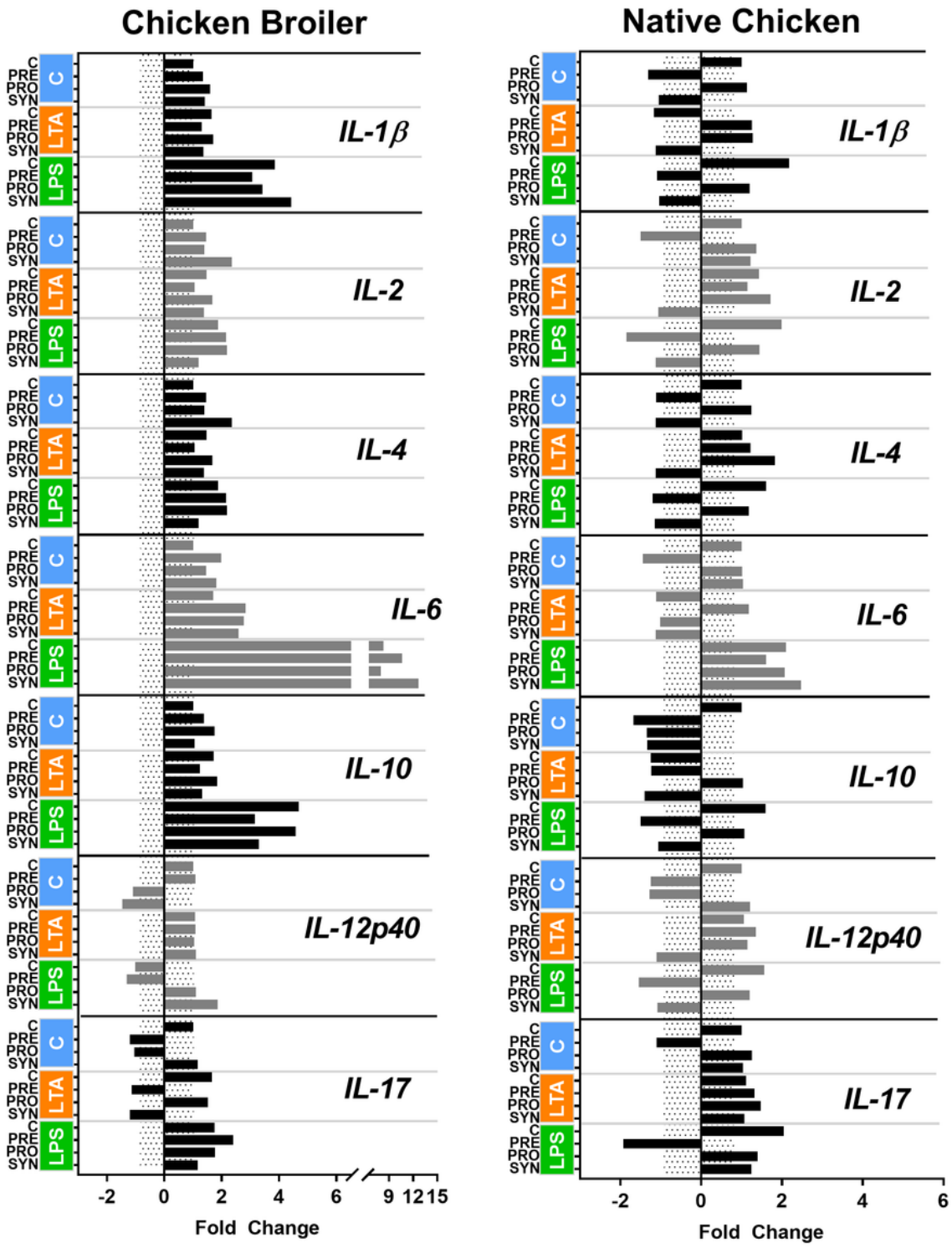

Figure 2

Mucosal immune responses to in ovo stimulation and immune challenge determined in caecal tonsils of chicken broilers and native chickens. In ovo stimulation carried out on day 12 of egg incubation using three bioactive compounds: prebiotic (GOS - galactooligosaccharides), probiotic (Lactococcus lactis subsp. cremoris) and synbiotic (GOS + L. lactis). Controls were mock-injected. Hatched chicks were reared in the group pens. On day 42 post-hatching immune challenge was applied (intraperitoneal injection of LTA – lipoteichoic acis and LPS – lipopolysaccharides). Controls were mock-injected. Samples of caecal tonsils were harvested 2 hours post-challenge fo relative gene expression analysis of cytokine genes. Relative gene expression performed using RT-qPCR and SYBR green chemistry. Cytokine genes: IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, and IL-17 were target genes. ACTB and UB were used as reference genes. Calculations were based on ΔΔCt method. Down-regulated data were transformed using a formula: 2/-FC. Graph prepared with GraphPad Prism 7 (GraphPad, La Jolla, CA, USA).

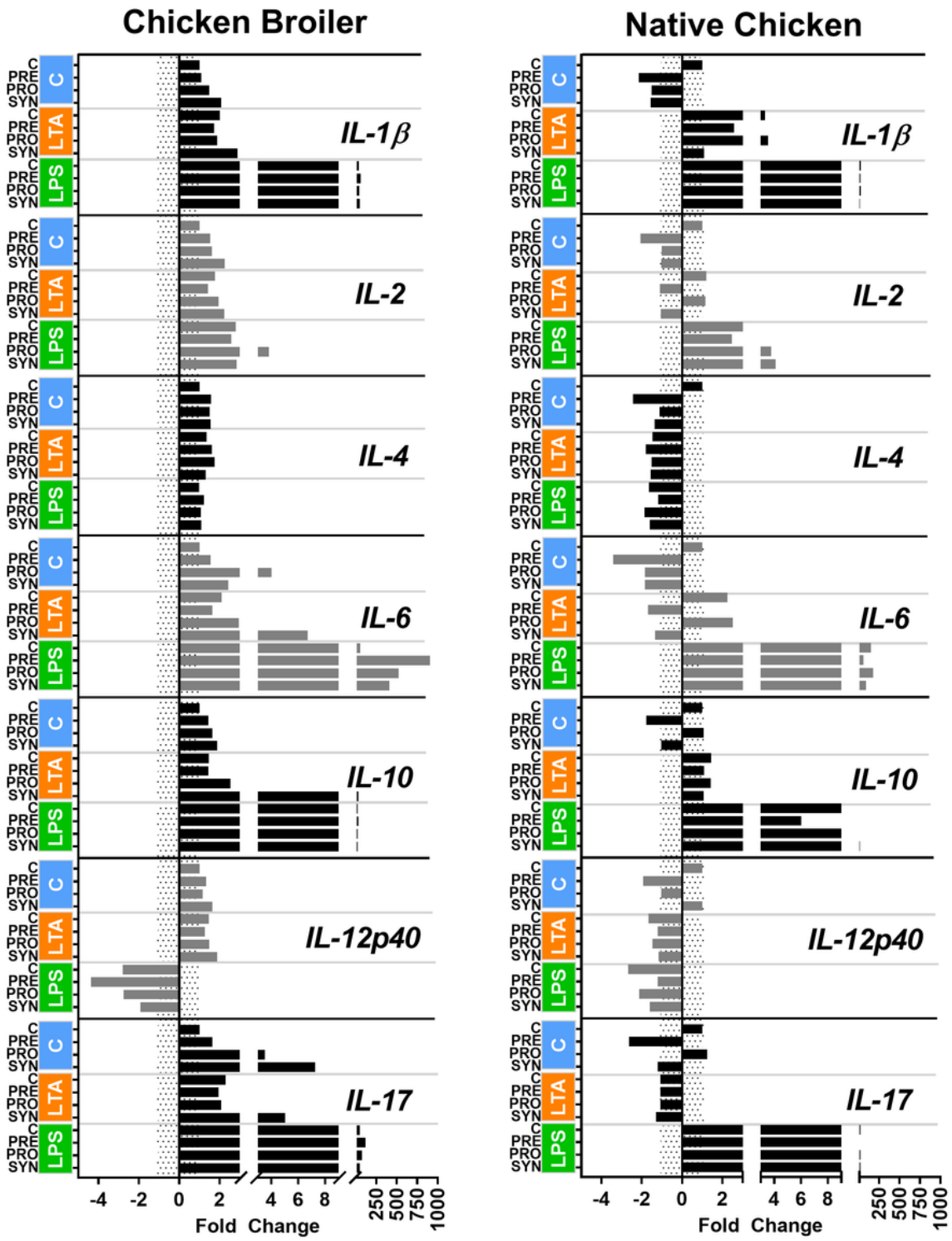

Figure 3

Systemic immune responses to in ovo stimulation and immune challenge determined in spleen of chicken broilers and native chickens. In ovo stimulation carried out on day 12 of egg incubation using three bioactive compounds: prebiotic (GOS - galactooligosaccharides), probiotic (Lactococcus lactis subsp. cremoris) and synbiotic (GOS + L. lactis). Controls were mock-injected. Hatched chicks were reared in the group pens. On day 42 post-hatching immune challenge was applied (intraperitoneal

injection of LTA – lipoteichoic acis and LPS – lipopolysaccharides). Controls were mock-injected. Samples of spleen were harvested 2 hours post-challenge fo relative gene expression analysis of cytokine genes. Relative gene expression performed using RT-qPCR and SYBR green chemistry. Cytokine genes: IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, and IL-17 were target genes. ACTB and UB were used as reference genes. Calculations were based on ΔΔCt method. Down-regulated data were transformed using a formula: 2/-FC. Graph prepared with GraphPad Prism 7 (GraphPad, La Jolla, CA, USA).