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Influence of Synbiotics Delivered *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 *in ovo* administration of synbiotics on immune system development in Ross (broiler) and Green-legged Partridgelike (GP, dual-purpose fowl) chickens. For *in ovo* delivery on the 12th day of the eggs incubation, two strains of lactic acid bacteria (LAB) were used, i.e. *Lactococcus lactis* subsp. *lactis* IBB SL1 (S1) and *Lactococcus lactis* subsp. *cremoris* IBB SC1 (S2), combined with raffinose family oligosaccharides (RFO) prebiotic. Other treatments included *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 (3rd and 6th week of life). The results indicate that the development of the lymphatic organs was significantly affected by *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 3rd 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). *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, *in ovo* technology.

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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 16th day of incubation. This explains the positive effect of RFO (raffinose family oligosaccharides) prebiotic's *in ovo* inoculation at the 12th day of embryonic development on shaping the microbiome in newly hatched chicks (PILARSKI *et al.* 2005; VILLALUENGA *et al.* 2004). The beneficial effects of *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 BEDNARCZYK *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% (PILARSKI *et al.* 2005) increased body weight (BEDNARCZYK *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-*

Table 1

Overview of the chicken experimental groups based on *in ovo* treatment

Group	Category	Content	Type
C	Control	Physiological salt	Commercial
P	Prebiotic	RFOs[a]	In-house
S1	Synbiotic	RFOs + *Lactococcus lactis* IBB SL1	In-house
S2	Synbiotic	RFOs + *Lactococcus lactis* IBB SC1	In-house
S3	Synbiotic	Lactose + *Lactobacillus acidophilus, Streptococcus faecium*	Commercial

[a] RFOs dissolved in physiological saline (0.9% NaCl)

cus lactis subsp. *lactis* IBB SL1 (S1) or *Lactococcus lactis* subsp. *cremoris* IBB SC1 (S2), both developed in the Institute of Biochemistry and Biophysics in Warsaw (Polish Academy of Sciences). Group S3 received (arbitrarily selected) commercially available synbiotic, used typically as a food supplement in poultry, containing: lactose up to 1 g, *Lactobacillus acidophilus* 10^9 cfu, *Streptococcus faecium* 10^9 cfu (in 100g). C was a control group, and it was treated with an adequate volume of physiological saline (0.9% NaCl).

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 xg 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 $2.5x10^3$ 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 $0.5x10^3$ cfu of *Lactobacillus acidophilus* and $0.5x10^3$ cfu of S*treptococcus faecium* (10^3 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, Ecublens, 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 25-28ºC for 18h under aerobic conditions. *Lactococcus lactis* subsp. *lactis* IBB SL1 was grown on GM17 agar (Oxoid, Thebarton, 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* SL1strain 212F (GATGCAATTGCATCACTCAAAG) and 1406R (ACGGGCGGTGTGTRC) primers were used (SALAMA *et al.* 1991), and for *Lactococcus lactis* subsp. *cremoris* IBB SC1 – TetMF (GAYACNCCNGGNCAYRTNGAYTT) and TetMR (CACCGAGCAGGGATTTCTCCAC) – 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 (ŻYLINSKA, 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

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

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.

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

Group / Trait	GP, 3rd week							GP, 6th week						
	C	P	S1	S2	S3	RSME	P- value	C	P	S1	S2	S3	RSME	*P*-value
BW	150[a]	177	183[b]	182	183[b]	15	<0.01	447	380[a]	517[b]	387	429	70	<0.05
TI	0.54	0.49	0.43	0.55	0.62	0.19	>0.1	0.55	0.75	0.69	0.72	0.75	0.17	>0.1
BI	0.26	0.30	0.36	0.34	0.31	0.09	>0.1	0.26	0.28	0.35	0.34	0.31	0.06	<0.05
SI	0.18[a]	0.17[a]	0.19[a]	0.25[b]	0.19[a]	0.03	<0.01	0.23	0.19	0.24	0.26	0.23	0.04	>0.05
B/S	1.73	2.16	2.34	1.51	1.78	0.51	>0.1	1.15	1.50	1.49	1.51	1.46	0.31	>0.1

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.

Table 4

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

Week 3	Bursa of Fabricius	Symbol[1]	Thymus	Symbol[1]
C	Normal lymphocyte density	+	Normal structure and ratio of cortex to medulla	+
P	Slight lymphocytic depletion in medulla	-	Slightly extended medulla	+
S1	Normal lymphocyte density	+	High density of thymocytes in the cortex	+++
S2	Slight lymphocytic depletion in medulla	-	Extended cortex; high density of thymocytes in medulla	+++
S3	Normal lymphocyte density	+	Extended cortex; high density of thymocytes in medulla	+++
Week 6	**Bursa of Fabricius**	**Symbol[1]**	**Thymus**	**Symbol[1]**
C	Slight lymphocytic depletion in medulla	-	Normal structure and ratio of cortex to medulla	+
P	Lymphocytic depletion in medulla	--	Extended medulla; slight decrease of thymocytes in cortex	-
S1	Slight lymphocytic depletion in cortex and medulla	-	Extended cortex	+
S2	Slight lymphocytic depletion in medulla	-	Extended cortex, slight increase of thymocytes in cortex	++
S3	Slight lymphocytic depletion in medulla	-	Extended medulla, slight increase of thymocytes in cortex	++

[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 – synbiotic 1, RFOs + *L.lactis* subsp. *lactis*; S2 – synbiotic 2, *L.lactis* subsp. *cremoris*; S3 – commercial synbiotic.

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 5

Histological analysis of lymphatic organs in general-purpose chicken (GP) stimulated *in ovo* with pre- and synbiotics

Week 3	Bursa of Fabricius	Symbol[1]	Thymus	Symbol[1]
C	Even lymphocyte density	+	Normal structure and ratio of cortex to medulla	+
P	Slight lymphocytic depletion in the medulla	-	Slight decrease of thymocytes in the cortex	-
S1	High, even lymphocyte density	++	Dense packing of thymocytes in the cortex	++
S2	High, even lymphocyte density	++	Very dense packing of thymocytes in the cortex, extended medulla	+++
S3	High, even lymphocyte density	++	Very dense packing of thymocytes in the cortex, extended medulla	+++
Week 6	**Bursa of Fabricius**	**Symbol[1]**	**Thymus**	**Symbol[1]**
C	Even, quite high lymphocyte density	++	Normal structure and ratio of cortex to medulla	+
P	Even, quite high lymphocyte density	++	Extended medulla, slight increase of thymocytes in the cortex	++
S1	Slight lymphocytic depletion in cortex and medulla	-	Extended medulla, slight increase of thymocytes in the cortex	++
S2	Slight lymphocytic depletion in cortex and medulla	-	Extended medulla, high increase of thymocytes in the cortex	+++
S3	Distinct lymphocytic depletion in medulla	- -	Extended medulla, high increase of thymocytes in the cortex	+++

[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 – synbiotic 1, RFOs + *L.lactis* subsp. *lactis*; S2 – synbiotic 2, *L.lactis* subsp. *cremoris*; S3 – commercial synbiotic.

Discussion

The impact of dietary supplementation with pre- and probiotics on the immune system in chickens is well documented (KOENEN *et al.* 2004; FARNELL *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 track 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 synbiotics (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 synbiotics 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

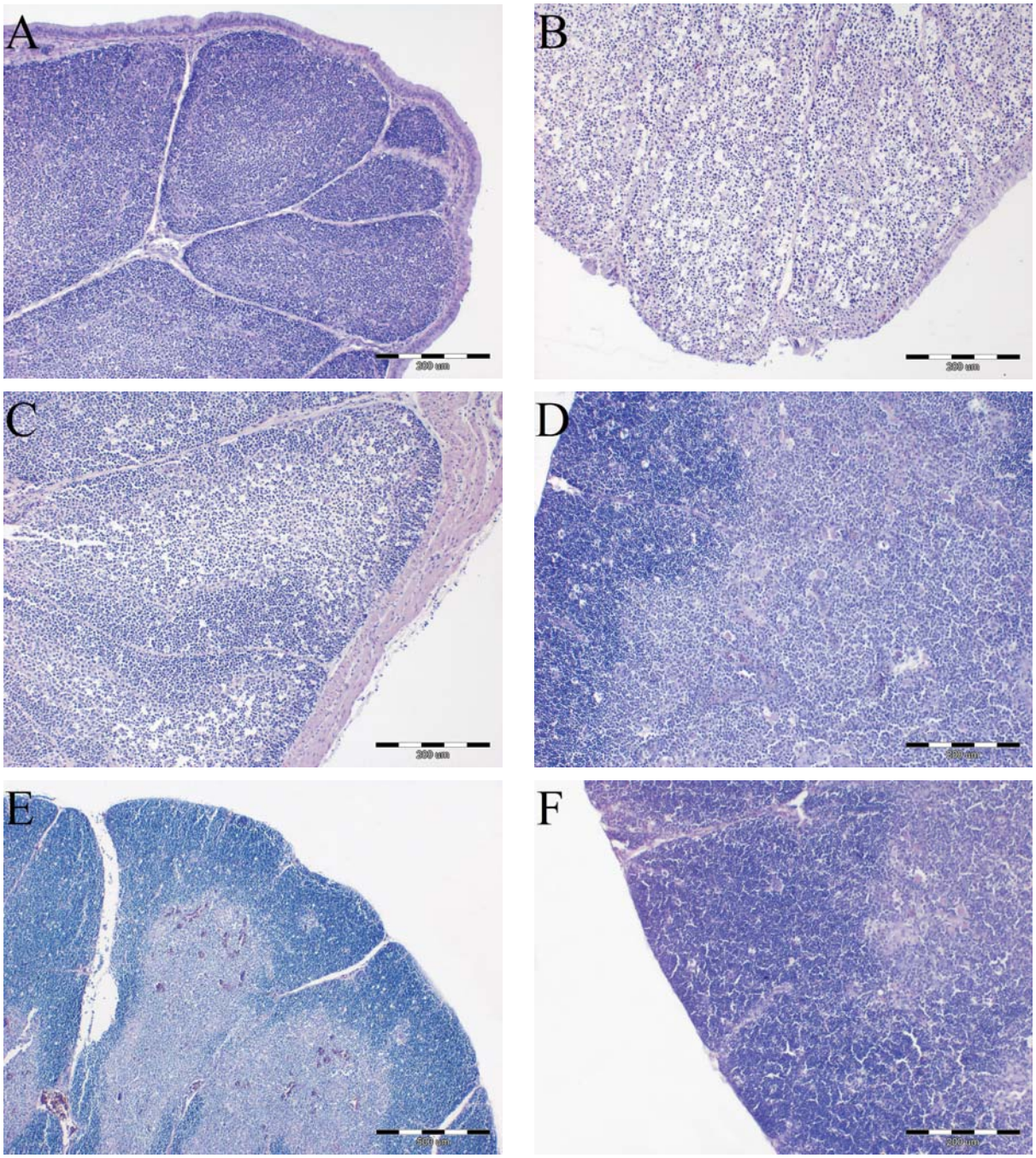

Fig. 1. Examples of histological patterns of the lymphoid organs in chickens that underwent *in ovo* stimulation with pre- and synbiotics: A – the typical chicken bursa of Fabricius showing normal structure and even density of the bursal lymphocytes in cortical and medullar parts of the bursal follicles (Ross, 3rd week, C group) 40× magnification; B – distinct lymphocytic depletion in the cortex and medulla of the bursal follicles (Ross, 6th week, S3 group) 100× magnification; C – slight lymphocytic depletion in the medulla of the bursal follicles (Ross, 6th week, S2 group) 100× 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), 100× magnification; E – the normal structure and ratio of cortex to medulla in the thymus (GP, 3rd week, C group), 40× magnification; F – dense packing of thymocytes in the cortex of the thymus (GP, 3rd week, S2 group), 100× 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.

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

it quickly transforms into a predominant lymphocytic organ that serves as a storage site for lymphocytes (POWERS 2000). In spleen and other secondary lymphoid tissues, differentiation of the immunologically competent T and B cells into antigen specific effector cells is completed (ROSE 1979). The size of the spleen is heritable (JOHN 1994) and it has been proven to be directly correlated with the immune response. European starlings with larger spleens mounted stronger immune 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 system to immunomodulatory environmental factors, such as *Lactococcus lactis* subsp. *cremoris* IBB SC1.

In avian thymus, the cortex contains a population of small, immature T lymphocytes, which migrate to medulla during maturation and stay there (PEARSE 2006). The results of the histological examination 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 synbiotic-treated animals in comparison to control ones suggests increased lymphocyte proliferation in the thymus and activation 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 stimulated through TLR signaling.

The impact of *in ovo* injection of synbiotics on the immune system of neonatal chickens is indirect. 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 optimal protection and regulate immune responses (CLANCY 2003). GALT of the neonate chickens contains functionally immature T and B lymphocytes. Their function is attained up to two weeks after hatching (MIYAZAKI *et al.* 2007). Thus, early activation of the innate immune responses by immunomodulatory probiotics delivered *in ovo* is considered crucial for proper maturation of GALT and attaining overall immunocompetence. However, 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 contrast to probiotics which affect the gastrointestinal tract only.

Conclusions

In this study we reported that *in ovo* administration of synbiotics into the developing chicken embryo is an effective way to provide stimulus for the immune organs of the growing chickens. *Lactococcus lactis* probiotics survived in the chicken guts throughout their lifespan. In-house developed probiotic bacteria, in combination with RFO prebiotic of a known function, displayed better effects than randomly selected commercial synbiotics manufactured for oral administration. We observed 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 lymphocyte 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 immune system enhancement, as it combines merits of prebiotics and probiotics and by early administration into the embryo, supports development of their immune organs.

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