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

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Accepted July 07, 2016 Published October 2016

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 Bi2tos (PB) and two synbiotics: inulin + L. lactis subsp. lactis (SI) or Bi2tos + 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.

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 (MCRUDER 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 allanto-chorion serves as a more efficient transport route from the air cell to the blood (FÁNCSI & FEHÉR

*Supported by grants No. UMO – 2011/01/B/NZ9/00642 from the National Science Centre in Cracow (Poland), and partially by project “THRIVE RITE – Natural Compounds to enhance Productivity, Quality and Health in Intensive Farming Systems”, an EU-funded research collaboration, FP7 Project No. 315198.
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 includes the extension of the small intestine in length, accompanied by rapid development. The small intestine is crucial. On the day of hatching, 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 secrete 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 sucrase 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 (Gorska 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 symbiotics 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 symbiotics 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 mm² 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, Solce 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 Bi2tos (Clasado Ltd) – commercially developed non-digestive transgalacto-oligosaccharides, respectively. The injection solution for both symbiotic groups (SI) and (SB) consisted of 1.76 mg Inulin or 0.528 mg Bi2tos, 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

The composition and nutritive value of feed for chickens

<table>
<thead>
<tr>
<th>Raw materials (%)</th>
<th>Starter</th>
<th>Grower</th>
<th>Finisher</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>26.73</td>
<td>29.19</td>
<td>30.66</td>
</tr>
<tr>
<td>Maize</td>
<td>30.00</td>
<td>30.00</td>
<td>30.00</td>
</tr>
<tr>
<td>Post-extraction soybean meal</td>
<td>32.50</td>
<td>28.20</td>
<td>25.33</td>
</tr>
<tr>
<td>Canola</td>
<td>5.00</td>
<td>6.00</td>
<td>7.00</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>2.10</td>
<td>1.33</td>
<td>1.80</td>
</tr>
<tr>
<td>Lard</td>
<td>-</td>
<td>2.00</td>
<td>2.50</td>
</tr>
<tr>
<td>Feed salt</td>
<td>0.30</td>
<td>0.30</td>
<td>0.28</td>
</tr>
<tr>
<td>Mel stern</td>
<td>1.09</td>
<td>0.95</td>
<td>0.85</td>
</tr>
<tr>
<td>Phosphate 1-Calcium</td>
<td>1.15</td>
<td>0.94</td>
<td>0.63</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.25</td>
<td>0.18</td>
<td>0.13</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.32</td>
<td>0.32</td>
<td>0.27</td>
</tr>
<tr>
<td>L-threonine</td>
<td>0.06</td>
<td>0.09</td>
<td>0.05</td>
</tr>
<tr>
<td>Premix vit.- mineral 1)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>The nutritional value</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>E.M, kcal/kg</td>
<td>2980</td>
<td>3100</td>
<td>3200</td>
</tr>
<tr>
<td>Total protein, %</td>
<td>22.00</td>
<td>20.50</td>
<td>19.50</td>
</tr>
<tr>
<td>Lysine, %</td>
<td>1.35</td>
<td>1.25</td>
<td>1.15</td>
</tr>
<tr>
<td>Methionine, %</td>
<td>0.57</td>
<td>0.49</td>
<td>0.43</td>
</tr>
<tr>
<td>Methionine + Cystine, %</td>
<td>0.95</td>
<td>0.85</td>
<td>0.78</td>
</tr>
<tr>
<td>Calcium, %</td>
<td>0.90</td>
<td>0.80</td>
<td>0.70</td>
</tr>
<tr>
<td>Phosphorus, %</td>
<td>0.40</td>
<td>0.35</td>
<td>0.28</td>
</tr>
<tr>
<td>Sodium, %</td>
<td>0.14</td>
<td>0.14</td>
<td>0.13</td>
</tr>
</tbody>
</table>

1) 1 kg of premix contains: Vitamin A – 5 000 000 IU, Vitamin D3 – 1 400 000 IU, Vitamin E – 18 200 mg, Vitamin K3 – 1200 mg, Vitamin B1 – 600 mg, Vitamin B2 – 2000 mg, Vitamin B6 – 1200 mg, Vitamin B12 – 8000 mg, biotin (H) – 80 000 mg, Fe – 20 000 mg, Mn – 40 000 mg, Zn – 36 000 mg, 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% CaCO₃ 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): \( \frac{1}{2} \times (VW / 2) \times (VH) \), 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 mm² 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 (\( \text{SEM} \)) 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>
<thead>
<tr>
<th>Item</th>
<th>Day 1</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>42.2±3.2</td>
<td>71.0±7.6</td>
</tr>
<tr>
<td>2. Prebiotic 1 (inulin)</td>
<td>41.9±3.2</td>
<td>73.6±9.3</td>
</tr>
<tr>
<td>3. Prebiotic 2 (Bi2tos)</td>
<td>41.3±3.0</td>
<td>69.9±8.2</td>
</tr>
<tr>
<td>4. Synbiotic 1 (inulin + Lactococcus lactis subsp. lactis 2955)</td>
<td>41.8±3.4</td>
<td>71.2±6.2</td>
</tr>
<tr>
<td>5. Synbiotic 2 (Bi2tos + Lactococcus lactis subsp. cremoris 477)</td>
<td>41.0±3.4</td>
<td>71.2±4.9</td>
</tr>
</tbody>
</table>

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

**Villus morphology**

**Duodenum.** 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 crypt depth was 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<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 Bi2tos (PB) and inulin (PI) prebiotics, 124.4±11.1 i 75.8±9.6 µm, respectively.

**Jejunum.** 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<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<0.05)
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.

Ileum. 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.

Goblet cells. In 1 day old chickens, a significantly higher (P<0.05) number of goblet cells per 1 mm² 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...
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; SLAWINSKA et al. 2014a; SLAWINSKA et al. 2014b).

In the present study, the influence of commercial prebiotics was investigated: inulin and Bi2tos, and synbiotics formed on the following basis: inulin + Lactococcus lactis subsp. lactis IBB SL1 and Bi2tos + 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 Bi2tos 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 Bi2tos + 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 2935 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 chicks 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 Bifidobacterium longum subsp. breve and B. 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<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 symbiotic 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, 1995; REBOULÉ 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 intesti-
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 \textit{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 \textit{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 (SLAWIŃSKA et al. 2014b) and affect the expression of several genes associated with the functioning of the immune system (SLAWIŃSKA 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 \textit{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 \textit{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 \textit{et al.} 2000).

Comparing the results of our study regarding the number of neutral goblet cells on the 1\textsuperscript{st} day of life of chicks with the results published by FORDER \textit{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 \textit{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 \textit{Lactobacillus}, while reducing the number of bacteria of \textit{Escherichia coli}, \textit{Campylobacter} and \textit{Salmonella} genus (YUSRIZAL \\& CHEN 2003; XU \textit{et al.} 2003). Similarly, TAKO and GLAHN (2012) reported the effect of inulin added to the amniotic fluid on the 17\textsuperscript{th} 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 \textit{et al.} (2011) which shows that a large number of goblet cells on the 1\textsuperscript{st} day after hatching is indicative of a good condition of chicken intestine injected \textit{in ovo} with bioactive substances. Similar results were obtained in our study in which the groups injected \textit{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<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 (Bι’τος + 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 mm² 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 (REBOLE 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 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 Bι’τος + 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.

Acknowledgments

The research was performed using equipment purchased within the frame of “Implementation of the 2nd stage of Regional Centre of Innovation”, project co-financed by the European Regional Development Fund under the Regional Operational Program for Cuaviian-Pomeranian Voivodeship for the years 2007-2013. We hereby show our gratitude to Goerge Tzortis (Clasado Ltd., Malta) for providing us with the commercial prebiotic Βι’τος. Jacek BARDOWSKI and Joanna ŻYLIŃSKA from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw are kindly acknowledged for the bacteria strains.

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