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# Tissue‐specific effects of in ovo prebiotic stimulation on cytokine gene expression in broiler chickens

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# Tissue-specific effects of *in ovo* prebiotic stimulation on cytokine gene expression in broiler chickens.

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## Abstract

The modulation of the immune system during the early life of broiler chickens has been shown to have a beneficial effect on disease resistance and overall health. The present study investigated the effects of *in ovo* administration of xylo- and mannooligosaccharides (XOS and MOS) on cytokine gene expression in two immune-relevant tissues: the spleen and cecal tonsils. The injection of fertilized eggs was conducted on embryonic day 12, with the substances xylotriose (XOS3), xylotetraose (XOS4), mannotriose (MOS3), mannotetraose (MOS4), or saline (control) utilised for this purpose. The gene expression of cytokines representing general immune regulation (*IL1β, IL2, IL10*), Th1 (*IL8, IL12, IFNβ, IFNγ*), Th2 (*IL4, IL6*), and Th17 (*IL17*) pathways was quantified in 42-day-old birds using RT-qPCR. A significant immunomodulatory effect was observed in the spleen, where XOS3 consistently increased the expression of cytokines associated with Th1-, Th2-, and Th17-mediated responses, indicating enhanced systemic immune activation. Conversely, no statistically significant differences were detected in the cecal tonsils, although numerical variation was present. This muted mucosal response is likely to reflect the inherent heterogeneity and tolerance-oriented function of gut-associated lymphoid tissue, consistent with the concept of acquired immunotolerance to commensal microbiota. Collectively, these findings demonstrate that *in ovo* prebiotic stimulation exerts tissue-specific effects, promoting systemic immune readiness while maintaining mucosal homeostasis, and highlight XOS3 as a particularly effective modulator of splenic cytokine expression in broiler chickens.



Key words: cecal tonsils, spleen, xylooligosaccharides, mannooligosaccharides

## Introduction

The avian immune system is comprised of innate and adaptive components that undergo rapid development during embryogenesis and the early post-hatch period. In chickens, the thymus, bursa of Fabricius, and spleen are considered to be central immune organs. These organs are responsible for the development of T- and B-cells, as well as for coordinating immune responses (Wlaźlak et al. 2023). It is imperative to establish a balanced equilibrium between T helper cell responses in order to achieve effective protection against pathogens and to maintain immune homeostasis. Th1 responses are primarily associated with cell-mediated immunity and defence against intracellular pathogens, Th2 responses promote humoral immunity and antibody production, and Th17 responses play a crucial role in mucosal defence and inflammation (Wang et al. 2024; Slawinska et al. 2024). The early modulation of these immune pathways has the potential to enhance disease resistance and improve poultry health.

Recent evidence highlights the bidirectional cross-talk between the gut microbiota and immune organs, which is critical for shaping immune development and responsiveness. The intestinal microbiota has been demonstrated to influence gene expression in lymphoid tissues, affecting immune cell differentiation and cytokine production, while the immune system modulates microbial composition through immunoglobulins, cytokines, and antimicrobial peptides (Shehata et al. 2021; Zhao et al. 2025). This interaction suggests that targeted manipulation of the gut microbiota may lead to systemic immunological effects beyond the intestine.

Prebiotics, defined as non-digestible carbohydrates that selectively stimulate beneficial intestinal microorganisms, have emerged as promising modulators of both the microbiota and the host immune system. Mannan-oligosaccharides (MOS) and xylo-oligosaccharides (XOS) have been demonstrated to enhance gut morphology, modulate microbiota composition, and stimulate both innate and adaptive immune responses in chickens (Singh et al. 2022; Bełdowska

et al. 2024). These compounds have been demonstrated to reduce pathogen colonization, enhance barrier function, and influence cytokine expression, thereby contributing to enhanced health and performance independent of antibiotic growth promoters.

*In ovo* administration of bioactive compounds constitutes an innovative strategy that allows for early stimulation of the gut and immune system prior to hatch. As demonstrated in previous studies, the provision of prebiotics *in ovo* has been shown to influence intestinal development, microbiota composition, and local gene expression in chickens (Siwek et al. 2018). The present study investigates how xylose- and mannose-based prebiotics administered during embryonic development influence gene expression in cecal tonsils and spleen, building on these findings. This work constitutes a follow-up to the study by Bełdowska et al. (Bełdowska et al. 2024) and aims to provide novel insights into the microbiota–immune axis in broiler chickens.

## Material and methods

### *Experimental setup and sample collection*

Experimental setup and sample collection was already described by Bełdowska et al., 2024. In brief, a total of 700 hatching eggs from Ross 308 broiler hens were obtained from a commercial hatchery. On day 7 of incubation, candling identified 300 fertilized eggs, which were selected for the study. On day 12, these eggs were randomly assigned to five groups (60 eggs per group). Each group received an injection of either 0.2 mL saline (control) or a prebiotic solution (xylotriose, xylotetrose, mannotriose, or mannotetrose) at a dose of 0.5 mg/0.2 mL. The injection site was sealed with non-toxic glue.

Eggs were incubated in a single-stage incubator set to 37.7°C, 55–60% humidity, and 50–60% ventilation until day 18, with automatic 45° tray rotation every hour. On day 19, eggs

were transferred to a hatcher maintained at 37.5°C, 70% humidity, and 80% ventilation. Eggs were candled on days 7 and 18 to remove unfertilized or non-viable eggs.

After hatching, chicks were housed in pens with chopped wheat-straw bedding and provided ad libitum access to feed and water. Starter, grower, and finisher feeds, with protein contents of 22%, 20.5%, and 19%, respectively, and metabolizable energy of 12.50 MJ/kg, were supplied according to nutritional standards for broiler chickens. The feed included essential vitamins and minerals as declared by the manufacturer.

***Total RNA isolation and RT-qPCR***

Total RNA was extracted from approximately 100 mg of spleen and cecal tonsils. Tissue samples were homogenized in 0.2 mL of chloroform and 1 mL of RNA Extracol reagent (EURx, Gdańsk, Poland) using a TissueRuptor homogenizer (Qiagen GmbH, Hilden, Germany). RNA purification was performed using the GeneMATRIX Universal RNA Purification Kit (EURx, Gdańsk, Poland) according to the manufacturer's protocol. The quantity and quality of RNA were assessed using the NanoDrop 2000 spectrophotometer (Thermo Scientific).

Gene expression analysis was conducted for a panel of genes representing: general regulatory cytokines (*IL1-β, IL-2, IL-10*), Th1 pathway related cytokines *(IL-8, IL-12, IFNγ, IFNβ*), Th2 pathway related cytokines (*IL-4, IL-6*), Th17 pathway cytokine (*IL-17*). *ACTB* and *G6PDH* were used as reference genes (Table 1).

Table 1. Primers used for the qPCR to determine the relative gene expression in spleen and cecal tonsils

| **Gene name** | **Gene symbol** | **Primer sequence[1] (5'→ 3')** | **Reference** |
|---|---|---|---|
| **House-keeping genes** | | | |
| Actin, beta | *ACTB* | F: CACAGATCATGTTTGAGACCTT | (Slawinska et al. 2019) |

| | | | |
|---|---|---|---|
| | | R: CATCACAATACCAGTGGTACG | |
| Glucose-6-Phosphate Dehydrogenase | *G6PDH* | F: CGGGAACCAAATGCACTTCGT | (Slawinska et al. 2019) |
| | | R: GGCTGCCGTAGAGGTATGGGA | |
| **General regulatory cytokines** | | | |
| Interleukin 1 beta | *IL1-β* | F: GGAGGTTTTTGAGCCCGTC | (Dunislawska et al. 2017) |
| | | R: TCGAAGATGTCGAAGGACTG | |
| Interleukin 2 | *IL2* | F: GCTTATGGAGCATCTCTATCATCA | (Pietrzak et al. 2020) |
| | | R: GGTGCACTCCTGGGTCTC | |
| Interleukin 10 | *IL10* | F: CATGCTGCTGGGCCTGAA | (Rothwell et al. 2004) |
| | | R: CGTCTCCTTGATCTGCTTGATG | |
| **Th1 pathway related cytokines** | | | |
| Interleukin 8 | *IL8* | F: AAGGATGGAAGAGAGGTGTGCTT | (Sławinska et al. 2014) |
| | | R: GCTGAGCCTTGGCCATAAGT | |
| Interleukin 12 | *IL12* | F: TTGCCGAAGAGCACCAGCCG | (Slawinska et al. 2019) |
| | | R: CGGTGTGCTCCAGGTCTTGGG | |
| Interferon gamma | | F: ACACTGACAAGTCAAAGCCGC | (Biesek et al. 2021) |
| | | R: AGTCGTTCATCGGGAGCTTG | |
| Interferon beta | | F: ACCAGATCCAGCATTACATCCA | (Biesek et al. 2021) |
| | | R: CGCGTGCCTTGGTTTACG | |
| **Th2 pathway related cytokines** | | | |
| Interleukin 4 | *IL4* | F: GCTCTCAGTGCCGCTGATG | (Biesek et al. 2021) |
| | | R: GGAAACCTCTCCCTGGATGTC | |
| Interleukin 6 | *IL6* | F: AGGACGAGATGTGCAAGAAGTTC | (Sławinska et al. 2014) |
| | | R: TTGGGCAGGTTGAGGTTGTT | |
| **Th17 pathway related** | | | |
| Interleukin 17 | *IL17* | F: CCGTCTTCTGCTGAGAGGAGTG | (Biesek et al. 2021) |
| | | R: ACCGTTGTTCCGTCCCATCAC | |

For quantitative PCR (qPCR), complementary DNA (cDNA) was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific/Fermentas, Vilnius, Lithuania). The qPCR reactions were performed on the LightCycler 480 II system using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA). Each reaction contained 1 μM of forward and reverse primers specific to the target gene (synthesized by Sigma-Aldrich, Schnelldorf, Germany) and 70 ng of cDNA.

The thermal cycling conditions included an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 15 seconds, and elongation at 72°C for 45 seconds. A melting curve analysis was performed to confirm reaction specificity. Relative gene expression was quantified using the ΔΔCT method, with the relative abundance of target genes calculated using the 2^-ΔΔCT formula.

***Statistical analysis***

Statistical analyses were performed based on the normalized gene expression values, calculated as the difference between the Ct value of the target gene and the geometric mean of the Ct values of the reference genes (ΔCt method). The distribution of data within each experimental group was assessed using the Shapiro–Wilk test for normality. Homogeneity of variances across groups was verified separately for each gene using Levene's test. Following these preliminary tests, a generalized linear model (GLM) procedure was applied, using the Kruskal–Wallis test due to deviations from normality or unequal variances. When a statistically significant effect of the experimental factor was detected ($p < 0.05$), post hoc multiple comparisons were conducted using Dunn's test. All statistical analyses were performed using GraphPad Prism, version 9.0.0 (GraphPad Software, San Diego, CA, USA).

## Results and discussion

In the present study, gene expression profiling was employed to analyze tissue-specific immunomodulation in broiler chickens that had been subjected to *in ovo* stimulation with selected prebiotics. XOS3, XOS4, MOS3 and MOS4. The present study focused on the analysis of two immune-related tissues: the spleen and the cecal tonsils. The spleen is the largest peripheral lymphoid organ. It plays a significant role in antibacterial and antiviral immune responses (Zhang et al. 2019). Cecal tonsils constitute an element of the gut-associated lymphoid tissue (GALT). The gut mucosa is exposed to microbiota and commensal bacteria that play a pivotal role in nutrient processing. Furthermore, it is imperative that the mucosal immune system fosters tolerance towards the extensive non-pathogenic microbiota, while concurrently maintaining responsiveness to potentially pathogenic microbes (Honda and Takeda 2009).

The *in ovo* administration of disparate prebiotic formulations resulted in measurable differences in the expression levels of selected cytokine genes in the spleen of 42-day-old broiler chickens. The initial gene panel that was analyzed in this study comprised general regulatory cytokines: *IL1β, IL2* and *IL10. IL1β* is a potent pro-inflammatory cytokine. The substance under discussion is implicated in the processes of T-cell activation and cytokine production, as well as B-cell activation and antibody production. Furthermore, it has been demonstrated to promote Th17 differentiation of T cells (Stelzer et al. 2016). *IL2* plays a crucial role in regulating the adaptive immune responses by controlling the proliferation of regulatory T-cells. Furthermore, it has been demonstrated to participate in the differentiation and homeostasis of effector T-cells (Stelzer et al. 2016). As Stelzer et al. (Stelzer et al. 2016) have demonstrated, *IL10* has a profound anti-inflammatory function. *IL10* has been shown to inhibit the activity of pro-inflammatory cytokines, such as *IL1β*, and to play a role in preventing cell

damage caused by inflammation (Lee et al. 2018). Furthermore, *IL10* has been demonstrated to contribute to the maintenance of immune homeostasis.

The second gene panel analyzed in this study contained Th1-related cytokines: *IL8, IL12, IFNβ, IFNγ*. *IL8, IL12, IFNβ* and *IFNγ*. *IL8* has been identified as chemotactic factor that plays a pivotal role in the mediation inflammatory responses (Stelzer et al. 2016). It has been demonstrated that this interleukin attracts neutrophils to the site of the inflammation during infection (Kogut 2002; Elnagar et al. 2021). *IL12* has been demonstrated to play a significant role as a pro-inflammatory molecule, regulating cell-mediated immune responses in response to intracellular pathogens (Majid et al. 2020). It has been demonstrated that this results in a Th1-type immune response, which is characterized by the production of IFNγ (Degen et al. 2004). Interferons have been identified as playing a crucial role in combating viral infections. *IFNβ* belongs to the IFN type I, which plays a pivotal role in the host organism's antiviral response. This response encompasses the inhibition of cell proliferation, the modulation of cell fate, and the mediation of cell differentiation and migration (Santhakumar et al. 2017). *IFNγ* has been identified as a critical mediator that connects the innate and adaptive immune systems. It has been demonstrated that the substance plays a role in activating Th-1-type immune responses. *IFNγ* is the member of type II IFN in birds (Santhakumar et al. 2017).

The third gene panel analyzed in this study contained Th2-related cytokines: *IL4* and *IL6*. *IL4* has been demonstrated to be involved in the Th-2 type of immune response. The phenomenon has been observed to induce a differentiation of naïve T cells to Th-2 cells, B cell antibody production, isotyping switching to IgE, expansion of basophils and eosinophils, mast cell activation, and skewing of macrophages towards the M2 type (Heeb et al. 2020). *IL6* is a pleiotropic cytokine that plays a role in inflammation and the acute phase immune response. It is also involved in haematopoiesis (Yu et al. 2019).

The final gene for which expression was analyzed in the spleen and cecal tonsils was *IL17*. This interleukin plays a pivotal role in the differentiation of the two distinct Th-1 and Th17 cell lineages. As posited by Boodhoo et al., (Boodhoo et al. 2025), *IL17* may exert an influence on the function of *IFNγ*.

Gene expression is presented as fold change (FC) values, calculated using the 2^−ΔΔCt method relative to the control group. FC values greater than 1 indicate upregulation, whereas values below 1 reflect downregulation. The data are presented in the form of bar graphs (Figures 1–8), with statistically significant differences between groups ($p < 0.05$) indicated by asterisks. Statistically significant differences were observed for several genes, with the most pronounced contrasts occurring between the XOS3 and XOS4 groups.

***Spleen***

The spleen is a vital organ that plays a crucial role in the immune system. It is the primary site of lymphocyte differentiation and proliferation. The avian spleen is responsible for triggering both innate and adaptive immune responses. It has been demonstrated that the subject under discussion also plays a role in hormonal and cell-mediated responses (Vali et al. 2023). Given the absence of lymph nodes in birds, the role of the spleen in mounting immune responses is of particular significance (Lowenthal et al. 2013).

Among the general immune regulators (Figure 1), *IL1β* expression was significantly lower in birds treated with XOS4 (FC = 0.26) compared to those receiving XOS3 (FC = 1.18) or MOS3 (FC = 1.24). A similar pattern was observed for *IL2*, with higher transcript levels in the XOS3 group (FC = 2.11) compared to XOS4 (FC = 0.23) and MOS3 (FC = 0.57). The expression profiles of general regulatory cytokines reveals a distinct immunomodulatory effect of the XOS3 upon an *in ovo* administration. The data indicates that XOS3 shows effect on both innate (*IL1β*) and adaptive (*IL2*) immune responses, without inducing compensatory anti-

inflammatory mechanisms (*IL10*). The increase in *IL1β* suggests enhanced early inflammatory signaling, which is likely to contribute to pathogen recognition and immune cell recruitment. The upregulation of *IL2* implies robust T cell activation and potential support for regulatory T cell maintenance, reflecting a balanced immune stimulation. In contrast, the expression of *IL10* remained constant across all groups, indicating that under these conditions, anti-inflammatory feedback mechanisms were not significantly activated (Lee et al. 2018).

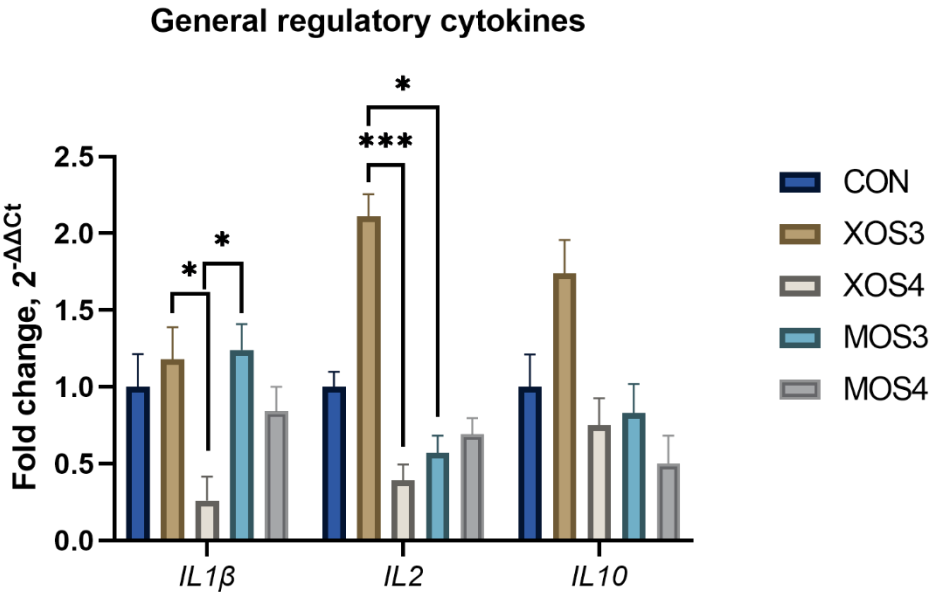



**Figure 1.** *Relative expression of cytokine genes involved in general immune regulation (interleukin 1 beta – IL1β, interleukin 2 – IL2, and interleukin 10 – IL10) in the spleen of broiler chickens from experimental groups subjected to early in ovo stimulation on embryonic day 12 (via air chamber administration). CON indicates the control group (physiological saline), XOS3 – xylotriose, XOS4 – xylotetraose, MOS3 – mannotriose, and MOS4 – mannotetraose (prebiotics). Gene expression is presented as fold change (FC), calculated using the 2^−ΔΔCt method. Error bars represent the standard error of the mean (SEM). Statistically significant differences are indicated by asterisks: $p < 0.05$ (*), $p < 0.001$ (***). Statistical analysis was performed using the Kruskal–Wallis test, followed by Dunn's post hoc test to identify group-wise differences.*

As illustrated in Figure 2, there were discrepancies observed in the expression levels of Th1-associated cytokines in the spleen. IL12 expression was significantly reduced in the MOS3 (FC = 0.19) and MOS4 (FC = 0.20) groups relative to both the control (FC = 1.00) and XOS3 (FC = 0.71) groups. For *IFNβ*, transcript levels were elevated in XOS3 (FC = 2.29) in comparison to XOS4 (FC = 0.32). A similar trend was observed for *IFNγ*, with lower expression in XOS4 (FC = 0.67) relative to XOS3 (FC = 1.95) and MOS4 (FC = 2.73). In the group of Th1-associated cytokines, XOS3 has been shown to possess promising immunomodulatory properties. XOS3 appears to function as a potent immunostimulatory prebiotic that selectively amplifies antiviral and Th1-related cytokine responses. The same set of Th1 pathway-related cytokines was analyzed in the cecum mucosa. As demonstrated in the research by Bełdowska et al. (Bełdowska et al. 2024), the upregulation of *IL12* has been determined for the Th1 cytokines in the XOS4 and MOS3 experimental groups. The more pronounced response in the spleen determined in the present study might be explained by accelerated and strengthened immune maturation.

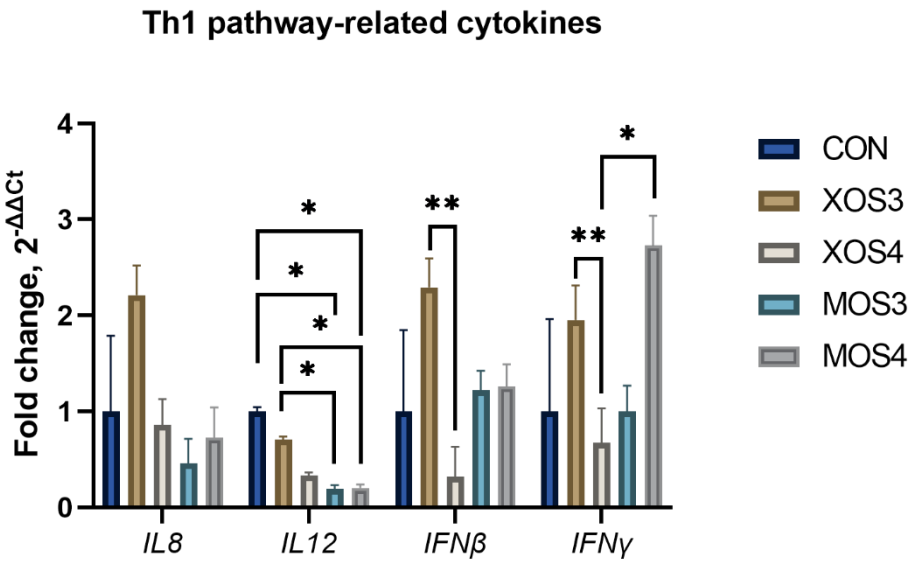



**Figure 2.** *Relative expression of cytokine genes associated with the Th1-type immune response (interleukin 8 – IL8, interleukin 12 – IL12, interferon beta – IFNB1, and interferon gamma – IFNG) in the spleen of 42-day-old broiler chickens following in ovo administration of different prebiotic formulations (delivered on embryonic day 12 into the air chamber). CON indicates the control group (physiological saline), XOS3 – xylotriose, XOS4 – xylotetraose, MOS3 – mannotriose, and MOS4 – mannotetraose (prebiotics). Gene expression is presented as fold change (FC), calculated using the 2^−ΔΔCt method. Error bars represent the standard error of the mean (SEM). Statistically significant differences are indicated by asterisks: $p < 0.05$ (*), $p < 0.01$ (**). Statistical analysis was performed using the Kruskal–Wallis test, followed by Dunn's post hoc test to identify group-wise differences.*

Regarding Th2-related cytokines (Figure 3), *IL4* expression was significantly lower in MOS3 (FC = 0.18) compared to XOS3 (FC = 1.03). For *IL6*, expression was higher in the XOS3 group (FC = 2.99), particularly in comparison to XOS4 (FC = 0.57). An elevated level of *IL4* indicates shift towards a Th2-type response (Zhao et al. 2020), which in turn supports B-cell activation, class switching and enhanced humoral immunity. This finding suggests that XOS3, may potentially prime the adaptive immune system towards antibody-mediated defenses during the early stages of life. Increased *IL6* in the context of concurrent *IL4* upregulation, most likely reflects a coordinated enhancement of immune readiness rather than overt inflammation. This may support the maturation and functional activation of splenic immune cells. As previously

demonstrated by Madej et al. (Madej et al. 2020), prebiotic administration *in ovo* has a pronounced effects on the number of B and T cells in spleen.

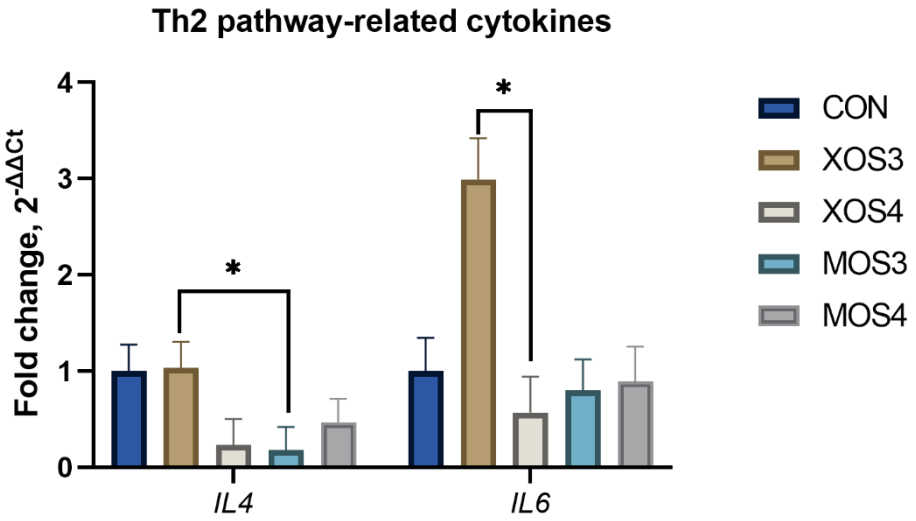



**Figure 3.** *Relative expression of cytokine genes associated with the Th1-type immune response (interleukin 8 – IL8, interleukin 12 – IL12, interferon beta – IFNB1, and interferon gamma – IFNG) in the spleen of 42-day-old broiler chickens following in ovo administration of different prebiotic formulations (delivered on embryonic day 12 into the air chamber). CON indicates the control group (physiological saline), XOS3 – xylotriose, XOS4 – xylotetraose, MOS3 – mannotriose, and MOS4 – mannotetraose (prebiotics). Gene expression is presented as fold change (FC), calculated using the 2^−ΔΔCt method. Error bars represent the standard error of the mean (SEM). Statistically significant differences are indicated by asterisks: p < 0.05 (*). Statistical analysis was performed using the Kruskal–Wallis test, followed by Dunn's post hoc test to identify group-wise differences.*

The analyzed in this study contained Th17-related cytokine *IL17*. With respect to the Th17-associated cytokine *IL17* (Figure 4), transcript levels were significantly lower in the XOS4 group (FC = 0.30) compared to XOS3 (FC = 1.21). Increased IL17 indicates stimulation of the Th17 axis, which is involved in neutrophil recruitment, mucosal/barrier defense, and early protection against extracellular bacteria and fungi. In the spleen, this may reflect an enhanced capacity for rapid pro-inflammatory responses when required. As demonstrated by

Yu at al. (Yu et al. 2021), there is an increase in *IL17* expression in the spleen in relation to *Eimeria tenella* infection. The XOS3 *in ovo* administration appears to program the splenic immune system towards a more responsive Th17 phenotype.

It has been demonstrated that XOS3 exerts a dual effect on the Th2 and Th17 arms, potentially engendering an environment conducive to both humoral support and the initiation of vigorous early inflammatory responses (Kim et al. 2014). It is evident that the process under scrutiny may involve a form of immune system "training" *in ovo*, as opposed to the manifestation of pathological inflammation. The hypothesis can be substantiated by the observation that no signs of infection were found during the rearing period. This finding is consistent with the results of the study by Wishna-Kadawarage et al. (Wishna-Kadawarage et al. 2025), which investigated the mitigation of *Campylobacter jejuni* infection through the training of the innate immune system by *in ovo* injection of probiotics and prophybiotics.

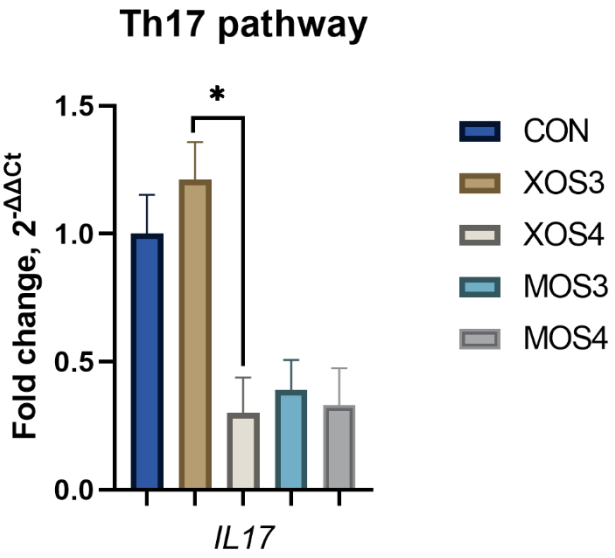



**Figure 4.** *Relative expression of interleukin 17 – IL17, a cytokine representative of the Th17-type immune response in the spleen of 42-day-old broiler chickens following in ovo administration of different prebiotic formulations (delivered on embryonic day 12 into the air chamber). CON indicates the control group (physiological saline), XOS3 – xylotriose, XOS4 – xylotetraose, MOS3 – mannotriose, and MOS4 – mannotetraose (prebiotics). Gene expression*

*is presented as fold change (FC), calculated using the 2^−ΔΔCt method. Error bars represent the standard error of the mean (SEM). Statistically significant differences are indicated by asterisks: $p < 0.05$ (*). Statistical analysis was performed using the Kruskal–Wallis test, followed by Dunn's post hoc test to identify group-wise differences.*

Although fold change values were moderate, the statistical significance and reproducibility of several pairwise differences suggest that the expression profile of certain cytokines in the spleen varied depending on the type of prebiotic used. Notably, discrepancies between the XOS3 and XOS4 groups were consistent across multiple cytokines with distinct immunological roles. XOS3 and XOS4 differ in their degree of polymerization and therefore the specific prebiotics effect on gut health. Xylotriose (XOS3) being shorter exhibit stronger prebiotic activity compared to longer xylotetraose (XOS4) (Ren et al. 2025).

***Cecal tonsils***

Cecal tonsils are the largest gut - associated lymphoid tissues in chicken. They serve as a part of the immune system being at the same time constantly exposed to the intestinal microbiota (Dunislawska et al. 2023).

A parallel analysis conducted in the cecal tonsils did not reveal any statistically significant differences in cytokine gene expression among the groups ($p > 0.05$). Nevertheless, numerical variation in transcript levels was observed. These data are presented in Figures 5–8. This variability is likely attributable to the biological heterogeneity of the tissue, as cecal tonsils are a component of gut-associated lymphoid tissue (GALT), characterized by dynamic cellular composition and considerable inter-individual variation, particularly at this stage of development.

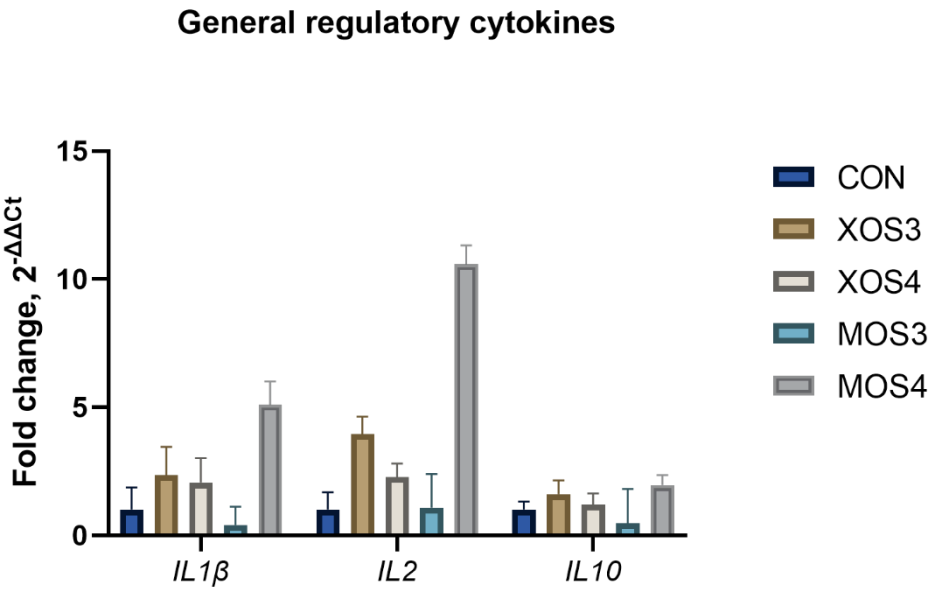



**Figure 5.** *Relative expression of cytokine genes involved in general immune regulation (interleukin 1 beta – IL1β, interleukin 2 – IL2, and interleukin 10 – IL10) in the cecal tonsils of 42-day-old broiler chickens following in ovo administration of different prebiotic formulations (delivered on embryonic day 12 into the air chamber). CON indicates the control group (physiological saline), XOS3 – xylotriose, XOS4 – xylotetraose, MOS3 – mannotriose, and MOS4 – mannotetraose (prebiotics). Gene expression is presented as fold change (FC), calculated using the 2^−ΔΔCt method. Error bars represent the standard error of the mean (SEM). Statistical analysis was performed using the Kruskal–Wallis test. No statistically significant differences were observed between groups (p > 0.05).*

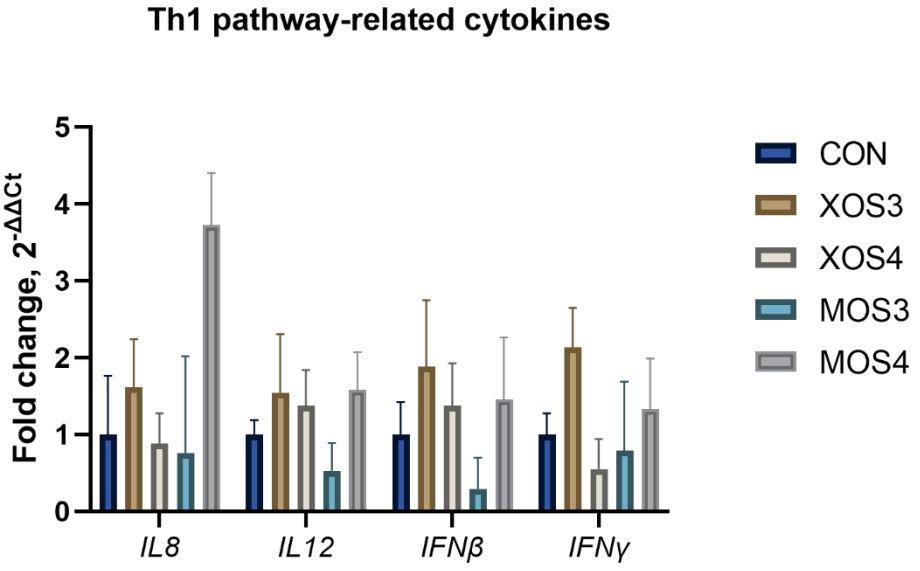


**Figure 6.** *Relative expression of cytokine genes associated with the Th1-type immune response (interleukin 8 – IL8, interleukin 12 – IL12, interferon beta – IFNβ, and interferon gamma – IFNγ) in the cecal tonsils of 42-day-old broiler chickens following in ovo administration of different prebiotic formulations (delivered on embryonic day 12 into the air chamber). CON indicates the control group (physiological saline), XOS3 – xylotriose, XOS4 – xylotetraose, MOS3 – mannotriose, and MOS4 – mannotetraose (prebiotics). Gene expression is presented as fold change (FC), calculated using the 2^−ΔΔCt method. Error bars represent the standard error of the mean (SEM). Statistical analysis was performed using the Kruskal–Wallis test. No statistically significant differences were observed between groups (p > 0.05).*

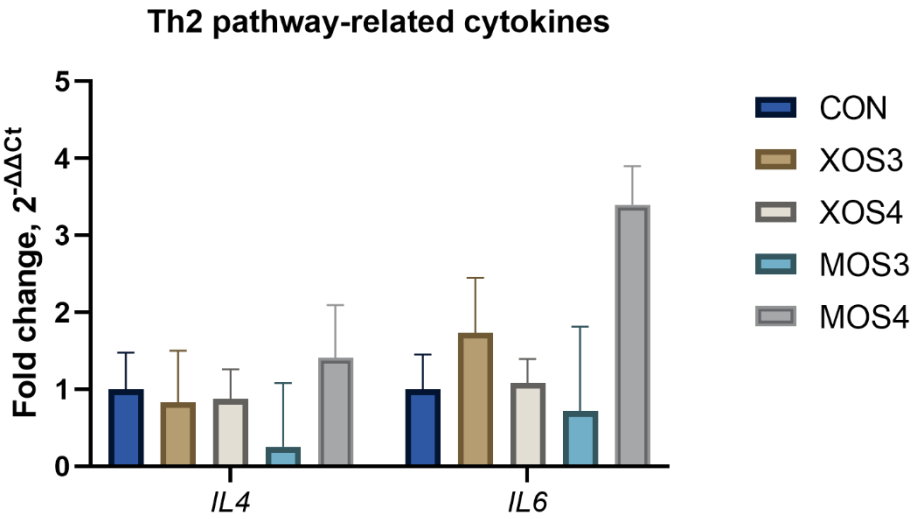



**Figure 7.** *Relative expression of cytokine genes associated with the Th2-type immune response (interleukin 4 – IL4 and interleukin 6 – IL6) in the cecal tonsils of 42-day-old broiler chickens following in ovo administration of different prebiotic formulations (delivered on embryonic day 12 into the air chamber). CON indicates the control group (physiological saline), XOS3 – xylotriose, XOS4 – xylotetraose, MOS3 – mannotriose, and MOS4 – mannotetraose (prebiotics). Gene expression is presented as fold change (FC), calculated using the 2^−ΔΔCt method. Error bars represent the standard error of the mean (SEM). Statistical analysis was performed using the Kruskal–Wallis test. No statistically significant differences were observed between groups (p > 0.05).*

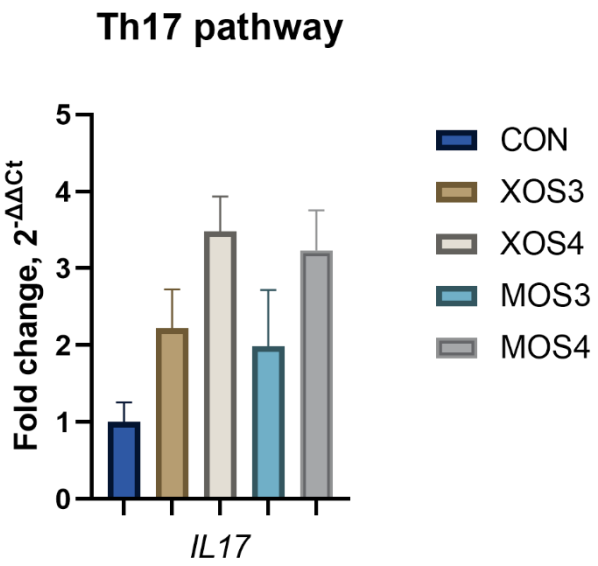



**Figure 8.** *Relative expression of interleukin 17 – IL17, a cytokine associated with the Th17-type immune response, in the cecal tonsils of 42-day-old broiler chickens following in ovo administration of different prebiotic formulations (delivered on embryonic day 12 into the air chamber). CON indicates the control group (physiological saline), XOS3 – xylotriose, XOS4 – xylotetraose, MOS3 – mannotriose, and MOS4 – mannotetraose (prebiotics). Gene expression is presented as fold change (FC), calculated using the 2^−ΔΔCt method. Error bars represent the standard error of the mean (SEM). Statistical analysis was performed using the Kruskal–Wallis test. No statistically significant differences were observed between groups (p > 0.05).*

The divergent cytokine responses observed between the spleen and cecal tonsils are consistent with the distinct immunological roles of these tissues and are also consistent with the framework proposed by Sławińska et al. (Slawinska et al. 2016). In the spleen, *in ovo* administration of prebiotics—particularly XOS3—showed a clear modulation of Th1-, Th2-, and Th17-related cytokines, reflecting systemic immune training during early development. Conversely, the cecal tonsils demonstrated no statistically significant alterations, despite observable numerical trends. This phenomenon is presumably attributable to the inherent heterogeneity and dynamic maturation of gut-associated lymphoid tissue.

This phenomenon is also consistent with the results of biochemical parameters of cecal digesta at the time of sampling (42 day of life). *In ovo* stimulation with XOS3, XOS4, MOS3

and MOS4 had no effect on ileal and cecal digesta pH. The levels of SCFA concentration in the cecum did not deviate from the control group. The study revealed also no observable impact on ammonia concentration within the ceca of broiler chickens (Bełdowska et al. 2024).

As Sławińska et al. (Slawinska et al. 2016) argue, early exposure to beneficial microbial signals is conducive to the establishment of acquired immunotolerance within the gut, thereby enabling commensal colonization while preventing excessive inflammatory activation. It is evident that cecal tonsils constitute an integral component of the GALT, thereby rendering them susceptible to exposure to the intestinal microflora. It is postulated that a tolerant environment of this nature will diminish or render less visible prebiotic-induced shifts in cytokine levels within the mucosa, particularly during the accelerated post-hatch remodeling of GALT.

The pronounced systemic effects observed in the spleen, in contrast to the subtle and variable responses seen in the cecal tonsils, suggest that *in ovo* prebiotics enhance systemic immune readiness while preserving mucosal tolerance mechanisms that support healthy microbiome establishment (Płowiec et al. 2015). As demonstrated by Sławińska et al. (Slawinska et al. 2024), a comparable pattern was observed, with gene expression being more pronounced in the spleen than in the cecal tonsils upon prebiotic stimulation *in ovo*.

In relation to the results of the same study presented by Bełdowska et al. (Bełdowska et al. 2024), in which the *IL2, IL12, IL1β* gene signature was detected on cecum mucosa. XOS4 and MOS3 exerted their effects locally, enhancing *IL2, IL12,* and *IL1β* expression in the cecal mucosa, while XOS3 primarily induced systemic immune programming, resulting in elevated *IL1β, IL2*, and *IL12* expression in the spleen. These mucosal effects are presumably the result of microbial interactions occurring in the early post-hatch phase, influenced by prebiotic exposure.

## Conclusions

The *in ovo* administration of xylo- and mannooligosaccharides on embryonic day 12 produced a lasting, tissue-specific immunomodulation that was detectable at 42 days of age. XOS3 predominantly enhanced systemic immune markers in the spleen across Th1, Th2 and Th17 axes. The cecal tonsils demonstrated stability in their transcription, consistent with the tolerance-oriented function of GALT and the presence of significant interindividual variability during the post-hatch maturation process. The results of the present study demonstrate that targeted *in ovo* prebiotic delivery has the capacity to program systemic and mucosal immune compartments differentially, thereby enhancing immune readiness without disrupting mucosal homeostasis. These findings support the potential use of embryonic prebiotic stimulation as a strategy to improve immune competence in broiler production.


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## Competing Interests

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

## Author contributions

All authors contributed to the study conception and design. Conducting *in-ovo* experiment, data collection and analysis were performed by Maria Siwek, Elżbieta Pietrzak and Aleksandra Dunisławska. Supervising the *in-vivo* experiment was done by Maria Siwek. The first draft of the manuscript was written by Maria Siwek, Elżbieta Pietrzak and Aleksandra Dunisławska.

Funding acquisition, supervision, review and editing was by Maria Siwek. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Data Availability

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

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