Influence of Synbiotics Delivered \textit{in ovo} on Immune Organs Development and Structure*

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Prebiotics and probiotics applied alone or together (synbiotics) can influence the intestinal microbiota and modulate the immune response. We analyzed the impact of \textit{in ovo} administration of synbiotics on immune system development in Ross (broiler) and Green-legged Partridgelike (GP, dual-purpose fowl) chickens. For \textit{in ovo} delivery on the 12$^{th}$ day of the eggs incubation, two strains of lactic acid bacteria (LAB) were used, i.e. \textit{Lactococcus lactis} subsp. \textit{lactis} IBB SL1 (S1) and \textit{Lactococcus lactis} subsp. \textit{cremoris} IBB SC1 (S2), combined with raffinose family oligosaccharides (RFO) prebiotic. Other treatments included \textit{in ovo} delivery of commercial synbiotic (S3), RFO prebiotics alone (P) and physiological saline (C). Immune system development was analyzed by relative weight (indices) and histology of the lymphatic organs (bursa of Fabricius, thymus and spleen) at two time points (3$^{rd}$ and 6$^{th}$ week of life). The results indicate that the development of the lymphatic organs was significantly affected by \textit{in ovo} treatment. The bursa and bursa to spleen index was higher in P and S2 groups of broilers (P<0.05) when compared to S3. In GP at the 3$^{rd}$ week of age, the spleen index was significantly higher in S2 (P<0.05). The histological image of the thymus displayed an increase of thymocytes in the cortex in all synbiotic-treated groups (S1, S2, S3). \textit{In ovo} delivery of synbiotics is an efficient mode of immune system stimulation in chickens but its efficiency depends on chicken genotype.

Key words: chickens, synbiotics, immune system, \textit{in ovo} technology.

The gut microbiota and gut-associated lymphoid tissue (GALT) are fundamental components of the both immune and digestive system function and homeostasis. For a long time it was believed that avian species develop a microbiome after hatching. However, PEDROSO (2009) discovered that the chicken embryo intestinal tract is far from sterile and microbiome colonization starts at the 16$^{th}$ day of incubation. This explains the positive effect of RFO (raffinose family oligosaccharides) prebiotic’s \textit{in ovo} inoculation at the 12$^{th}$ day of embryonic development on shaping the microbiome in newly hatched chicks (PILARSKI et al. 2005; VILLALUENGA et al. 2004). The beneficial effects of \textit{in ovo} application of RFOs on the post-hatching development of chickens were already confirmed under field conditions (BEDNARCZYK et al. 2011),

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proving that this mode of delivery can replace the common prebiotics additives in the chicken diet.

Prebiotics and probiotics applied alone or together (synbiotics) can influence the intestinal microbiota and modulate the immune response. Their mode of action includes competitive exclusion that allows for growth of the physiological intestinal microflora and limits pathogens and their toxins (FOOKS & GIBSON 2002; GIBSON et al. 1995). Prebiotics can also directly modulate immunity by interacting with the immune cells receptors and stimulating endocytosis, phagocytosis, respiratory burst as well as production of numerous cytokines and chemokines (reviewed by DI BARTOLOMEO et al. 2013). Probiotics are able to cross the intestinal barrier through intestinal epithelial cells. After being processed and presented to the immune system, they modulate the innate and adaptive responses (DOBSON et al. 2012). Apart from stimulation of the intestinal immune system through the gastrointestinal tract (BRISBIN et al. 2011; HAGHIGHI et al. 2008), probiotics can also affect immune responses in other lymphatic organs through the common mucosal immune system (MALT) (CESTA 2006).

In ovo technology, based on mechanical delivery of substances directly into the incubating egg, is well established for vaccination of 18 day old embryos against multiple infectious agents, including Marek’s disease virus and infectious bursal disease (reviewed by RICKS et al. 1999; WILLIAMS & ZEDEK 2010). Apart from vaccination, in ovo technology has been applied to stimulate embryonic development, to select for the sexual phenotype, to inject genetically modified cells and to stimulate a beneficial bacterial profile, as reviewed by BEDNARCYK et al. (2010). Our group used in ovo technology for RFO prebiotic administration under experimental and field conditions. It was revealed that RFO injection into the air cell during embryogenesis decreased mortality by 50% (PIŁARSKI et al. 2005) increased body weight (BEDNARCYK et al. 2011) and improved meat quality in terms of collagen content (MAIORANO et al. 2012). All of these results clearly show measurable effects of RFO prebiotics injected in ovo.

However, to our best knowledge, this is the first report that provides insight into the effects of in ovo injection of RFO prebiotic enriched with probiotic lactic acid bacteria (LAB), on immune organs development and structure in the hatched chicks. The general aim of the study was to analyze the impact of the RFO prebiotic applied alone or in combination with strictly selected and characterized LAB strains, applied in ovo on the 12th day of chicken embryonic development, on the development of the main lymphatic organs in different chicken types (meat-type, dual-purpose fowl). We also addressed specific issues: (1) does the RFO prebiotic show synergistic effects when applied together with probiotic bacteria? (2) is the effect dependent on the synbiotic used? (in-house developed and characterized vs. randomly selected, commercial synbiotics). Here we present the results of this study.

Material and Methods

Biological material

The experiment was performed on 600 fertilized chicken eggs of two breeds, Ross (meat-type chicken) (300 eggs) and Green-legged Partridge-like (GP) (dual-purpose fowl) (300 eggs). The animals were divided into five experimental groups, denoted as P, S1, S2, S3 and C, based on the substance delivered in ovo during embryonic development. An overview of the experimental groups and in ovo treatment applied is presented in Table 1. Briefly, group P was treated with a solution of RFO prebiotic, prepared according to GULEWICZ et al. (2000). The same RFO prebiotic was used as in previous experimental and field studies. Groups S1 and S2 were treated with two in-house prepared synbiotics which consisted of a mixture of RFOs and a strain of probiotic bacteria, either Lactococ-
Preparation of synbiotics for in ovo injection

Bacterial cultures of both strains of Lactococcus lactis were incubated at 25-28°C under aerobic conditions for 18 hours. Harvested cultures were centrifuged at 7000 x g for 1 min. The bacterial pellet was washed twice and resuspended in a prebiotic solution to a final concentration of 10^5 cfu/ml. In order to provide 10^3 cfu per embryo, 190 μl of prebiotic solution and 10 μl of bacterial suspension (10^5 cfu/ml) were used. The commercial synbiotic was obtained as a lyophilized mixture which was resuspended in distilled water and adjusted to a final concentration of 2.5x10^5 cfu/ml with respect to each strain of bacteria included in the synbiotics. Each embryo was injected with a total volume of 200 μl including 0.5x10^5 cfu of Lactobacillus acidophilus and 0.5x10^5 cfu of Streptococcus faecium (10^5 cfu of LAB in total).

In ovo treatment during embryonic development

The initial stages of the experiment were carried out in a commercial hatchery Drobex Agro sp. z o.o. (Solec Kujawski, Poland). At the first day, 600 hatching eggs were placed into automatic incubators (Petersime, vision) with electronically controlled conditions of egg incubation (temperature 37.8°C and relative humidity 60%). On the 12th day of incubation the eggs were treated with different pre- and synbiotics by in ovo administration. Prior to the injection, the hatching eggs were candled and unfertilized eggs were discarded. An aqueous injection solution at equal volumes of 0.2 ml was delivered manually into the air cell with the use of self-refilling syringes (Socorex, Ecbulens, Switzerland). Experimental groups P, S1 and S2 were injected with 0.2 ml of RFO solution containing 1.9 mg of RFOs per egg. In experimental groups S1 and S2 the RFO solution was enriched with probiotic bacteria as described above. After injection, each hole was sealed with an adhesive tape and incubation of the eggs was continued until hatching. Post hatch treatment of animals and sampling

Chickens were raised for 6 weeks according to the animal welfare recommendations of European Union directive 86/609/EEC in an experimental poultry house that provided good husbandry conditions (e.g. stocking density, litter, ventilation). The birds were grown for six weeks in pens, separate for each experimental group. They were fed and watered ad libitum. Commercial diets were used according to the age of chickens. At three time points (1st day, 3rd and 6th week), a total number of 150 faecal samples were taken (five samples per experimental group in a given time point), using sterile swabs. The chickens were sacrificed at 3 and 6 weeks of age and the immune organs (bursa of Fabricius, thymus and spleen) were taken for measurement and histological analysis. The number of animals sacrificed amounted to five chickens per experimental group in the 3rd week (50 chickens in total) and six chickens per experimental group in the 6th week (60 chickens in total). Measurement of the immune organs was done directly after dissection, and prior to histological analyses the organs were preserved in 10% buffered formalin. All treatments were accepted by the Local Animal Research Ethics Committee at the University of Technology and Life Sciences in Bydgoszcz, Poland.

Bacteriological control of probiotics in the faeces

To confirm the presence of probiotic bacteria strains applied in ovo in the guts of the growing chickens, microbiological analysis was performed at three time points: after hatching, and at 3 and 6 weeks post hatching. The faecal swabs were dispersed in 500ml of physiological saline (0.9% NaCl). The samples were serially diluted to 10^1 and 10^2 dilutions, poured into agar plates and incubated at 25-28°C for 18h under aerobic conditions. Lactococcus lactis subsp. lactis IBB SL1 was grown on GM17 agar (Oxoid, Thebwort, Australia) and Lactococcus lactis subsp. cremoris IBB SC1 – on GM17 agar supplemented with tetracycline (Sigma-Aldrich GmbH, Schnelldorf, Germany) at a concentration of 10 μg/ml. Genomic DNA was isolated from the bacterial colonies (A&A Biotechnology, Gdynia, Poland) and PCR with specific primers was performed to confirm the presence of a given Lactococcus lactis strain. For identification of Lactococcus lactis subsp. lactis SL1 strain 212F (GATGCAATTGCATCACTCAAAG) and 1406R (ACGGGCGGTGTGTRC) primers were used (SALAMA et al. 1991), and for Lactococcus lactis subsp. cremoris IBB SC1 – TetMF (GAYACNCNGGNCAAYTTNGAYTT) and TetMR (ACCGGACGGATTTTCCAC) – encoding tet M gene fragment (GEVERS et al. 2003).
The results were visualized with agarose gel electrophoresis stained with ethidium bromide.

### Immune organ measurement

The immune organs under study included bursa of Fabricius, thymus and spleen. Animals were weighed and dissected post mortem for preparation of the immune organs. Spleen, thymus and bursa of Fabricius were excised and weighed. The data were presented in the form of the relative weight (index) of the given immune organ weight in the total body weight. The indices were calculated as follows: immune organ weight divided by body weight and multiplied by 100%. In the same way, the ratio of the bursa of Fabricius to spleen was calculated. The data were analyzed statistically with the Statistica 7.0 package (StatSoft Inc., Tulsa, OK, USA). Experimental groups were tested for significant differences with the GLM model. For a univariate test of significance, Wilk’s lambda was used and the means were compared with the post hoc Scheffe test.

### Histological analysis of the immune organs

Fragments of the immune organs for histological analyses were fixed in 10% formalin and embedded in paraffin blocks. The samples were cut into sections and stained with hematoxylin and eosin (HE). The histological structure of the organs was analyzed with an Axiophot microscope (Carl Zeiss, Stuttgart, Germany) and MultiScanBase V 14.04 software (Computer Scanning Systems, Warsaw, Poland).

### Results

Colonization of chicken guts with *in ovo* administered *Lactococcus lactis* strain of LAB was controlled at three time points (1st day, 3rd and 6th week) using PCR based on template DNA isolated from chicken faeces. The respective DNA fragments were detected in experimental groups S1 and S2, which proved successful *in ovo* treatment and survivability of *Lactococcus lactis* in the chicken guts. Both *in ovo* injected LAB strains (*Lactococcus lactis* subsp. *lactis* IBB SL1 and *Lactococcus lactis* subsp. *cremoris* IBB SC1) survived in chicken guts throughout the experiment (42 days). Moreover, during passage through the gastrointestinal tract, the LAB strains proliferated and altered their metabolism (expressed by API tests), as a form of adaptation to the environment of the chicken guts (ZYLINSKA, personal communication).

Detailed results of immune organ measurement are presented in Table 2 (Ross) and Table 3 (GP). Briefly, in broiler chickens stimulated *in ovo* with pre- and synbiotics, major effects were observed in the relative weight of bursa of Fabricius and in the bursa to spleen index. At the 6th week of age both values were higher in P and S2 groups (P<0.05) when compared to the S3 group. In GP chickens at the 3rd week of age, the relative spleen weight was significantly higher in S2 (P<0.05) in comparison to the control group (C).

The results of the histological analysis of bursa of Fabricius and thymus are summarized in Table 4 (Ross) and Table 5 and presented in Figure 1. (GP). Briefly, at the 3rd week of age in GP chickens, the histological pattern of bursa of Fabricius showed a delayed involution in all synbiotic-treated groups (S1, S2, S3) in comparison to control and

### Table 2

**Effect of *in ovo* treatment with pre- and synbiotics on immune system organ measurements in broiler chicken (Ross) at 3rd and 6th weeks of age**

<table>
<thead>
<tr>
<th>Group</th>
<th>Trait</th>
<th>Ross, 3rd week</th>
<th>Ross, 6th week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>P S1 S2 S3 RSME P-value</td>
<td>C</td>
</tr>
<tr>
<td>BW</td>
<td>728</td>
<td>683 715 680 700 76 &gt;0.1</td>
<td>2276 2344 2488 2445 2358 229 &gt;0.1</td>
</tr>
<tr>
<td>TI</td>
<td>0.53</td>
<td>0.47 0.53 0.64 0.54 0.14 &gt;0.1</td>
<td>0.46 0.45 0.40 0.49 0.47 0.15 &gt;0.1</td>
</tr>
<tr>
<td>BI</td>
<td>0.24</td>
<td>0.29 0.24 0.27 0.19 0.09 &gt;0.1</td>
<td>0.16 0.24A 0.13 0.23A 0.09B 0.06 &lt;0.001</td>
</tr>
<tr>
<td>SI</td>
<td>0.10</td>
<td>0.10 0.14 0.09 0.10 0.03 &lt;0.05</td>
<td>0.09 0.10 0.14 0.09 0.09 0.03 &lt;0.05</td>
</tr>
<tr>
<td>B/S</td>
<td>2.60</td>
<td>2.99 1.76 3.09 1.91 1.09 &gt;0.1</td>
<td>1.81 2.47A 0.93B 2.43A 1.10 0.78 &lt;0.01</td>
</tr>
</tbody>
</table>

Traits: BW – body weight, TI – thymus index, BI – bursa index, SI – spleen index, B/S – bursa to spleen ratio; Groups (treatments) denoted as in Table 1. Means in the same row that are marked with different values differ significantly at a, b P<0.05 and A, B P<0.01; n=6/group.
prebiotic-treated groups (C, P). Furthermore, all synbiotic-treated groups displayed a higher density of thymocytes in the cortex or medulla of the thymus, in comparison to the control group (C). An increase in lymphocyte density in the cortex was observed in synbiotic-treated groups of 6 week old Ross (S1, S2, S3) and in both time points (3rd and 6th week) in GP chickens (S1, S2, S3).

Table 3
Effect of in ovo treatment with pre- and synbiotics on immune system organ measurements in dual-purpose fowl (GP) at 3rd and 6th weeks of age

<table>
<thead>
<tr>
<th>Group Trait</th>
<th>Trait</th>
<th>GP, 3rd week</th>
<th>GP, 6th week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>P</td>
</tr>
<tr>
<td>BW</td>
<td></td>
<td>150a</td>
<td>177</td>
</tr>
<tr>
<td>TI</td>
<td></td>
<td>0.54</td>
<td>0.49</td>
</tr>
<tr>
<td>BI</td>
<td></td>
<td>0.26</td>
<td>0.30</td>
</tr>
<tr>
<td>SI</td>
<td></td>
<td>0.18a</td>
<td>0.17a</td>
</tr>
<tr>
<td>B/S</td>
<td></td>
<td>1.73</td>
<td>2.16</td>
</tr>
</tbody>
</table>

Table 4
Histological analysis of lymphatic organs in broiler chicken (Ross) stimulated in ovo with pre- and synbiotics

<table>
<thead>
<tr>
<th>Week 3</th>
<th>Bursa of Fabricius</th>
<th>Symbol</th>
<th>Thymus</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Normal lymphocyte density</td>
<td>+</td>
<td>Normal structure and ratio of cortex to medulla</td>
<td>+</td>
</tr>
<tr>
<td>P</td>
<td>Slight lymphocytic depletion in medulla</td>
<td>-</td>
<td>Slightly extended medulla</td>
<td>+</td>
</tr>
<tr>
<td>S1</td>
<td>Normal lymphocyte density</td>
<td>+</td>
<td>High density of thymocytes in the cortex</td>
<td>+++</td>
</tr>
<tr>
<td>S2</td>
<td>Slight lymphocytic depletion in medulla</td>
<td>-</td>
<td>Extended cortex; high density of thymocytes in medulla</td>
<td>+++</td>
</tr>
<tr>
<td>S3</td>
<td>Normal lymphocyte density</td>
<td>+</td>
<td>Extended cortex; high density of thymocytes in medulla</td>
<td>+++</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Week 6</th>
<th>Bursa of Fabricius</th>
<th>Symbol</th>
<th>Thymus</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Slight lymphocytic depletion in medulla</td>
<td>-</td>
<td>Normal structure and ratio of cortex to medulla</td>
<td>+</td>
</tr>
<tr>
<td>P</td>
<td>Lymphocytic depletion in medulla</td>
<td>--</td>
<td>Extended medulla; slight decrease of thymocytes in cortex</td>
<td>-</td>
</tr>
<tr>
<td>S1</td>
<td>Slight lymphocytic depletion in cortex and medulla</td>
<td>-</td>
<td>Extended cortex</td>
<td>+</td>
</tr>
<tr>
<td>S2</td>
<td>Slight lymphocytic depletion in medulla</td>
<td>-</td>
<td>Extended cortex, slight increase of thymocytes in cortex</td>
<td>++</td>
</tr>
<tr>
<td>S3</td>
<td>Slight lymphocytic depletion in medulla</td>
<td>-</td>
<td>Extended medulla, slight increase of thymocytes in cortex</td>
<td>++</td>
</tr>
</tbody>
</table>

1 Minus (–) / plus (+) represents decrease/increase in the cell density. The number of (−)/(+) symbols refers to the magnitude of the change in the cell density in comparison to the control group. Experimental groups denoted as follows (based on in ovo treatment): C – control, physiological saline; P – prebiotic, RFOs; S1 – symbiotic 1, RFOs + L. lactis subsp. lactis; S2 – symbiotic 2, L. lactis subsp. cremoris; S3 – commercial symbiotic.

Traits: BW – body weight, TI – thymus index, BI – bursa index, SI – spleen index, B/S – bursa to spleen ratio; Groups (treatments) denoted as in Table 1. Means in the same row that are marked with different values differ significantly at a, b P<0.05 and A, B P<0.01; n=5/group at 3rd week and n=6/group at 6th week.
The impact of dietary supplementation with pre- and probiotics on the immune system in chickens is well documented (KOENEN et al. 2004; FAR-NELL et al. 2006). Inclusion of probiotics in the diet is expected to mimic the natural situation in which the newly hatched chick is equipped with protective bacteria from its mother’s faeces. To fully imitate this process, external probiotic bacteria should be administered as early as possible (KABIR 2009). We claim that in ovo technology is the best solution for pre/pro/synbiotic delivery since it ensures that the embryo’s gastrointestinal tract is protected as early as from the first hour after hatching. In ovo injection into the air cell of the chicken egg is not only an effective route of delivery, but it also enables further development and hatchability of in ovo treated eggs (COX et al. 1992). We have already proven that in ovo technology works well for prebiotic delivery and effectively improves hatchability (PILARSKI et al. 2005) and body weight (BEDNARCZYK et al. 2011). Moreover, prebiotic properties of RFOs extracted from lupine (Lupinus album L) and applied in ovo are known to stimulate chickens towards proliferation of their natural intestinal microflora, as measured by Bifidobacterium count in the faeces (VILLALUENGA et al. 2004). In this study we went one step further and evaluated the effects of in ovo delivery of symbiotics (a composite of pre- and probiotics) on immune organ development and structure in chickens. In other words, the previously used RFO prebiotic was combined here with two strains of Lactococcus lactis bacteria and applied in ovo.

The results of immune organ development upon in ovo delivery of symbiotics are in concordance with the literature; WILLIS et al. (2007) found that the relative weight of bursa was significantly higher in probiotic-fed broiler chickens at 49 days of age (but not at 21 days of age). There is a strong correlation between the relative size of bursa and the average levels of IgG antibody expression (GLICK et al. 1956; YONASH et al. 2002). KABIR et al. (2004) evaluated the dynamics of probiotics on the immune response of broilers and they reported significantly higher antibody production (P<0.01) in experimental birds as compared to control ones. They also demonstrated that the differences in the weight of spleen and bursa of broilers that were conventionally fed vs. supplemented with probiotics, could be attributed to different levels of antibody production in response to

### Table 5

<table>
<thead>
<tr>
<th>Week 3</th>
<th>Bursa of Fabricius</th>
<th>Symbol</th>
<th>Thymus</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Even lymphocyte density</td>
<td>-</td>
<td>Normal structure and ratio of cortex to medulla</td>
<td>+</td>
</tr>
<tr>
<td>P</td>
<td>Slight lymphocytic depletion in the medulla</td>
<td>-</td>
<td>Slight decrease of thymocytes in the cortex</td>
<td>-</td>
</tr>
<tr>
<td>S1</td>
<td>High, even lymphocyte density</td>
<td>++</td>
<td>Dense packing of thymocytes in the cortex</td>
<td>++</td>
</tr>
<tr>
<td>S2</td>
<td>High, even lymphocyte density</td>
<td>++</td>
<td>Very dense packing of thymocytes in the cortex, extended medulla</td>
<td>+++</td>
</tr>
<tr>
<td>S3</td>
<td>High, even lymphocyte density</td>
<td>++</td>
<td>Very dense packing of thymocytes in the cortex, extended medulla</td>
<td>+++</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Week 6</th>
<th>Bursa of Fabricius</th>
<th>Symbol</th>
<th>Thymus</th>
<th>Symbol</th>
</tr>
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<td>++</td>
<td>Normal structure and ratio of cortex to medulla</td>
<td>+</td>
</tr>
<tr>
<td>P</td>
<td>Even, quite high lymphocyte density</td>
<td>++</td>
<td>Extended medulla, slight increase of thymocytes in the cortex</td>
<td>++</td>
</tr>
<tr>
<td>S1</td>
<td>Slight lymphocytic depletion in cortex and medulla</td>
<td>-</td>
<td>Extended medulla, slight increase of thymocytes in the cortex</td>
<td>++</td>
</tr>
<tr>
<td>S2</td>
<td>Slight lymphocytic depletion in cortex and medulla</td>
<td>-</td>
<td>Extended medulla, high increase of thymocytes in the cortex</td>
<td>+++</td>
</tr>
<tr>
<td>S3</td>
<td>Distinct lymphocytic depletion in medulla</td>
<td>-</td>
<td>Extended medulla, high increase of thymocytes in the cortex</td>
<td>+++</td>
</tr>
</tbody>
</table>

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**Discussion**

The impact of dietary supplementation with pre- and probiotics on the immune system in chickens is well documented (KOENEN et al. 2004; FAR-NELL et al. 2006). Inclusion of probiotics in the diet is expected to mimic the natural situation in which the newly hatched chick is equipped with protective bacteria from its mother’s faeces. To fully imitate this process, external probiotic bacteria should be administered as early as possible (KABIR 2009). We claim that in ovo technology is the best solution for pre/pro/synbiotic delivery since it ensures that the embryo’s gastrointestinal tract is protected as early as from the first hour after hatching. In ovo injection into the air cell of the chicken egg is not only an effective route of delivery, but it also enables further development and hatchability of in ovo treated eggs (COX et al. 1992). We have already proven that in ovo technology works well for prebiotic delivery and effectively improves hatchability (PILARSKI et al. 2005) and body weight (BEDNARCZYK et al. 2011). Moreover, prebiotic properties of RFOs extracted from lupine (Lupinus album L) and applied in ovo are known to stimulate chickens towards proliferation of their natural intestinal microflora, as measured by Bifidobacterium count in the faeces (VILLALUENGA et al. 2004). In this study we went one step further and evaluated the effects of in ovo delivery of symbiotics (a composite of pre- and probiotics) on immune organ development and structure in chickens. In other words, the previously used RFO prebiotic was combined here with two strains of *Lactococcus lactis* bacteria and applied in ovo.

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sheep red blood cells (SRBC). Finally, SATO et al. (2009) found that the relative weights of spleen and bursa of Fabricius in chicks fed the immunobiotic diets were slightly higher than the control values at 1 and 3 days of age, suggesting that the probiotic bacteria used in that study was most effective in neonatal chicks.

In birds, the spleen is a fundamental immune organ since they lack lymph nodes. When chickens hatch, the spleen is a granulocytopoietic organ, but

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Fig. 1. Examples of histological patterns of the lymphoid organs in chickens that underwent in ovo stimulation with pre- and synbiotics: A – the typical chicken bursa of Fabricius showing normal structure and even density of the bursal lymphocytes in cortical and medullar parts of the bursal follicles (Ross, 3rd week, C group) 40x magnification; B – distinct lymphocytic depletion in the cortex and medulla of the bursal follicles (Ross, 6th week, S3 group) 100x magnification; C – slight lymphocytic depletion in the medulla of the bursal follicles (Ross, 6th week, S2 group) 100x magnification; D – A microscopic image of the typical chicken thymus. Dense thymocytes in the cortex (darker color), lower density of thymocytes in the center of the lobules (lighter color). In the center of the lobules few thymic corpuscles (i.e. Hassall’s corpuscles) are visible (GP, 3rd week, C group), 100x magnification; E – the normal structure and ratio of cortex to medulla in the thymus (GP, 3rd week, C group), 40x magnification; F – dense packing of thymocytes in the cortex of the thymus (GP, 3rd week, S2 group), 100x magnification; H&E stain. Experimental groups denoted as follows (based on in ovo treatment): C – control, physiological saline; P – prebiotic, RFOs; S1 – synbiotic 1, RFOs + L.lactis subsp. lactis; S2 – synbiotic 2, L.lactis subsp. cremoris; S3 – commercial synbiotic.
it quickly transforms into a predominant lympho-
cytic organ that serves as a storage site for lympho-
cytes (POWERS 2000). In spleen and other
secondary lymphoid tissues, differentiation of the
immunologically competent T and B cells into anti-
gen specific effector cells is completed (ROSE
1979). The size of the spleen is heritable (JOHN
1994) and it has been proven to be directly cor-
related with the immune response. European starlings
with larger spleens mounted stronger immune re-
response as measured by PHA responsiveness,
which provides direct evidence that larger spleens
harbour a larger amount of resting T-cells that are
mobilized upon exposure to PHA (ARDIA 2005).
Enhanced development of spleen in GP chickens
shows a good responsiveness of their immune sys-
tem to immunomodulatory environmental factors,
such as Lactococcus lactis subsp. cremoris IBB
SC1.

In avian thymus, the cortex contains a popula-
tion of small, immature T lymphocytes, which mi-
grate to medulla during maturation and stay there
(PEARSE 2006). The results of the histological ex-
amination of the thymus samples are in line with
the known impact of probiotics on T cell-mediated
immune responses via activation of dendritic cells
in the guts (CLANCY 2003). A larger density of
thymocytes of the symbiotic-treated animals in
comparison to control ones suggests increased
lymphocyte proliferation in the thymus and activa-
tion of the cellular response. Supporting evidence
was obtained by SATO et al. (2009), who – based
on the gene expression study of GALT in neonatal
chickens fed with immunobiotic LAB – concluded
that the T cell-related immune system was stimu-
lated through TLR signaling.

The impact of in ovo injection of synbiotics on
the immune system of neonatal chickens is indi-
rect. It works through stimulation of microbiome
development in the chicken guts and activation of
the common mucosal system through interaction
with gut antigen-presenting cells to provide opti-
mal protection and regulate immune responses
(CLANCY 2003). GALT of the neonate chickens
contains functionally immature T and B lympho-
cytes. Their function is attained up to two weeks
after hatching (MIYAZAKI et al. 2007). Thus, early
activation of the innate immune responses by im-
munomodulatory probiotics delivered in ovo is
considered crucial for proper maturation of GALT
and attaining overall immunocompetence. How-
ever, activation of the immune system in growing
chickens can lead to growth depression effects and
worse feed utilization (KLASING & KORVER 1997).
Therefore, balance must be maintained between
immune and growth trait stimulation in livestock.
In the light of discoveries of the new functions of
probiotics, CLANCY (2003) introduced a term –
immunobiotics – to define bacteria strains which
modulate mucosal immune mechanisms in con-
trast to probiotics which affect the gastrointestinal
tract only.

Conclusions

In this study we reported that in ovo administration
of synbiotics into the developing chicken em-
bryo is an effective way to provide stimulus for the
immune organs of the growing chickens. Lacto-
coccus lactis probiotics survived in the chicken
guts throughout their lifespan. In-house developed
probiotic bacteria, in combination with RFO pre-
biotic of a known function, displayed better effects
than randomly selected commercial synbiotics
manufactured for oral administration. We ob-
served synergistic effects of the RFO prebiotic and
Lactococcus lactis subsp. cremoris IBB SC1 on
the development of the immune organs, i.e. bursa
of Fabricius (in meat-type chickens) and spleen (in
general-purpose chickens) as well as on lympho-
cyte proliferation in the thymus in both chicken
genotypes. In the light of the results obtained we
suggest that the in ovo administration of selected
synbiotics is a promising approach in chicken im-
mune system enhancement, as it combines merits
of prebiotics and probiotics and by early admini-
stration into the embryo, supports development of
their immune organs.

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