In ovo injection of prebiotics and synbiotics affects the digestive potency of the pancreas in growing chickens

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ABSTRACT The purpose of the study was to examine the effect of 2 prebiotics and 2 synbiotics on the digestive potency of pancreas in 1-, 3-, 7-, 14-, 21-, and 34-day-old cockerels. Prebiotics (inulin and Bi^{2}tos) and synbiotics (inulin + *Lactococcus lactis* subsp. *lactis* and Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris*) were injected in ovo into the air cell on the 12th d embryonic development. Their application increased the activity of amylase, lipase, and trypsin in the pancreas. The most pronounced changes were observed at the end of the investigated rearing period (d 34). The strongest stimulative effects on amylase were shown by both synbiotics, on lipase synbiotic Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris*, and on trypsin all the used prebiotics and synbiotics. Simultaneously, neither the absolute nor the relative mass of the pancreas in comparison to control group were changed. Also, the injected in ovo compounds did not cause a deterioration in the posthatching condition of the chicken liver, as determined by measurement of the activity of marker enzymes in the blood (alanine aminotransferase and aspartate aminotransferase). Treatment with the prebiotics and synbiotics did not change the feed conversion ratio but Bi^{2}tos (galacto-oligosaccharide) and inulin (fructan) + *Lactococcus lactis* subsp. *lactis* significantly increased final BW.

Key words: prebiotic, synbiotic, in ovo, broiler chicken, pancreas enzymatic activity

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INTRODUCTION

Since 2006, when the European Union banned the use of antibiotic growth promoters in poultry nutrition, many alternatives have been investigated to replace antimicrobials without any loss of productivity or negative influence on health.

One possible way to solve this problem is to control the microbiota of the gastrointestinal tract. Two kinds of feed additives are of interest. The first method is the direct introduction of live bacteria into the digestive tract, the second is the creation of conditions for the development of beneficial bacteria. Prebiotics have been utilized to improve chicken performance via direct impact on the microflora of host animals and in this way effect a reinforcement of the intestinal mucosal barrier against deleterious agents (Fioramonti et al., 2003). Prebiotics (mostly fructans, e.g., inulin and fructooligosaccharides, but also raffinose family oligosaccharides and galactooligosaccharides) selectively stimulate the growth of beneficial gut microbiota, especially bifidobacteria and lactobacteria (Roberfroid, 2001; Villaluenga et al., 2004; Baffoni et al., 2012).

The beneficial bacteria inhabit the intestines of chickens immediately after hatching. In the wild, this process occurs through contact with maternal feces (Kabir et al., 2004). In artificial hatching, the settlement of the intestinal beneficial bacteria is delayed, even if this process is induced by feed additives. Therefore, direct administration of the given substance into the egg has been used and the results are promising (Villaluenga et al., 2004; Pilarski et al., 2005; Maiorano et al., 2012; Sławinska et al., 2014). Compared to dietary prebiotic inclusion, in ovo injection increases the population of beneficial microflora on the day of hatch, and leads to a high and stable level of Biffidobacteria throughout the broiler chickens growing period (Villaluenga et al., 2004). Also, the substances injected in a very low doses are effective compared to administration of antibiotics in the diet (Bednarczyk et al., 2011).

Some studies have indicated that the best option to potentiate this effect is to combine prebiotics and probiotics (Sławińska et al., 2014). Such a mixture can have

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a beneficial effect both by improving survival and implantation of live microbial dietary supplements and by affecting the microflora which is already present in the gastrointestinal tract (Gibson and Roberfroid, 1995). Synergistic effects of prebiotics and probiotics can be useful in improving gut health, which in turn may increase feed utilization, and thus affect growth.

Improving the health status of the intestine may also be reflected in the general metabolism. Fructans supplementation of the diet for broilers results in a decrease in body fat deposition (Ammerman et al., 1989) and serum cholesterol concentration (Velasco et al., 2010). Dietary inulin supplementation has a beneficial effect on blood serum lipids by decreasing triglyceride concentrations and increasing the capacity of sunflower oil for enhancing the polyunsaturated fatty acid to saturated acid ratio of intramuscular fat in broilers (Velasco et al., 2010). Although it is difficult to specify the mechanisms linking the microbiota of the gastrointestinal tract with the metabolic changes mentioned above, it must be assumed that there is crosstalk between gut microbiome and systemic metabolism. Pre-, pro-, and synbiotics in mice reduce lipogenesis in liver and kidney, hepatic glycogen and glutamine, and adrenal ascorbate which proves that many metabolic processes are under symbiotic homeostatic control (Martin et al., 2009).

One of the most important factors determining food efficiency is the activity of pancreatic enzymes. Xu et al. (2003) observed that a diet for broilers supplemented with fructooligosaccharide changed the protease, lipase, and amylase activity in the small intestinal digesta. They suggest that the increase in enzyme activity is related to the delivery of additional portions of the enzyme by intestinal bacteria. However, the possibility cannot be excluded that the observed changes are also the result of an increase in the activity of the exocrine pancreas. Thus, the purpose of this study was to examine how the in ovo application of 2 prebiotics and prebiotics together with probiotics (synbiotics) can imprint the enzymatic potency of the pancreas during the fattening period. Additionally, we performed a study if the changes in enzymatic activity may influence the production performance of broilers.

MATERIALS AND METHODS

The study had the approval of the Polish Local Ethical Commission (Nos. 22/2012 and 21.06.2012) and was undertaken in accordance with the animal welfare recommendations of European Union Directive 125 86/609/EEC.

Treatment in ovo

Eggs of Ross 308 hens (ca 60 g), collected from the same 32-weeks-old breeder flock, were incubated at a commercial hatchery (Drobex, Solec Kujawski, Poland) in a Petersime incubator. On d 12, both infertile eggs and those with dead embryos were discarded. Those eggs containing live embryos were randomly allotted to 5 groups (1,800 eggs/group), and treated in ovo with pre- and synbiotic solution. The procedure of in ovo injection was performed with the use of a dedicated automatic system (Bednarczyk et al., 2011). The control group (Group C) was injected with physiological saline. The prebiotic groups (Groups PI and PB) were injected with solutions of either inulin (1.76 mg) (Sigma–Aldrich, St. Louis, MO) and Bi^{2}tos (0.528 mg) (Clasado Ltd), nondigestive transgalacto-oligosaccharides, respectively. The synbiotic groups (Groups SI and SB) were injected with 1.76 mg inulin or 0.528 mg Bi^{2}tos, respectively, enriched with different probiotic bacteria. Group SI received 1,000 cfu *L. lactis* ssp. *lactis* IBB SL1 and group SB received 1,000 cfu *L. lactis* ssp. *cremoris* IBB SC1. These synbiotics were selected on the basis of previous trials (Bednarczyk et al., 2013; Slawinska et al., 2015).

Animals

After hatch, 3,250 cockerels (initial BW ca 42.0 g) were chosen for rearing and physiological investigations at the study farm of the University of Warmia and Mazury in Olsztyn (Poland). The rearing conditions including diet formulation and feeding periods were consistent with Ross broiler management recommendations. The chickens were housed in pens (3.75 m^2) on litter, ans initial (at d 1) density was 17.33 birds per 1 m^2. The study was performed in 10 repetitions within groups. Animals were fed, ad libitum, standard commercial fodders obtained from Agrocentrum (Kałęczyn, Poland): starter (d 1 to 14), grower (d 15 to 30), and finisher (d 31 to 34). The detailed composition of diets is shown in Table 1. During the rearing period, the BW of all cockerels (d 14 and 34) was checked and feed intake was monitored.

Tissue Sampling for Physiological Measurements

Birds were chosen randomly for physiological investigations. On days 1, 3, 7, 14, 21, and 34, 10 cockerels were slaughtered by cervical dislocation, blood was collected in vials, and blood serum obtained by centrifugation. Immediately after blood collection, the whole pancreas from each bird was cut out and frozen in liquid nitrogen. Pancreases and blood serum were stored at −80°C until being assayed.

Analyses of Pancreatic Enzymes

Frozen pancreases were weighed and next briefly homogenized in ice in an appropriate volume of Tris Buffered Saline (TBS) to achieve 20% homogenates. The obtained homogenates were next centrifuged

Table 1. Composition and calculated analysis of diets (grams/kilogram as-fed basis).

	Starter 1 to 14 d	Grower 15 to 30 d	Finisher 31 to 34 d
Ingredient			
Wheat	267.3	291.9	306.6
Maize	300.0	300.0	300.0
Soybean meal	325.0	282.0	253.3
Rapeseed	50.0	60.0	70.0
Soybean oil	21.0	13.3	18.0
Lard	-	20.0	25.0
Salt	3.0	3.0	2.8
Limestone	10.9	9.5	8.5
Monocalcium phosphate	11.5	9.4	6.3
DL-Methionine 99	2.5	1.8	1.3
L-Lysine 78	3.2	3.2	2.7
L-Threonine	0.6	0.9	0.5
Vitamin–mineral premix[1]	5.0	5.0	5.0
Calculated nutrient level[2]			
AME, kcal/kg	2,980	3,100	3,200
CP	220.0	205.0	195.0
Crude fat	60.9	77.0	90.4
Lysine	13.5	12.5	11.5
Methionine + cysteine	9.5	8.5	7.8
Calcium	9.0	8.0	7.0
Available P	4.0	3.5	2.8
Na	1.4	1.4	1.3

[1]Supplied the following per kilogram diets: vitamin A 12,500 IU, vitamin D_3 4,500 IU, vitamin E 45 mg, vitamin K_3 3 mg, vitamin B_1 3 mg, vitamin B_2 6 mg, vitamin B_6 4 mg, pantothenic acid 14 mg, nicotinic acid 50 mg, folic acid 1.75 mg, choline 1.6 g, vitamin B_{12} 0.02 mg, biotin 0.2 mg, Fe 50 mg, Mn 120 mg, Zn 100 mg, Cu 15 mg, I 1.2 mg, Se 0.3 mg, fitase 500 FTU, diclazuril 1 mg (only in starter and grower diets).

[2]Estimation based on the Polish feedstuff analysis tables (Smulikowska and Rutkowski, 2005).

(10,000 × *g* for 30 min at 4°C). For investigation of lipase, supernatants were diluted 100 times using TBS and lipase activity measured with a lipase activity colorimetric assay kit (BioVision, Milpitas, United States). For investigation of amylase, supernatants were diluted 1,000 times with commercially supplied buffer and amylase activity measured with an amylase activity colorimetric assay kit (BioVision, Milpitas, United States). Trypsin activity was measured using a trypsin activity colorimetric assay kit (BioVision, Milpitas, United States). Supernatants were diluted 100 times using TBS and incubated (30 min at 37°C) with 1% enterokinase (Sigma–Aldrich, St. Louis, MO); diluted in 0.1 M Tris-HCl with 0.1 M $CaCl_2$, pH 7.2) to convert trypsinogen.

The incubation temperature was as described in manuals; −25°C for amylase and trypsin, and 37°C for lipase. The results of enzyme activity were calculated as amounts of glycerol (lipase), nitrophenol (amylase), and *p*-nitroaniline (trypsin) released from substrates and expressed per minute/whole pancreas.

Analyses of Blood Serum Enzymes

The activities of alanine aminotransferase (**ALT**) and aspartate aminotransferase (**AST**) were measured using commercial Pointe Scientific kits (Canton, United States) and are expressed in IU.

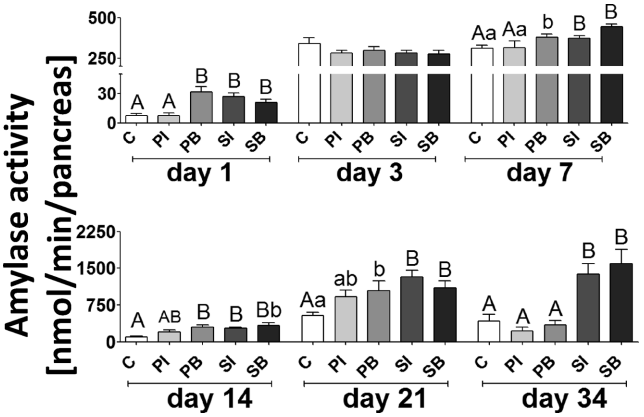

Figure 1. The influence of prebiotics and synbiotics on total activity of pancreatic amylase. C, Control; PI, Prebiotic 1 (inulin); PB, Prebiotic 2 (Bi^2tos); SI, Synbiotic 1 (inulin + *Lactococcus lactis* ssp. *lactis*), SB−Synbiotic 2 (Bi^2tos + *Lactococcus lactis* ssp. *cremoris*). Values presented are means and SEM for n = 10; the statistically significant differences between means are marked for $P < 0.05$ (different small letters) and $P < 0.01$ (different capital letters).

Statistical Analysis

All data were analyzed using one-way ANOVA (SPSS Inc., 2010). The means were compared using the Duncan's multiple range test. Data are presented as means ± SEM, and a value of $P < 0.05$ was considered statistically significant.

RESULTS

During the period from hatching to adulthood, we have noticed an elevation of the total activity of the intrapancreatic enzymes (activity counted per pancreas) involved in food digestion. In ovo treatment with the applied bioactive substances additionally elevated the total activity of hydrolases stored in the pancreas.

The influence of prebiotics and synbiotics on total amylase activity (Figure 1) was well-pronounced on d 1, 7, 14, 21, and 34. The positive effect of inulin (PI) was visible only in a single day (21st), whereas Bi^2tos (PB) enhanced the total activity on d 1 and between d 7 and 21. Simultaneously, alterations caused by both used synbiotics (SI and SB) lasted longer, up to the 34th d life. The exception was the third day in which there were no deviations between groups.

In the case of lipase (Figure 2), statistically significant changes started from d 3. On that day the influence of in ovo treatment with PB and both synbiotics could be seen. After d 3, a statistically significant influence of both prebiotics was noticed only on the 34th d life. Changes caused by the prebiotics were, however, rather mild and even statistically important differences between mean values were below 11%. In comparison, the effects of in ovo treatment with synbiotics were strongly emphasized. On d 3 and 7 after hatching the effects of both synbiotics were visible. Afterward, on d 21 and 34 imprinting with SB definitely dominates.

Also, total trypsin activity, similar to amylase and lipase, was altered by prebiotics and synbiotics

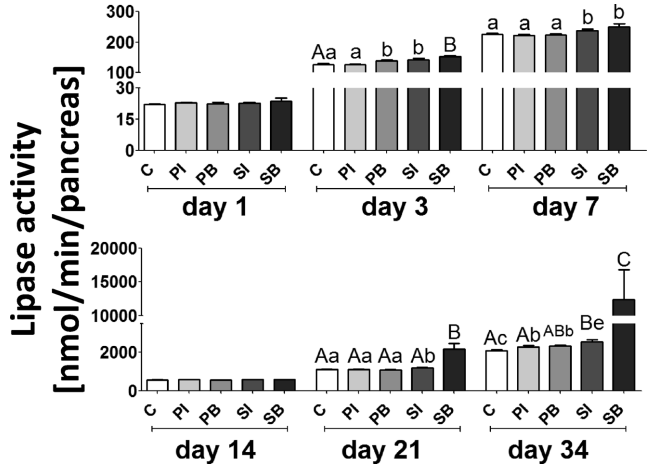

Figure 2. The influence of prebiotics and synbiotics on total activity of pancreatic lipase. C, Control; PI, Prebiotic 1 (inulin); PB, Prebiotic 2 (Bi^2tos); SI, Synbiotic 1 (inulin + *Lactococcus lactis* ssp. *lactis*); SB, Synbiotic 2 (Bi^2tos + *Lactococcus lactis* ssp. *cremoris*). Values presented are means and SEM for n = 10; the statistically significant differences between means marked for $P < 0.05$ (different small letters) and $P < 0.01$ (different capital letters).

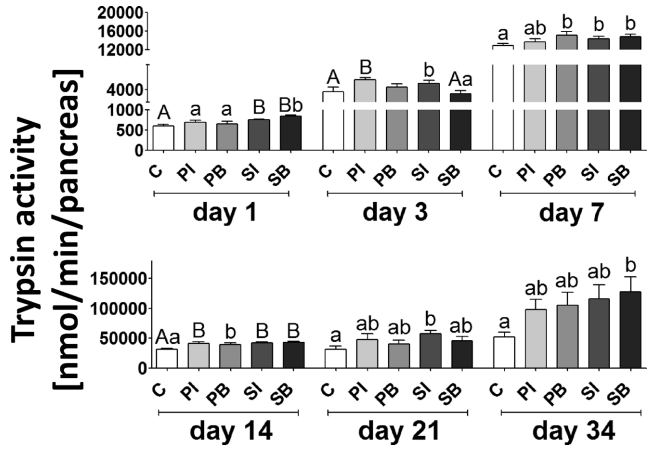

Figure 3. The influence of prebiotics and synbiotics on total activity of pancreatic trypsin. C, Control; PI, Prebiotic 1 (inulin); PB, Prebiotic 2 (Bi^2tos); SI, Synbiotic 1 (inulin + *Lactococcus lactis* ssp. *lactis*); SB, Synbiotic 2 (Bi^2tos + *Lactococcus lactis* ssp. *cremoris*). Values presented are means and SEM for n = 10; the statistically significant differences between means are marked for $P < 0.05$ (different small letters) and $P < 0.01$ (different capital letters).

(Figure 3) almost during the whole posthatching time. The elevation was particularly pronounced at the end of the rearing period (d 34). Groups receiving bioactive compounds showed the values at levels higher than those for the control: PI almost 90%, PB over 100%, SI over 120%, and SB over 140%.

The observed increased digestive potency of pancreas after pre- and synbiotics treatment was not the effect of the elevated weight of this gland. During the whole investigated period the bioactive substances significantly altered neither the absolute nor the relative weight of the pancreas (Table 2). While causing an elevation of pancreatic enzyme activity, none of the pre- and synbiotics changed the feed conversion ratio (**FCR**) or decreased chickens' BW. During the first

Table 2. Absolute weight (AW, mg), relative weight (RW, mg/100 g BW) chickens' pancreas[1], final BW (g)[2], and feed conversion ratio (FCR, kg/kg).[2,3]

Group/item	1 d	3 d	7 d	14 d	21 d	34 d	BW[2]	FCR[2]
C, Control							2.064 ± 0.213[a]	1.569 ± 0.033
AW	56.1 ± 4.0	319.7 ± 12.4	538.3 ± 31.0	1,397.3 ± 93.9	2,952.3 ± 206.5[a,b]	4,637.2 ± 315.0		
RW	133.7 ± 10.2	392.1 ± 27.5	406.3 ± 23.5	406.4 ± 26.8	329.6 ± 16.2	185.7 ± 11.1		
PI, Prebiotic 1 (inulin)							2.061 ± 0.229[a]	1.554 ± 0.038
AW	57.2 ± 6.4	318.2 ± 16.2	497.6 ± 43.0	1,460.7 ± 86.2	2,581.2 ± 111.6[a]	4,451.3 ± 257.1		
RW	137.6 ± 14.4	416.3 ± 19.8	391.3 ± 34.7	443.9 ± 30.4	304.1 ± 6.9	176.2 ± 13.2		
PB, Prebiotic 2 (Bi^2tos)							2.140 ± 0.232[b]	1.557 ± 0.038
AW	57.2 ± 5.0	312.7 ± 18.3	572.5 ± 48.5	1,378.7 ± 66.1	3,144.8 ± 174.0[b]	4,273.0 ± 170.2		
RW	142.2 ± 11.3	436.4 ± 31.8	434.4 ± 41.0	389.3 ± 20.5	339.4 ± 16.5	167.4 ± 9.4		
SI, Synbiotic 1 (inulin + *Lactococcus lactis* ssp. *lactis*)							2.120 ± 0.240[b]	1.566 ± 0.048
AW	54.6 ± 5.3	284.2 ± 22.3	557.7 ± 34.2	1,319.8 ± 105.8	2,618.7 ± 150.3[a]	4,390.4 ± 233.9		
RW	141.9 ± 13.4	379.2 ± 30.6	401.9 ± 20.9	401.3 ± 21.9	286.9 ± 26.6	195.8 ± 9.3		
SB, Synbiotic 2 (Bi^2tos + *Lactococcus lactis* ssp. *cremoris*)							2.093 ± 0.248[a,b]	1.561 ± 0.031
AW	55.7 ± 4.0	288.4 ± 10.6	542.8 ± 39.0	1,337.3 ± 82.2	2,653.7 ± 110.4[a]	4,184.3 ± 127.3		
RW	136.2 ± 10.7	325.3 ± 31.5	418.7 ± 26.3	403.1 ± 23.0	289.6 ± 10.6	176.4 ± 11.0		

[1]Values are means and SEM; n = 10.

[2]BW measured on d 34, FCR measured d day 1 to 34. Values are means and SEM for 442 (C), 434 (PI), 436 (PB), 439 (SI), and 435 (SB) animals.

[3]Saline and dissolved in saline compounds were injected in ovo on 12th d incubation.

[a,b]Statistically significant differences (vertically) are marked for $P < 0.05$.

Table 3. Serum activity of alanine (ALT) and aspartate aminotransferase (AST) in broiler chickens (IU/L).[1]

ALT	C	PI	PB	SI	SB
d 1	4.00 ± 0.30	3.93 ± 0.65	4.55 ± 0.79	3.89 ± 0.41	3.45 ± 0.48
d 3	9.84 ± 1.32	12.11 ± 0.99	13.33 ± 0.93	12.57 ± 1.35	13.87 ± 1.45
d 7	8.91 ± 0.42^{B,b}	8.46 ± 0.49^{b,d}	5.15 ± 0.71^{A,a,e}	6.74 ± 0.75^{a,d}	4.69 ± 0.68^{A,b,c}
d 14	2.53 ± 0.56	2.56 ± 0.33	1.64 ± 0.31	2.76 ± 0.41	2.92 ± 0.51
d 21	4.58 ± 0.46^{B}	3.74 ± 0.50	4.18 ± 0.41	2.85 ± 0.14^{A}	3.01 ± 0.53^{A}
d 34	2.65 ± 0.42^{b}	1.79 ± 0.49^{a,b}	1.55 ± 0.40^{a}	1.35 ± 0.31^{a}	1.72 ± 0.30^{a}
AST	C	PI	PB	SI	SB
d 1	29.72 ± 2.87^{a}	27.63 ± 1.30^{a}	32.75 ± 1.91^{a,b}	37.81 ± 2.60^{b}	33.00 ± 1.44^{a,b}
d 3	26.19 ± 1.95^{a}	29.57 ± 1.69^{a,b}	29.88 ± 0.75^{a,b}	27.87 ± 1.59^{a,b}	32.55 ± 2.02^{b}
d 7	37.99 ± 1.48	35.38 ± 1.05	39.60 ± 1.55	37.66 ± 1.59	39.80 ± 3.52
d 14	29.66 ± 1.62	33.08 ± 1.35	27.40 ± 1.21	29.55 ± 1.35	30.50 ± 0.98
d 21	37.19 ± 1.89^{a,b}	35.60 ± 2.22^{a}	33.68 ± 1.93^{a}	33.33 ± 1.84^{a}	42.70 ± 3.25^{b}
d 34	42.37 ± 2.44^{b}	39.69 ± 3.33^{a,b}	46.98 ± 4.82^{b}	35.85 ± 1.55^{a}	35.23 ± 1.11^{a}

[1]Saline and dissolved in saline compounds were injected *in* ovo on 12th d incubation. C, Control; PI, Prebiotic 1 (inulin); PB, Prebiotic 2 (Bi^2tos); SI, Synbiotic 1 (inulin + *Lactococcus lactis* ssp. *lactis*); SB, Synbiotic 2 (Bi^2tos + *Lactococcus lactis* ssp. *cremoris*). Values presented are means and SEM for n = 10.

[A,B]Statistically significant differences (horizontally) are marked for $P < 0.01$.

[a–e]Statistically significant differences (horizontally) are marked for $P < 0.05$.

phase of the study (d 14) no statistical differences were noticed for BW (Control group−0.473 ± 0.009 g; PI group−0.464 ± 0.012 g; PB group−0.478 ± 0.007 g; SI group−0.475 ± 0.011g; SB group−0.472 ± 0.011 g). However, 2 of the in ovo used bioactive additives (PB and SI) significantly increased the final weight (d 34; Table 2). Simultaneously, some of the additives, especially PB and SI caused slight elevation (statistically not significant) of the mean daily feed intake per bird (d 1 to 34; Control group, 91.08 ± 2.32 g; PI group, 89.71 ± 2.02 g; PB group, 93.07 ± 2.36 g; SI group, 93.22 ± 2.81g; SB group, 92.043 ± 3.80 g).

Generally, no significant changes were noticed in the activity of blood serum ALT or AST, which could be regarded as indicators of liver function disturbances (Table 3). Some statistically significant elevations in comparison to the control were noticed only on d 1 (AST: SI group) and on d 3 (AST: SB group). More often, a statistically significant decrease in activity was demonstrated: d 7 (ALT: PB, SI, and SB groups), d 21 (ALT: SI and SB groups), and d 34 (ALT: PB, SI and SB groups; AST: SI and SB groups).

DISCUSSION

It is widely accepted that symbiosis between intestinal bacteria and animals has a positive effect on the maintenance of homeostasis. Modern data show at least several areas which can be affected by disorders of the intestinal microflora; for example, the functioning of the liver, fat tissue, kidney, and pancreas (Martin et al., 2009). Therefore, it is especially important to create a proper environment for microorganisms in the gut by prebiotic supplementation or to provide beneficial bacteria themselves as a probiotic. On the one hand, there is little research on bioactive substances administered early during embryogenesis, but previous experiments documented that in ovo inoculations exerted beneficial effects on the development of appropriate gut microbiome (Villaluenga et al., 2004; Pilarski et al., 2005; Sławińska et al., 2014). The mechanisms of action of in ovo injected bioactive substances are complex (Sławinska et al., 2014), but researchers still predict their positive effects on organism growth and BW. Synbiotics used in this paper were selected from the several combinations of pre- and probiotics by in vitro tests, followed by validation with animal model (Bednarczyk et al., 2013; Slawinska et al., 2015).

The results presented in the current study showed that some in ovo injected PB and SI increased the final BW of the investigated chicken (Table 2), whereas others (PI and SB) were ineffective. It can be presumed that the observed elevation of body weight after PB and SI is the cumulative effect of small, statistically not significant, differences in FCR (decrease) and the mean daily feed intake (increase). Simultaneously, described in the paper elevated activity of pancreatic enzymes might be the positive factor influencing feed consumption, FCR, and final BW. Earlier investigations with in ovo administered prebiotics and probiotics do not provide conclusive results indicating growth-promoting action. Sławińska et al. (2014), demonstrating immunomodulatory effects, did not state any influence of synbiotics (composed of prebiotic raffinose family oligosaccharides and probiotic *Lactobacillus lactis* or *Lactobacillus* acidophilus plus *Streptococcus faecium*) on chicken body weight. Previously, Maiorano et al. (2012) did not report any influence of raffinose family oligosaccharides (**RFO**) and synbiotics (RFO plus *Lactococcus lactis ssp. lactis* SL1, RFO plus *Lactococcus lactis ssp. cremoris* IBB SC1, lactose plus *Lactobacillus acidophilus* and *Streptococcus faecium*) on final BW. Moreover, field experiments carried out on a total

of 222,400 Cobb 500, Ross 708, and Hubbard chickens did not unequivocally prove better growth rates after RFO injected in ovo (Bednarczyk et al., 2011).

Results obtained using in ovo technology may be compared to studies in which bioactive substances are added directly to feed. Often, the last type of experiments does not provide conclusive results but shows contradictory effects. Some of studies prove that broilers fed a diet with fructooligosaccharides showed better body gains in comparison to control animals (Ammerman et al., 1989; Xu et al., 2003; Rebole et al., 2010). However, in contrast to these studies, in some other experiments the supplementation of a diet with inulin or fructooligosaccharides proved to be ineffective (Waldroup et al., 1993; Biggs et al., 2007; Williams et al., 2008). There is little information regarding the effect of galactooligosaccharides on weight gain in chickens. Jung et al. (2008) showed their beneficial effect as dietary additives on selective stimulation of the fecal microflora of broilers; however, no significant differences in BW, feed intake, or FCR were noticed.

The efficacy of prebiotics as growth promoters may also be influenced by other factors not directly related to their contents in the feed. We believe that the method of bioactive substance administration could also be important. The colonization of the gastrointestinal tract by beneficial microflora should occur as soon as possible after hatching. Inoculations of one day old chicken with prebiotics or probiotics have been shown to promote beneficial microbial population and to reduce *Salmonella* and *Campylobacter* (Nisbet, 1998; Fukata et al., 1999). However, the in ovo injection technology we applied in our study allows us to influence the microbiological status before hatching. The results concerning the BW of broilers presented in our study confirm the efficacy of this method of administration.

The efficient utilization of feed ingredients requires the presence of appropriate enzymes in the gastrointestinal tract. Enzymes of the highest activity in the digestion of starch, protein and triglycerides are synthesized in the pancreas and stored in zymogen granules. Pancreatic enzyme activity may reflect the potential ability of digestion. Kadhim et al. (2011) compared the activity of amylase, trypsin, and chymotrypsin in 2 breeds of chicken: red jungle fowl and commercial broiler chickens. They observed that in faster growing broilers the activities of enzymes were significantly higher both in the pancreas and in the intestine. In our study all pancreatic enzyme activity increased in broilers after in ovo injection (Figures 1–3). As for amylase and trypsin, increased enzyme activity was observed on the first day life, which may indicate stimulation of pancreatic development already during the incubation of eggs. However, the most significant elevation of enzyme activity was observed on d 34 after hatch. Given these results, one reason for the higher BW of chickens from PB and SI groups might be better digestion of feed ingredients; however, FCR reduction was not statistically significant. It should be emphasized that the increase in the total activity of pancreatic enzymes was not associated with an increase in pancreas absolute and relative weight (Table 2). This may indicate that the additives used do not cause pancreatic hyperplasia or hypertrophy and are in agreement with investigations made by Awad et al. (2009), who demonstrated that the weight of the pancreas was even lower after feeding with the addition of synbiotic (Biomin IMBO) and slightly diminished by probiotic (*Lactobacillus sp.*). Also, Abdel-Raheem and Abd-Allah (2011) reported that prebiotic mannan oligosaccharide and probiotic *Saccharomyces cerevisiae* given separately reduced the mass of the pancreas, although used together as a synbiotic, they increased the weight of this organ. The latter effect did not occur in our study when other methods of administration and other kinds of synbiotics were used. Generally, little is known about the influence of pre-, pro-, and synbiotics on exocrine pancreas function and pancreatic enzyme activity. So far, there are no similar studies which consider the influence of pre- and probiotics given in ovo on pancreatic enzymes activity; hence, our studies have an innovative character. The mechanism by which synbiotics may affect the activity of the enzymes is not established. However, our study supports the concept that major metabolic processes are under symbiotic homeostatic control (Martin et al., 2009).

We also examined whether the applied bioactive substances did not adversely affect liver metabolism. AST and ALT are the common indicators the activity of which increase with liver damage and this is used to screen for and/or monitor liver diseases. The level of AST in birds is used to diagnose some disorders (Coleman, 1995). In our study, no significant elevation of either aminotransferase was observed and on some days (Table 2); the activity of the enzymes in blood serum was even diminished. This indicates the lack of any negative and even positive influence of the additives used, especially both injected synbiotics. Salarmoini and Fooladi (2011) demonstrated the impact of commercial probiotic Bioplus2 (*Bacillus licheniformis* and *Bacillus subtilis*) and *L. acidophilus* from fermented milk on ALT and AST activity. They did not notice any significant elevation on d 21 and 42, and so did not indicate any negative consequences of the use of these additives. Similar results (Shareef and Al-Dabbagh, 2009) were obtained when *Saccharomyces cerevisiae* were used as a probiotic. Chickens supplemented with this probiotic showed increased body BW and feed consumption as well as better feed conversion efficiency, but the activities of ALT and AST were not affected.

CONCLUSIONS

In conclusion, this study demonstrates the beneficial effects of prebiotics and synbiotics inoculated in ovo. Bi^{2}tos and inulin given with *Lactococcus lactis* ssp. when injected in ovo on the 12th d chicken embryo development elevated BW at the end of the

rearing period. Simultaneously, the investigated compounds significantly increased the total activity of pancreatic enzymes; amylase, lipase, and trypsin. This may explain the positive effect of additives on BW. In general, all injected mixtures increased the activity of the enzymes at different time-points, but most potent for the stimulation of amylase and trypsin activity were both synbiotics and for lipase synbiotic Bi^{2}tos plus bacteria. The increase in the activity of enzymes stored in the pancreas seemed not to be associated with any negative disturbances in pancreas such as hyperplasia or hypertrophy because of the non-elevated weight of this organ. Also, the activities of the 2 investigated enzymatic markers (ALT and AST) were not changed, which proves that the health status of the liver was not impaired.

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