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Effects of Prebiotics and Synbiotics Delivered *In Ovo* on Broiler Small Intestine Histomorphology During the First Days After Hatching*

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The objective of the study was to determine the effect of prebiotics and synbiotics administered *in ovo* on the 12th day of incubation, on the development of the intestinal villi and the number of goblet cells in the small intestine of broiler chickens on the 1st and the 4th days of life of chicks. Two prebiotics: inulin (PI) or Bi^{2}tos (PB) and two synbiotics: inulin + *L. lactis* subsp. *lactis* (SI) or Bi^{2}tos + *L. lactis* subsp. *cremoris* (SB) were injected *in ovo* on the 12th day of embryonic development. The control group of the embryos was injected with physiological saline (C). On the 1st day of life, an increase in the height of the villi in the jejunum was reported as a result of the injection of pre- and synbiotics, moreover an increase in the surface area of the villi in the jejunum and the duodenum in chicks from the SB group was also observed. A stimulatory effect of synbiotics on the morphology of the duodenum and the jejunum was also observed on the 4th day after hatching. Conversely, in the ileum, in the SB group, a reduction in the height of villi was found both on the 1st and the 4th days of life. In contrast, injection of inulin and synbiotic with the addition of inulin resulted in an increase in the number of goblet cells in the duodenum and the jejunum on the 1st day of life, and caused a significant decrease on the 4th day after hatching.

Key words: Prebiotics, probiotics, synbiotics, bioactive substances, small intestine, histomorphology.

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Supplementation with external probiotics may mimic the situation in wild species, whereby newly hatched chicks become equipped with beneficial bacteria from maternal feces (KABIR 2009). To ensure the best protection for the newly hatched individual, external supplementation should be given as early as possible. Hence, an *in ovo* technology has been used, enabling administration of the given substance in a solution directly into the incubating eggs. *In ovo* injection has successfully been used for administration of MOS (CHELED-SHOVAL *et al*. 2011), Salmonella enteritidis – immune lymphokines (MCGRUDER *et al*. 2011), amino acids (OHTA *et al*. 2001), IN (TAKO & GLAHN 2012) and L-carnitine (ZHAI *et al*. 2008). *In ovo* administration of probiotics, in terms of lactic acid bacteria (LAB), is a patented method for delivering viable microbial cells to animals *in ovo* (US Patent 5,458,875, 1995).

In the case of prebiotics, the time from injection to hatching necessary to promote the growth of the intestinal microflora has a significant influence on the count of bifidobacteria of the hatched chicks. A greater number of bifidobacteria appeared in the feces when the injection was done on the 12 day of incubation (VILLALUENGA *et al*. 2004). On the other hand, after 12 days of incubation, the completely developed and highly vascularized allantochorion serves as a more efficient transport route from the air cell to the blood (FÁNCSI & FEHÉR

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1979). Injection of FOS and RFO in increasing doses of 0.1763, 0.8815, 1.763 and 8.815 mg/egg on the 12th day of embryo development significantly influences embryo body weight and the number of bifidobacteria in the chicken colon. The number of bifidobacteria populations had a linear growth increase after *in ovo* administration of RFOs or FOS for all experimental doses (VILLALUENGA *et al*. 2004).

For the growth and development of birds, early morphological and functional maturity of the gastrointestinal tract is crucial. On the day of hatching, the chicken small intestine forms 3.5% of the body mass. The early stage of gastrointestinal development includes the extension of the small intestine in length, accompanied by rapid development of the intestinal epithelium with its digestive – absorptive function (UNI *et al*. 2003b). Following hatch, the intestinal villi, forming small insets located on the surface of the mucous membrane to increase the absorbing area, undergo a number of rapid morphological changes as well. The villi surface area increases with the age of the birds, which occurs most rapidly in the duodenum. UNI *et al*. (1998) reported that the development of the duodenum villi area extends up to the 7th day, while in the jejunum and in the ileum it progresses until the 14th day post – hatching. IJI *et al*. (2001) found the highest growth rate of jejunum villi to occur during the initial 7 days following the hatch. Moreover, in duodenum the length of villi is the highest in comparison to the remaining segments of the small intestine (GEYRA *et al*. 2001; IJI *et al*. 2001). At the time of hatching, the intestinal crypts are meager and their increase in size in the postnatal period may in effect increase the number of enterocytes in intestinal villi. Enterocytes are intestinal mucous membrane epithelium building cells which take part in the process of absorption from the small intestine to blood (UNI *et al*. 2000). Concurrent to the development of the intestinal epithelium, enterocytes grow in number. During the initial week following hatching, enterocytes undergo two development stages: they develop brush border membrane and they hypertrophy. The number of enterocytes increases concurrent to the growth in the length of the intestinal villi (GEYRA *et al*. 2001; IJI *et al*. 2001; UNI *et al*. 1998). At the base of the crypts, goblet cells develop as well, located over the entire gastrointestinal system. These cells secret glycoproteins of a high molecular mass called mucins, which allows them to take part in mucus production. (AYABE *et al*. 2000).

The digestive system plays an important role in individual development (STARCK 1993). Exogenous ingredients affect the maturation of the gastrointestinal tract (UNI *et al*. 1998), both the morphology of the intestinal and digestive enzyme activity. EDENS *et al.* (2003) reported that probiotics improve digestion, absorption and increase the availability of nutrients and increase the activity of intestinal enzymes. According to HU *et al*. (2010), fructooligosaccharides given in feed affected the activity of protease and amylase. Studies by DONG *et al*. (2012) showed that with the development of avian intestines, maltase and sucrose activity during the first 8 days of life is greatest in the jejunum. Bioactive substances have a positive effect on broiler's intestinal microstructure. They contribute to an increase in the number of goblet cells, length of intestinal villi and depth of crypts (REHMAN *et al*. 2007; ŚWIĄTKIEWICZ & ŚWIĄTKIEWICZ 2008; BAURHOO *et al*. 2009; MUNYAKA *et al.* 2012; MAJD *et al*. 2013). On the first days after hatching, the small intestine of chickens grows at a rate exceeding the rate of other organs (UNI *et al*. 1998; 1999). In young birds, this is a very active metabolic period and the gastrointestinal microflora is actively formed. This represents a major element affecting the health of the host (GÓRSKA *et. al*. 2009). It stimulates the maturation of intestinal and systemic immune response (STRZĘPA & SZCZEPANIK 2013) by inhibiting the growth of pathogenic bacteria.

The effects of the application of various bioactive substances on mucosa histomorphology of chicken small intestine in the form of fodder supplements are ambiguous (AWAD *et al*. 2009; FORDER *et al*. 2007; REHMAN *et al*. 2007; SOLIS DE LOS SANTOS *et al*. 2005). Moreover, most studies are based on delivery of prebiotics and/or synbiotics in the form of feed additives, not *in ovo,* as herein. Therefore, the objective of this study was to determine the effect of prebiotics and synbiotics administered *in ovo* on the 12th day of incubation on the development of the intestinal villi in the small intestine of broiler chickens in the first days after hatching, and on the number of goblet cells calculated per 1 mm^2 of the intestinal villi area.

Material and Methods

In ovo treatment

Hatching eggs were obtained from a 32-wk-old flock (Ross 308). The eggs were collected from the same breeder flock, and had approximately the same weight of 60 g. Eggs were incubated in a commercial hatchery (Drobex, Solec Kujawski, Poland) in a Petersime incubator. On the 12th day of incubation, the eggs were candled, and infertile ones and those containing dead embryos were discarded. Eggs containing live embryos were randomly divided into five experimental groups (1800 eggs per group), and treated with different bioactive compounds by *in ovo* injection.

An aqueous solution of an equal volume of 0.2 ml was injected into the air cell, and the hole in the egg shell was sealed with the use of a custom automatic system (BEDNARCZYK *et al.* 2011). The control group (C) was injected with physiological saline. The prebiotic group (PI and PB) was injected with a solution containing 1.76 mg of Inulin (Sigma-Aldrich) or with solution containing 0.528 mg of Bi^{2}tos, (Clasado Ltd) – commercially developed non-digestive transgalacto – oligosaccharides, respectively. The injection solution for both synbiotic groups (SI) and (SB) consisted of 1.76 mg Inulin or 0.528 mg Bi^{2}tos, respectively, enriched with different probiotic bacteria. Group SI received 1000 CFU of *Lactococcus lactis* subsp. *lactis* IBB SL1 and group SB received 1000 CFU of *Lactococcus lactis* subsp. *cremoris* IBB SC1. The prebiotics concentration and prebiotic/probiotic ratio were selected earlier (BEDNARCZYK *et al.* 2013). The bacterial cultures were prepared from fresh over-night cultures of the IBB SL1 and IBB SC1 strains in GM17 liquid medium. The number of bacteria was estimated at the level of $3x10^8$ living cells. Directly before the injection the bacterial cultures were diluted in a prebiotic solution to obtain a bacterial suspension of 1000 CFU in 20 µl. The synbiotics injected in SI and SB groups were comprised of 180 µl of the prebiotic solution and 20 µl of bacterial suspension.

Animals

The body weight of cockerels selected for histological analysis was similar with the average weight for the entire group. The experiment was conducted at the experimental farm of the University of Warmia and Mazury in Olsztyn. The animals were reared for 6 weeks upon the approval of the Polish Local Ethical Commission (No 22/2012. 21.06.2012) and in accordance with the animal welfare recommendations of European Union directive 86/609/EEC, providing adequate husbandry conditions with continuous monitoring of stocking density, litter, ventilation etc. The experiment was performed in 10 repetitions within the groups, each of which consisted of 650 chickens kept in collective pens (65 chickens/pen). They were fed and watered *ad libitum.* For feeding, commercial diets were used according to the age of chickens (Table 1).

Samples of the small intestine for histology were collected from 1 and 4 day old cockerels. Following hatching, the chickens were weighed individually, on the 1st and the 4th day of life; for each treatment 10 chickens were selected based upon the average

Table 1

The composition and nutritive value of feed for chickens

	Starter	Grower	Finisher
Raw materials (%)			
Wheat	26.73	29.19	30.66
Maize	30.00	30.00	30.00
Post-extraction soybean meal	32.50	28.20	25.33
Canola	5.00	6.00	7.00
Soybean oil	2.10	1.33	1.80
Lard	–	2.00	2.50
Feed salt	0.30	0.30	0.28
Mel stern	1.09	0.95	0.85
Phosphate 1-Calcium	1.15	0.94	0.63
DL-Methionine	0.25	0.18	0.13
L-Lysine	0.32	0.32	0.27
L-threonine	0.06	0.09	0.05
Premix vit.- mineral.[1)]	0.50	0.50	0.50
The nutritional value			
EM, kcal/kg	2980	3100	3200
Total protein ,%	22.00	20.50	19.50
Lysine, %	1.35	1.25	1.15
Methionine, %	0.57	0.49	0.43
Methionine + Cystine, %	0.95	0.85	0.78
Calcium, %	0.90	0.80	0.70
Phosphorus, %	0.40	0.35	0.28
Sodium, %	0.14	0.14	0.13

[1)] 1 kg of premix contains: Vitamin A – 5 000 000 IU, Vitamin D_3 – 1 400 000 IU, Vitamin E – 18 200 mg, Vitamin K_3 – 1200 mg, Vitamin B_1 – 600 mg, Vitamin B_2 – 2000 mg, Vitamin B_6 – 1200 mg, Vitamin B_{12} – 8000 mg, biotin ((H) – 80 000 mg, Fe – 20 000 mg, Mn – 40 000 mg, Zn – 36 000mg, Cu – 6000 mg, J – 400 mg, Se – 140 mg, calcium pantothenate – 4800 g, nicotinic acid – 20 000 mg, folic acid – 400 mg, choline chloride – 380 g, phytase – 500 FTU.

body weight of each group. Such selection was performed in order to eliminate the effect of body weight on the morphology of the small intestine. Overall, for histological assays, the samples of the small intestine were collected from 100 chickens (50 birds total on the 1st and the 4th day of life).

Histomorphological samples

Samples for histological assays (approximately 2 cm) were collected directly after euthanizing birds from three sections of the small intestine: duodenum, jejunum and ileum. Samples were taken from the midpoint of the duodenum, from the midpoint of the jejunum between the point of entry of the bile duct and Meckel's diverticulum and midway of the ileum between Meckel's diverticulum and the ileocecal junction.

Histomorphological examination

The individual sections of the intestine were flushed with 0.9% saline and then fixed in 4% $CaCO_3$ buffered formalin. The fixed samples were dehydrated, x-rayed with xylene followed by paraffin impregnation in a tissue processor (Thermo Shandon, Chadwick Road, Astmoor, Runcorn, Cheshire, United Kingdom), and subsequently embedded in paraffin blocks using an embedding system (Medite, Burgdorf, Germany). The resulting blocks were cut on 10 micron thick sections using a rotary microtome (Thermo Shandon, Chadwick Road, Astmoor, Runcorn, Cheshire, United Kingdom), which in turn were placed on microscope slides coated with egg protein with the addition of glycerin.

Staining methods

Before staining, preparations were deparaffinized and hydrated. Subsequently they were subjected to routine H+E staining (hematoxylin and eosin in order to conduct morphometric analyses of the small intestine) and PAS staining with Schiff's reagent (to stain and count goblet cells).

Histomorphological measurements

Measurements included height and width of intestinal villi and intestinal crypt depth. Numbers of neutral goblet cells in intestine were counted using a Carl Zeiss (Jena, Germany) microscope equipped with a digital camera ToupCam™ and a program for the analysis of microscopic images Multiscan 14.02 (Computer Scanning Systems II, Warsaw, Poland). In order to measure the height of the villi, ten villi per bird were randomly selected from a cross section. The length was measured from the tip of the villus to its base at the crypt-villus outlet. The width of the villus was measured at half of its length. Subsequently, the surface of the villi was calculated according to the formula given by SAKAMOTO *et al.* (2000): $(2\pi) \times (VW / 2) \times (VH)$, where VW = villus width, and VH = villus height. The depth of intestinal crypts was defined as the invagination depth between neighboring intestinal villi and was measured between 10 villi (UNI *et al.* 1998). The number of PAS – positive cells was calculated per 1 mm^2 of the intestinal villi area.

Statistics

Data was analysed by means of one way analysis of variance (ANOVA) using the SAS program (SAS Institute Inc. 2013).

The arithmetic mean ($\bar{x}$) and the standard error of the mean (*SEM*) were calculated. The significant differences between the groups were tested using Duncan's multiple range test.

Results

Body weight

Body weight of chicks on the 1st day of life ranged from 41.0 ± 3.4 to 42.2 ± 3.2 g, and on the 4th day of life from 69.9 ± 8.2 to 73.6 ± 9.3 g (Table 2). No statistically significant differences between the groups in terms of body weight of chickens on the 1st and the 4th day of life were recorded.

Table 2

Body weight of chickens at 1 and 4 d of age depending on bioactive substances injected *in ovo* on the 12th day of incubation[1]

Item	Day 1	Day 4
Body weight (g)		
1. Control	42.2±3.2	71.0±7.6
2. Prebiotic 1 (inulin)	41.9±3.2	73.6±9.3
3. Prebiotic 2 (Bi^2tos)	41.3±3.0	69.9±8.2
4. Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis* 2955)	41.8±3.4	71.2±6.2
5. Synbiotic 2 (Bi^2tos + *Lactococcus lactis* subsp. *cremoris* 477)	41.0±3.4	71.2±4.9

[1] Means ±SEM representing 10 birds per group and 10 measurements per parameter per bird.

Table 3

Effect of pre- and synbiotics injected *in ovo* on the duodenal morphology of chickens at 1 and 4 day of age[1]

Item	Day 1	Day 4
Villus height (μm)		
C – Control	408.6±85.3	756.0^{b}±95.0
PI – Prebiotic 1 (inulin)	417.0±59.7	739.2^{b}±63.4
PB – Prebiotic 2 (Bi^2tos)	414.7±81.8	749.1^{b}±98.8
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	400.9±76.8	852.0^{a}±89.6
SB – Synbiotic 2 (Bi^2tos + *Lactococcus lactis* subsp. *cremoris 477)*	471.1±95.9	727.8^{b}±54.8
Villus width (μm)		
C – Control	76.6^{a}±13.7	105.1^{bc}±13.2
PI – Prebiotic 1 (inulin)	57.3^{b}±7.5	98.5^{c}±9.5
PB – Prebiotic 2 (Bi^2tos)	72.0^{a}±9.5	114.9^{b}±19.1
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	69.0^{a}±10.5	111.4^{b}±10.2
SB – Synbiotic 2 (Bi^2tos + *Lactococcus lactis* subsp. *cremoris 477)*	76.0^{a}±5.4	130.3^{a}±18.9
Villus surface area (μm^2)		
C – Control	$100,813.4^{a}$±35,008.6	$249,229.5^{bc}$±49,117.2
PI – Prebiotic 1 (inulin)	$75,993.2^{b}$±18,997.6	$228,115.9^{c}$±30,572.8
PB – Prebiotic 2 (Bi^2tos)	$96,825.7^{a}$±26,700.1	$270,652.0^{b}$±56,442.8
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	$87,398.1^{a}$±23,873.2	$295,315.0^{a}$±37,496.9
SB – Synbiotic 2 (Bi^2tos + *Lactococcus lactis* subsp. *cremoris 477)*	$113,140.3^{a}$±26,444.2	$295,481.9^{a}$±40,190.0
Crypt depth (μm)		
C – Control	64.4^{a}±6.9	87.3^{c}±5.3
PI – Prebiotic 1 (inulin)	47.2^{c}±3.8	75.8^{d} ±9.6
PB – Prebiotic 2 (Bi^2tos)	64.7^{a}±4.3	124.4^{a}±11.1
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	56.0^{b}±11.1	77.0^{cd}±11.6
SB – Synbiotic 2 (Bi^2tos + *Lactococcus lactis* subsp. *cremoris 477)*	60.0^{ab}±1.9	105.7^{b}±14.0
No of neutral goblet cells		
1. Control	3630^{bx}±1295.2	1909^{ay}±465.9
2. Prebiotic 1 (inulin)	5725^{ax} ±1227.1	2389^{ay}±485.9
3. Prebiotic 2 (Bi^2tos)	3670^{bx}±1098.2	2058^{ay}±696.1
4. Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lacti 2955s)*	5812^{ax}±1356.3	1802^{by}±254.0
5. Synbiotic 2 (Bi^2tos + *Lactococcus lactis* subsp. *cremoris 477)*	3498^{bx} ±1317.0	1673^{by}±362.4

[a-d] Difference ($P \leq 0.05$) between treatments (vertical), [1] Means ±SEM representing 10 birds per group and 10 measurements per parameter per bird.
[x-y] Difference ($P<0.05$) between treatments (horizontal), [1] Means ±SEM representing 10 birds per group and 10 measurements per parameter per bird.
[1] Means ±SEM representing 10 birds per group and 10 measurements per parameter per bird.

Histomorphological measurements

Villus morphometry

D u o d e n u m. The average values of height, width, surface area of the villi and crypt depth are provided in Table 3. No influence of the investigated pre- and synbiotics was observed when administered *in ovo* on the height of the duodenal villus on the 1st day of life of the chicks. However, the surface area of the villi was variable, and it was affected by different width of the villi in the groups of birds. A reduction in the surface area of the villi and the depth of intestinal crypts ($P \leq 0.05$) as a result of inulin injection was found. The smallest surface area of the villi and crypt depth were detected in the group injected with inulin. On the 4th day after hatching, in the SI group, the highest duodenal villus height were observed, which differed ($P \leq 0.05$) from the values obtained in other groups of birds. Moreover, in this case the width of the villi measured in the middle of their length affected absorptive surface area. The highest surface of villi was found for the SI and SB groups, and the smallest for the PI group. The highest and the lowest crypt depth on the 4th day after hatching was found in the group injected with Bi^2tos (PB) and inulin (PI) prebiotics, 124.4±11.1 i 75.8±9.6 μm, respectively.

J e j u n u m. Morphometric parameters of the villi in the jejunum of chickens on the 1st and the 4th days after hatching are presented in Table 4. *In ovo* injection of prebiotics and synbiotics significantly ($P \leq 0.05$) affected the growth of the villi as compared to the control group on the 1st day of life. The largest surface area of the villi in the jejunum was observed in the PI, PB and SB groups on the 1st day, and in the SI and SB groups on the 4th day of life in comparison to SI (on the 1st day) and PI (on 4th day) groups. Moreover, the SB synbiotic affected the highest, significantly different ($P \leq 0.05$)

Table 4

Effect of pre- and synbiotics injected *in ovo* on the jejunal morphology of chickens at 1 and 4 day of age[1]

Item	Day 1	Day 4
Villus height (μm)		
C – Control	272.6^{b}±73.0	392.5±91.2
PI – Prebiotic 1 (inulin)	348.5^{a}±67.6	444.1±77.2
PB – Prebiotic 2 (Bi^{2}tos)	308.7^{a}±46.2	434.4±79.7
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	285.9^{a}±50.9	477.5±70.1
SB – Synbiotic 2 (Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris 477)*	302.2^{a}±79.9	441.1±75.9
Villus width (μm)		
C – Control	57.3^{bc}±9.5	110.5^{b}±17.9
PI – Prebiotic 1 (inulin)	57.1^{bc}±7.2	86.1^{c}±15.8
PB – Prebiotic 2 (Bi^{2}tos)	63.7^{b}±7.8	99.7^{bc}±9.5
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	50.2^{c}±5.9	110.3^{b}±19.6
SB – Synbiotic 2 (Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris 477)*	68.0^{a}±9.1	116.8^{a}±19.4
Villus surface area (μm^{2})		
C – Control	50,125.2^{ab}±20,067.2	136,663.4^{ab}±35,112.8
PI – Prebiotic 1 (inulin)	62,846.5^{a}±17,760.7	118,252.8^{b}±19,312.7
PB – Prebiotic 2 (Bi^{2}tos)	62,522.6^{a}±14,417.4	134,945.7^{ab}±41,846.3
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	45,417.4^{b}±11,901.6	163,227.3^{a}±42,384.9
SB – Synbiotic 2 (Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris 477)*	63,032.2^{a}±12,366.4	162,910.3^{a}±45,274.3
Crypt depth (μm)		
C – Control	50.0^{b}±5.7	74.1±3.1
PI – Prebiotic 1 (inulin)	50.0^{b}±3.3	76.9±7.9
PB – Prebiotic 2 (Bi^{2}tos)	53.6^{ab}±1.5	74.0±3.2
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	49.5^{b}±4.0	79.1±11.6
SB – Synbiotic 2 (Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris 477)*	55.2^{a}±5.5	75.2±13.1
No of neutral goblet cells		
1. Control	5666^{abx} ±1632.6	2872^{aby}±609.2
2. Prebiotic 1 (inulin)	4703^{bx}±688.9	3252^{ay}±610.8
3. Prebiotic 2 (Bi^{2}tos)	5264^{abx}±1997.8	3241^{ay}±656.3
4. Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lacti 2955s)*	6821^{ax}±1486.2	2488^{by}±828.6
5. Synbiotic 2 (Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris 477)*	4156^{bx}±1228.1	2456^{by}±591.2

$^{a-c}$ Difference (P≤0.05) between treatments (vertical), [1] Means ±SEM representing 10 birds per group and 10 measurements per parameter per bird.
$^{x-y}$ Difference (P<0.05) between treatments (horizontal), [1] Means ±SEM representing 10 birds per group and 10 measurements per parameter per bird.
[1] Means ±SEM representing 10 birds per group and 10 measurements per parameter per bird.

depth of crypts on the 1st day after hatching, as compared to the C, PI, and SI groups. On the other hand, on the 4th day of life of the chicks, no influence of injection of pre-and synbiotics on crypt depth was recorded.

I l e u m. Histomorphology of the ileum is presented in Table 5. The lowest height of the villi on both the 1st and 4th day of life was found in the SB group. However, in the SB group, the reduction of the height of villi in the ileum was compensated by an increase in crypt depth. As a result, no significant effect of the injection of pre- or synbiotics on the surface area of the villi of chicks was observed on the 1st day after hatching. However, on the 4th day of life, the lowest (P≤0.05) surface area of the villi in the ileum was observed in the SB and C group in comparison to the other groups.

G o b l e t c e l l s. In 1 day old chickens, a significantly higher (P≤0.05) number of goblet cells per 1 mm^{2} surface of the villi in the duodenum (Table 3) was found in the PI and SI groups. In the jejunum (Table 4) the number of goblet cells was higher (P≤0.05) only in the SI group compared to the PI and SB groups. However, in the ileum (Table 5) of all experimental groups, lower numbers of respondent cells in comparison with the control group were observed. However, a significantly lower number of these cells in the PI, PB, SI and SB groups was reported in comparison to the C group. With respect to the SI group, the difference was not confirmed statistically.

On the 4th day of life, both in the duodenum and the jejunum, a significantly lower number of goblet cells in the SI and SB groups was observed, and

Table 5

Effect of pre- and synbiotics injected *in ovo* on the ileal morphology of chickens at 1 and 4 day of age[1]

Item	Day 1	Day 4
Villus height (μm)		
C – Control	250.8^{a}±75.2	328.2^{ab}±83.4
PI – Prebiotic 1 (inulin)	294.2^{a}±32.9	414.1^{a}±96.1
PB – Prebiotic 2 (Bi^{2}tos)	262.9^{ab}±32.9	423.5^{a}±96.4
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	264.4^{a}±48.2	414.3^{a}±55.8
SB – Synbiotic 2 (Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris 477)*	234.2^{b}±49.2	301.8^{b}±75.2
Villus width (μm)		
C – Control	56.6 ^{ab}±10.6	84.11±13.7
PI – Prebiotic 1 (inulin)	49.5 ^{b}±6.2	116.1±37.7
PB – Prebiotic 2 (Bi^{2}tos)	59.6 ^{a}±9.7	97.2±10.4
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	48.6 ^{b}±9.5	95.6±18.0
SB – Synbiotic 2 (Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris 477)*	53.9 ^{ab}±5.2	92.2±17.7
Villus surface area (μm^{2})		
C – Control	46,006.9±21,460.1	84,525.8^{b}±16,006.9
PI – Prebiotic 1 (inulin)	46,018.2±9,500.2	118,252.8^{a}±19,312.7
PB – Prebiotic 2 (Bi^{2}tos)	50,070.7±13,071.3	128,632.9^{a}±41,961.6
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	40,061.9±9,984.6	122,696.6^{a}±23,369.5
SB – Synbiotic 2 (Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris 477)*	40,022.6±10,562.9	87,381.1^{b}±27,142.5
Crypt depth (μm)		
C – Control	48.3^{ab}±5.0	73.7^{b}±13.3
PI – Prebiotic 1 (inulin)	48.9^{ab}±3.9	77.1^{b}±11.5
PB – Prebiotic 2 (Bi^{2}tos)	46.8^{b}±0.9	77.9^{b}±1.5
SI – Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lactis 2955)*	45.5^{b}±6.5	60.3^{c}±9.0
SB – Synbiotic 2 (Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris 477)*	51.1^{a}±1.0	93.2^{a}±8.7
No of neutral goblet cells		
1. Control	9511^{ax}±2542.7	3877^{ay}±975.8
2. Prebiotic 1 (inulin)	6250^{bx}±1286.9	3040^{by}±487.4
3. Prebiotic 2 (Bi^{2}tos)	5402^{bx}±1851.7	3335^{aby}±987.7
4. Synbiotic 1 (inulin + *Lactococcus lactis* subsp. *lacti 2955s)*	7177^{bx}±1354.4	3027^{by}±657.3
5. Synbiotic 2 (Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris 477)*	5853^{bx}±1271.5	3550 ^{aby}±751.0

$^{a-b}$ Difference ($P \leq 0.05$) between treatments (vertical), [1] Means ±SEM representing 10 birds per group and 10 measurements per parameter per bird.
$^{x-y}$ Difference ($P<0.05$) between treatments (horizontal), [1] Means ±SEM representing 10 birds per group and 10 measurements per parameter per bird.
[1] Means ±SEM representing 10 birds per group and 10 measurements per parameter per bird.

significantly higher in the C, PB and PI groups (duodenum) and in the PI and PB groups (jejunum). Significantly fewer cells in the ileum were found in the SI and PI groups in comparison to the C group.

Discussion

In ovo technology used in this study is an innovative method of automatic injection of bioactive substances into the egg air chamber (BEDNARCZYK *et al.* 2010; 2011). Effective stimulation of the composition and functioning of the microbiome of chickens was the main objective of prebiotic injection (PILARSKI *et al.* 2005; VILLALUENGA *et al*. 2004). In our study, carefully selected prebiotics and synbiotics (BEDNARCZYK *et al*. 2013), which may assist the development of the immune system of birds already at the stage of embryonic development, were injected (BRUDNICKI 2012; SŁAWIŃSKA *et al*. 2014a; SŁAWIŃSKA *et al*. 2014b).

In the present study, the influence of commercial prebiotics was investigated: inulin and Bi^{2}tos, and synbiotics formed on the following basis: inulin + *Lactococcus lactis* subsp. *lactis* IBB SL1 and Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris* IBB SC1 on the histological features of the small intestine of broiler chickens. The microscopic images of the individual sections of the chicken small intestine (duodenum, jejunum and ileum) are presented in Fig. 1. Histological morphology of the small intestine mucosa of birds provides valuable information on its functionality (YAMAUCHI 2007).

The gastrointestinal tract of chickens is relatively short, and the digestive processes therein are very intense. At the time of hatching it is anatomically complete but physiologically immature

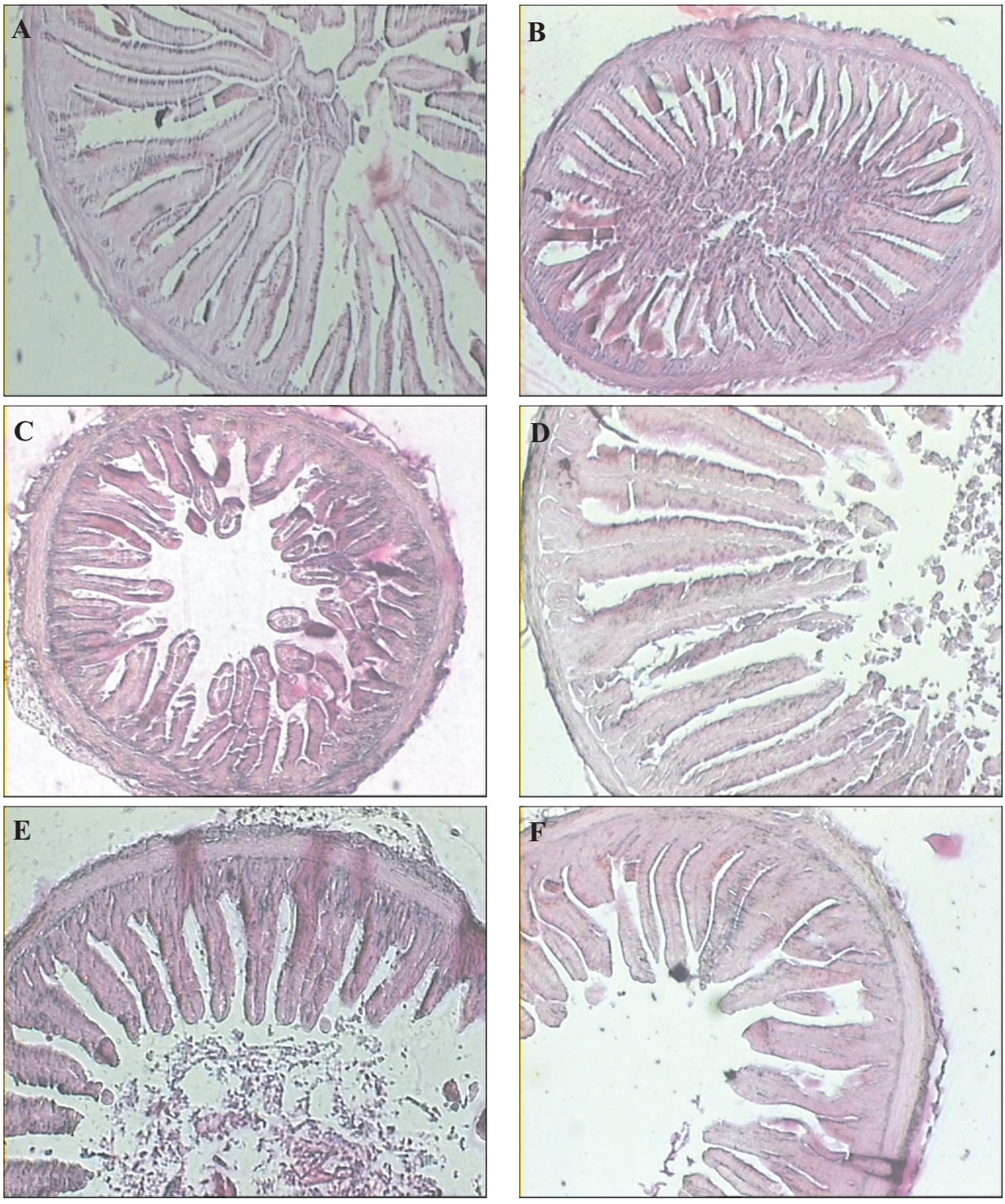

Fig. 1. Small intestine of chickens at 1 and 4 day of age. A – Microscopic photo of intestinal villi from the duodenum of broiler chick at one day of age in prebiotic inulin treatment group. PAS reaction, mag. 3.2 x 10; B – Microscopic photo of intestinal villi from the jejunum of broiler chick at one day of age in prebiotic Bi^{2}tos treatment group. PAS reaction, mag. 3.2 x 10; C – Microscopic photo of intestinal villi from the ileum of broiler chick at one day of age in control group. PAS reaction, mag. 3.2 x 10; D – Microscopic photo of intestinal villi from the duodenum of broiler chick at four day of age in synbiotic Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris 477* treatment group. PAS reaction, mag. 3.2 x 10; E – Microscopic photo of intestinal villi from the jejunum of broiler chick at four day of age in synbiotic inulin + *Lactococcus lactis* subsp. *lactis 2955* treatment group. PAS reaction, mag. 3.2 x 10; F – Microscopic photo of intestinal villi from the ileum of broiler chick at four day of age in control group. PAS reaction, mag. 3.2 x 10.

(NOY & SKLAN 2001; UNI *et al*. 2003b). The maturation of the gastrointestinal tract is associated mainly with an increase of the absorptive surface of the intestine, and with the transformation of the structure of the intestinal epithelium. The immaturity of the gastrointestinal tract of chickens in the first days after hatching may be a limiting factor in the development and growth of birds. This results from the fact that the ability to secrete enzymes, increasing the amount of intestinal absorption by an increase in length and height of intestinal villi has not yet taken place. The epithelium of the intestine must undergo numerous important changes, both morphological as well as physiological, which are essential to achieve functional maturity of the organ (UNI *et al*. 1998, BAR-SHIRA & FRIEDMAN 2006). The obtained results are consistent with previous studies of BRUDNICKI (2012). They show that a single *in ovo* injection of RFO on the 12th day of incubation increases the rate of yolk sac resorption, mainly on the histomorphological characteristics and structure of chicken intestine, especially in the first week of life. Similar research was provide by CHELED-SHOVAL *et al*. (2011) who investigated the effects of MOS injection on the 17th day of incubation on the microstructure of the jejunum in 1 day-old chickens Cobb 500. In the experimental group (MOS) they found a statistically significant increase of villi height and surface and crypt depth, as compared to the control group.

In the present study, beneficial effects of pre- and synbiotics were observed following *in ovo* injection which impacted the height of jejunum villi of chickens on the 1st day after hatching. In the 1st day of life of the chickens, an increase in the height of intestinal villi in the jejunum was found in the experimental groups in comparison to the C group ($P<0.05$). These results correspond to the study by CHELED-SHOVAL *et al*. (2011), who revealed that the group injected with MOS had larger and more equalized villi, indicating a more mature stage in their intestinal development. In this segment of the small intestine, the villi were the broadest, their area was characterized by the largest absorptive surface and the crypts were the deepest in the group of chickens injected with Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris* IBB SC1 synbiotic. On the 4th day of life, the largest surface area of the intestinal villi in the duodenum and the jejunum was found as a result of the injection of both synbiotics (SI or SB). In the case of the duodenum, the differences in comparison to the other groups were statistically significant ($P \leq 0.05$).

The effect of injected pre- or synbiotics was different in the ileum as compared to the duodenum and the jejunum. In one day-old chicks, among the examined bioactive substances, we recorded an impact on increase of intestinal villi found in C, SI and PI groups. On the 4th day of life of the chicks, an increase in the villi surface area was observed in the ileum in the PI, PB and SI groups ($P<0.05$). The effect of bioactive substances administered *in ovo* on the histomorphology of the small intestine is not as clear as in case of the chicks aged 1 day, and this may result from the co-occurrence of many factors. As indicated by PATTERSON and BURKHOLDER (2003) and VERDONK *et al*. (2005), these factors include: diet type, animal characteristics, farming hygiene, and environmental stress. However, in contrast to the duodenum and the jejunum, reductions were observed in height and area of ileal villi both on the 1st and the 4th day of life under the influence of the synbiotic SB. The reduction in height in villi influenced crypt deepening in the ileum, which may affect nutrient absorption because most of the digested nutrients were already absorbed in the upper parts of the small intestine, i.e. in the duodenum and the jejunum (YAMAUCHI *et al*. 2010).

In the duodenum of day-old chickens, no effect of *in ovo* injected substances on the height of the intestinal villi was observed. However, the negative effect of inulin on width, surface area and crypt depth was observed. This is quite surprising considering that many authors report that inulin supplementation to the diet of poultry causes an increase in the height of the intestinal villi, density of their network, as well as increase in crypt depth (YUSRIZAL & CHEN 2003; REHMAN *et al*. 2007). Perhaps this may result from a different route of administration of this prebiotic, as the majority of previous studies concern the impact of various bioactive substances added to feed (AWAD *et al*. 2009; SOLIS DE LOS SANTOS *et al*. 2005; 2007; UNI *et al*. 2003b). XU *et al*. (2003), who studied the effect of fructooligosaccharides (FOS) in the diet of broiler chicks, observed that FOS supplementation significantly increased the ileum and villi height and caused a shallowing of intestinal crypts in both of these intestines. Such changes facilitate nutrient absorption (CASPARY 1992; REBOLÉ *et al.* 2010). If the contrary occurs, i.e., the villi are shortened accompanied by deepening of the crypts, nutrient absorption may be degenerated, which leads to a worse functioning of the gastrointestinal tract (XU *et al*. 2003). On the 4th day of life, the injection of synbiotics to the air chamber of chicken eggs positively affected the surface area of the villi in two segments of the small intestine: duodenum and jejunum. Villum shortening with simultaneous deepening of the crypts was only observed in the duodenum in chicks injected with synbiotic (the SB group). A reversal of this was observed in the SI group, where the intestinal villi were extended in length while the crypt depth decreased.

The mechanisms of interaction of pre- and synbiotics on the histomorphology of the small intes-

tine of tested chickens are of considerable interest. In the case of synbiotics, an indirect effect is frequently assumed through the modification of the microbiome of the treated embryos, and in consequence the chickens after hatching. In the case of *in ovo* administered prebiotics, at least two possible mechanisms of interaction on the histomorphology of the small intestine are possible (BRUDNICKI 2012). A direct impact is based on partial absorption of prebiotics and their interactions with appropriate intestinal epithelial cell receptors and immune cells (SEIFERT & WATZL 2007). An indirect impact is based on the modification of the intestinal flora and production of short chain fatty acids (SCFA), which are the main energy source for intestinal epithelial cells (BOSSCHER 2009). The latter mechanism is possible because in contrast to the conventional paradigm, it is becoming increasingly evident that the gastrointestinal tract of the embryo is not sterile. DEEMING (2005) found that microorganisms may be internalized from yolk at 18 days of embryonic development. PEDROSO (2009) found that the embryo intestinal tract is not only sterile but that the pioneer microbial community demonstrates signs of evolution in the last 4-5 days before hatch.

Bioactive substances administered *in ovo* stimulate endogenous defense mechanisms of the host and affect not only the stability of the intestinal microflora, but also stimulate the development of immune organs (SŁAWIŃSKA *et al*. 2014b) and affect the expression of several genes associated with the functioning of the immune system (SŁAWIŃSKA *et al*. 2014a). The immune system plays an important role in regulating the increase of density of goblet cell and mucin production. This system mediates the physiological changes of the intestine, associated with the body's defense reaction against a pathogen.

An increased amount of secreted mucus prevents pathogens from adhering to the surface of intestinal epithelium, and also helps to remove pathogens from the intestine (KIM & KHAN 2013). According to UNI *et al*. (2003a) the number of goblet cells accounts for approximately 13%, 23%, and 26% of epithelial cells around the villus in the duodenum, the jejunum and the ileum, respectively. According to CORFIELD *et al*. (2000) and KIM & KHAN (2013), goblet cells apart from macrophages and dendritic cells, are the main components of the immune system, which together with the intestinal mucus layer constitute the main defense system of hatched chicks. Goblet cells are formed at the base of the crypts from pluripotent stem cells during the process of mitosis (AYABE *et al*. 2000).

Comparing the results of our study regarding the number of neutral goblet cells on the 1st day of life of chicks with the results published by FORDER *et al*. (2007), who did not administer any bioactive substances to birds, we confirm a larger number of these cells in the jejunum and the ileum. Most notably in the PI and SI groups, the numbers of goblet cells was twice as high as the numbers reported by FORDER *et al*. (2007). In our study, the injection of inulin (PI group) as well as synbiotics with the addition of inulin (SI group) resulted in an increase in the number of mucus-producing cells in the duodenum (Table 3).

These results were partially confirmed in the jejunum (Table 4), because the SI group had the highest number of goblet cells but were significantly higher only when compared to the PI and SB groups. Many authors claim that inulin indirectly stimulates the growth of pro-health intestinal bacterial strains and inhibits proliferation of pathogenic bacteria. For instance, the addition of inulin to the diet of poultry may contribute to changes in the intestinal microflora of chickens causing an increase in the population of *Lactobacillus,* while reducing the number of bacteria of *Escherichia coli*, *Campylobacter* and *Salmonella* genus (YUSRIZAL & CHEN 2003; XU *et al*. 2003). Similarly, TAKO and GLAHN (2012) reported the effect of inulin added to the amniotic fluid on the 17th day of incubation of eggs, as well as to the diet of broiler chicken, on the increase of intestinal bacterial population, improvement of functionality of the intestine and thereby improvement in iron absorption.

In general, the high number of goblet cells in chick small intestines on day one post-hatch may result from their immune system not being fully mature, and the main protective barrier of the intestine being the mucus layer lining the intestinal epithelium. Confirmation of this association was shown by the study of CHELED-SHOVAL *et al*. (2011) which shows that a large number of goblet cells on the 1st day after hatching is indicative of a good condition of chicken intestine injected *in ovo* with bioactive substances. Similar results were obtained in our study in which the groups injected *in ovo* with the prebiotic (PI) and with the synbiotic with inulin (SI): the chick duodenum was found to contain a significantly higher number of (PAS – positive) goblet cells in comparison to the remaining groups, and the same was found in the jejunum of the SI group. In the ileum (Table 5), the highest ($P \leq 0.05$) number of goblet cells was found in the C group in comparison to the other groups. It is difficult to unquestionably explain the reason for the high number of goblet cells in this section of the chick small intestine in the control group. Nevertheless, in the PI and SI groups, a significantly higher number of the goblet cells was maintained in comparison to PB and SB groups.

On the 4th day of life, a reduction in the number of neutral goblet cells was observed in the duodenum, the largest, 3 and 2.5 fold reduction in the SB (Bit^{2}tos + *Lactococcus lactis* subsp. *cremoris* SC1) and SI (inulin + *Lactococcus lactis* subsp. *lactis* IBB SL1) groups respectively, while in the other groups this number decreased by half (Table 3). Additionally in the jejunum, the highest reduction in the number of goblet cells was reported in the SI and SB groups. In the ileum, the largest decrease in the number of goblet cells was observed not only in the PI and SI groups, compared to the control group.

When analysing the number of goblet cells in the respective sections of the small intestine, regardless of the bioactive substance used (a pre- or a synbiotic), the number of intestinal villi per 1 mm^2 was observed to be the highest in the ileum of chicks in 1st to 4th day of life, followed by the jejunum, while the lowest was in the duodenum. The number of goblet cells is an exception, with an insignificantly lower number in the ileum of the chicks injected with prebiotic with inulin (PI group) than in their duodenum.

It is difficult to unquestionably indicate the advantageous effect of the bioactive substances used because the number of PAS – positive cells in chick gastrointestinal tracts may be influenced not only by the presence of pathogens (KIM & KHAN 2013) or diet (REBOLÉ *et al*. 2010; SOLIS DE LOS SANTOS *et al*. 2007; LANGHOUT *et al*. 1999; SHARMA *et al*. 1997, cited in UNI *et al*. 2003a), but also by the migration rate of epithelium cells from the crypts to the tips of villi (SHEA-DONOHUE *et al*. 1985, cited in UNI *et al*. 2003a), as well as changes in the intensity of differentiating precursor cells into mature goblet cells (DE RITIS *et al*. 1975; WATTEL *et al*. 1979; SHUB *et al*. 1983, cited in UNI *et al*. 2003a). Many authors had already indicated the beneficial effects of inulin, including preventing colonization by pathogenic bacteria (WALDROUP *et al*. 1993; XU *et al*. 2003).

To conclude, it may be assumed that in the groups injected with the prebiotic and synbiotic with inulin, a significantly higher number of goblet cells (and their secretion) in the 1st day of the life of chicks prevented pathogenic colonization in the intestine. In consequence, the potential reaction of the intestine increasing the number of goblet cells producing neutral mucins in response to an infection, did not occur by the 4th day of life. These results may indicate the good condition of the intestine in chickens from the abovementioned groups.

Conclusions

This study demonstrates a significant effect of *in ovo* injection of pre- or synbiotics on the histomorphology of the small intestine of chickens on the 1st and the 4th days of life. This effect was different depending on the investigated section of the small intestine and substance injected. On the 1st day of life, an increase in the height of the villi in the jejunum was found as a result of the injection of pre- and synbiotics. The stimulatory effect of synbiotics inulin + *Lactococcus lactis* subsp. *lactis* IBB SL1 on the morphology of the duodenum was also reported on the 4th day of life. A different phenomenon occurred in the ileum, where a reduction was observed in the height of villi on both the 1st and the 4th day of life under the influence of Bi^{2}tos + *Lactococcus lactis* subsp. *cremoris* IBB SC1 synbiotic (SB). The significantly higher number of goblet cells in chicks aged 1 day, injected with pre- and synbiotic with inulin, as well as their largest reduction on the 4th day of life, may indicate the beneficial effect of inulin on the condition of the small intestine in the birds. Further research is needed to select appropriate pre- and synbiotics affecting the functional features of the mucous membrane of different segments of the intestine.

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