The impact of synbiotic administration through in ovo technology on the microstructure of a broiler chicken small intestine tissue on the 1\textsuperscript{st} and 42\textsuperscript{nd} day of rearing

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Abstract

Background: Application the innovative method which is in ovo technology provides a means of modulating the immune system at early embryonic stages. The aim of study was to determine influence of the i

Results: On the 1\textsuperscript{st} day of chickens life, in the duodenum of both experimental groups (SYN1 and SYN2), a significantly higher and wider intestinal villi as well as a significantly larger absorbent surface of these villi were found in comparison with the Control group ($P \leq 0.01$). The duodenum in the SYN1 group compare to the Control group. In the jejunum on the 1\textsuperscript{st} day of life, in the SYN1 group, significantly higher villi than in the Control group, with a simultaneous decrease in the depth of crypts ($P \leq 0.01$), and also the largest width of villi and their absorbent area ($P \leq 0.01$) in comparison to the other groups were found. On the 42\textsuperscript{nd} day of life, in the jejunum, an increase in the height of the villi whilst reducing the crypt depth in the SYN2 group was found ($P \leq 0.01$). In turn, in the SYN1 group, there were significantly more neutral goblet cells observed compared with the control group ($P \leq 0.05$). In the ileum of 1-day-old chickens, the widest villi ($P \leq 0.05$) and the deepest crypts ($P \leq 0.01$) were found in the SYN2 group. In the same group, there was also the least amount of neutral goblet cells in comparison to the other groups ($P \leq 0.05$).

Conclusions: We observed that symbiotic 1 and 2 beneficially affected the examined characteristics on the 1\textsuperscript{st} and 42\textsuperscript{nd} day of life. The obtained results allow us to conclude that the use of symbiotics significantly affect gut structure which should contribute to improvement in nutrient absorption by the gut.

Keywords: Broiler chicken, In ovo, Small intestine, Synbiotics
Background
Synbiotics consist of a combination of synergistically interacting probiotics and prebiotics which can be aimed at improving the resistance and stability of health-promoting organisms in the gut of birds by providing a substrate for fermentation [1, 2]. Thus, synbiotics are factors that modulate the immune system of birds by acting on the bacterial flora of their gastrointestinal tract [3]. To ensure the greatest protection of the immune system for newly hatched chickens, external supplementation with bioactive substances, such as synbiotics, should occur as early as possible. Application of an innovative method, such as in ovo technology, provides a means of modulating the immune system at early embryonic stages. This technology involves the introduction - on the appropriate day of embryonic development - of the particular substance in solution form to the air chamber of eggs or directly into a developing embryo [4]. Of course, the effectiveness of the injection and the level of use of the injected bioactive substance by the avian embryo depend on various factors [5]. These can be the chemical and physical features of the injected substances, its dose and the egg surface where the injection was performed (i.e. the embryo, the amnion, the allantois, the air chamber egg or the yolk sac). A thorough understanding of the various stages of embryonic development in birds allows the optimal time of injection to be defined [6, 7]. According to Villaluenga et al. [8], the optimal time of prebiotic injection is on the 12th day of embryonic development. In comparison with injections on d 1, 8 and 17, a significantly higher number of Bifidobacteria was observed in the colon of two-day-old chickens. A similar result was obtained by Pilarski et al. [9], who studied the effect of alpha-galactosides (RFOs) administered on the 12th day of egg incubation on selected traits of chickens.

This is mainly due to the fact that on this day of bird embryonic development, allantochorion is already fully developed and vascularised. The embryo is surrounded by the amniotic fluid that remains in contact with the embryonic gastrointestinal tract, which permits the transport of substances from the air chamber into the intestine [10]. Thus, a highly vascularised allantochorion enables efficient transport of bioactive substances given by injection in ovo on the 12th day of egg incubation between the air chamber of the eggs and the digestive tract of chickens. Upon hatching, the in ovo modulated profile of the gut microflora has an influence on the good condition of health of a chick, eliminating the need for antibiotics. We presume that this beneficial condition of the GI (gastrointestinal) tract would be reflected in the morphology of the intestines and might be maintained throughout the life of the chicken.

The prebiotics used in this study as components of the synbiotics are: commercially developed non-digestive transgalacto-oligosaccharides (Bi2tos, Clasado Ltd.) and raffinose family oligosaccharides (RFOs), which was obtained from lupin seeds as a white powder.

In previous projects carried out by our team, we evaluated the impact of bioactive substances in the form of pre-, pro- and synbiotics injected on the 12th day of incubation into the air chamber of the egg on production traits and the macro- and microstructure of the small intestine. The best composition of these substances and their optimal dose was selected. On the basis of these studies, we found the most beneficial effect of synbiotics on the above-mentioned parameters [11, 12].

The aim of study was to determine the influence of the in ovo stimulation with synbiotics on the microstructure of the duodenum, jejunum and ileum on the 1st and 42nd day of rearing.

Methods
The experiment was conducted on hatching eggs of the Cobb 500 FF line incubated in commercial hatchery conditions (Drobex - Agro Ltd., Solec Kujawski, Poland) in Petersime incubators and on 1- and 42-day-old broilers. On d 12 of incubation, the eggs were candled, and the infertile ones or those containing dead embryos were discarded. Eggs containing living embryos were randomly divided into 3 groups. Bioactive substances were administered in an amount of 0.2 mL into the air chamber of the egg on the 12th day of embryonic development by the in ovo technique. The hole in the shell of the egg was sealed with the use of a special automatic system [13]. 5000 eggs were injected. The SY1 group received synbiotic L. salivarius IBB3154 + Bi2tos, Clasado Ltd. (2 mg of Bi2tos probiotic +10^7 bacteria/egg), and the SYN2 group received synbiotic L. plantarum IBB3036 + lupin RFOs (2 mg of RFO probiotic +10^7 bacteria/egg). The Control group was injected with physiological saline 0.9% NaCl.

Animals
The rearing experiment was conducted on the experimental farm of the PIAST company in Olszowa according to the technological recommendations and lasted for 42 d. All groups were fed and watered ad libitum. Commercial diets were used according to the age of the chickens: 1-10 d - starter, 10–20 d - grower, 20-41 d – finisher (Table 1).

Histomorphological examination
The material for the morphological and histological assays (approx. 2 cm) of the small intestine (duodenum, jejunum, ileum) were collected from 1- and 42-day-old chickens. Before slaughter, 680 chickens from each group were weighed, and their mean body weight was calculated. Subsequently, 15 birds per group, with a body weight similar to the mean for the group, were selected. Directly after slaughter, the small intestine was extracted,
and all sections of the small intestine was excised, measured and weighed. Samples were taken from the mid-point of the duodenum, from the midpoint of the jejunum between the point of entry of the bile duct and Meckel's diverticulum and the midpoint of the ileum between Meckel's diverticulum and the ileocecal junction. A total of 270 samples were collected (3 groups × 3 sections × 15 birds × 2 repetitions). 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, cleared and permeated with paraffin 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). Thus, the formed blocks were cut into 10 μm-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 glycerine. Before staining, the preparations were deparaffinised and hydrated. They were subsequently subjected to PAS

### Table 1 Composition of premix for starter, grower and finisher diets for chickens

| Vitamin/Element | Declared name | Additive Code | Units | Starter diet Quantity per tonne of finished feed | Grower diet Quantity per tonne of finished feed | Finisher diet Quantity per tonne of finished feed |
|-----------------|---------------|---------------|-------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Vitamin A       | Retinyl acetate | E672 | MIU   | 13.00 - | 10.00 - | 10.00 - |
| Vitamin D₃      | Cholecalciferol | E671 | MIU   | 5.00 - | 5.00 - | 5.00 - |
| Vitamin E       | alpha Tocopherol | 3a700 | g     | 80.00 - | 50.00 - | 50.00 - |
| Vitamin K       | Vitamin K      | -   | g     | 3.00 - | 3.00 - | 3.00 - |
| Vitamin B₁      | Vitamin B₁     | -   | g     | 3.00 - | 2.00 - | 2.00 - |
| Vitamin B₂      | Vitamin B₂     | -   | g     | 9.00 - | 8.00 - | 6.00 - |
| Vitamin B₃      | Vitamin B₃     | 3a831 | g     | 4.00 - | 3.00 - | 3.00 - |
| Vitamin B₁₂     | Vitamin B₁₂    | -   | mg    | 20.00 - | 15.00 - | 15.00 - |
| Biotin          | Biotin         | -   | g     | 0.15 - | 0.12 - | 0.12 - |
| Cal-D-Pan       | Calcium Pantothenate | - | g   | 15.00 - | 12.00 - | 10.00 - |
| Nicotinic acid  | Nicotinic acid | -   | g     | 60.00 - | 50.00 - | 50.00 - |
| Folic acid      | Folic acid     | -   | g     | 2.00 - | 2.00 - | 1.50 - |
| Choline         | Choline chloride | - | g   | 0.50 - | 0.40 - | 0.35 - |
| Methionine      | Methionine     | 3.1.1 | g     | 3405.00 - | 3018.00 - | 2514.00 - |
| Threonine       | Threonine      | 3.3.1 | g     | 745.00 - | 726.00 - | 361.00 - |
| Lysine          | Lysine         | 3.2.3 | g     | 2812.00 - | 2831.00 - | 1779.00 - |
| Iodine          | Calcium iodate | E2 | g   | 1.00 1.59 | 1.00 1.59 | 1.00 1.59 |
| Selenium        | Sodium selenite | E8 | g   | 0.35 7.78 | 0.35 7.78 | 0.35 7.78 |
| Iron            | Ferrous sulphate | E1 | g   | 40.00 133.33 | 40.00 133.33 | 40.00 133.33 |
| Molybdenum      | Sodium molybdate | E7 | g   | 0.50 1.27 | 0.50 1.27 | 0.50 1.27 |
| Manganese       | Manganous oxide | E5 | g   | 100.00 161.29 | 100.00 161.29 | 100.00 161.29 |
| Copper          | Cupric sulphate | E4 | g   | 15.00 60.00 | 15.00 60.00 | 15.00 60.00 |
| Zinc            | Zinc oxide     | E6 | g   | 100.00 138.89 | 100.00 138.89 | 100.00 138.89 |
(Periodic acid–Schiff) staining with Schiff’s reagent to conduct morphometric analyses of the small intestine and to stain and count neutral goblet cells. Measurements were made using a DELTA EVOLUTION 300 microscope equipped with a digital camera by ToupuCam™. Measurements included: height and width of intestinal villi and intestinal crypt depth, and the number of neutral goblet cells in the intestine were calculated using the MultiScan-Base v. 18.03 (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 perimeter length of the villus was measured to calculate the surface area using the formula cited by Sakamoto et al. [14]. The depth of intestinal crypts was defined as the invagination depth between neighbouring intestinal villi and was measured between 10 villi [15]. The number of PAS- positive cells was calculated per 1 mm² of the intestinal villi surface area.

The data were analysed by means of a one-way analysis of variance using STATISTICA 10.0 PL. The arithmetic mean (X) and the standard error of the mean (SEM) were calculated. Significant differences between the groups were tested using Duncan’s Multiple Range Test.

Results

Symbiotic L. plantarum IBB3036 + lupin RFO (SYN2 group) significantly increased the body weight of chickens on the 1st day of life (P ≤ 0.05) (Table 2), while significantly higher feed intake was found in both injected groups compared to the Control group (P ≤ 0.05) (Table 3). FCE (Feed Conversion Ratio) did not significantly increase after synbiotics throughout the entire rearing period (Table 3). In all study groups, we also observed a very low mortality rate, which was below 2% [16].

While macroscopically evaluating the small intestines of the chickens, we found that the use of synbiotic 2 reduced the length (P ≤ 0.05 and P ≤ 0.01) and weight (P ≤ 0.01) of the duodenum on the 1st day of life of the chickens in relation to the other groups (Table 4). However, it did not affect the microstructure of the analysed section at this age, because in the duodenum, in both experimental groups (SYN1 and SYN2), a significantly higher and wider intestinal villi as well as a significantly larger absorbent surface of these villi were found in comparison with the Control group (P ≤ 0.01) (Table 5). Simultaneously, in the

Table 2 Effect of synbiotics treatment injected in ovo on d 12 of incubation on the body weight of broiler chickens

| Item              | Day | 1   | 42  |
|-------------------|-----|-----|-----|
| Body weight, g    |     |     |     |
| Control           |     | 40.3b| 3146 |
| SYN1              |     | 41.6a| 3111 |
| SYN2              |     | 41.6a| 3111 |
| SEM               |     | 0.1803| 9.7890 |

**Table 3** Effect of synbiotics treatment injected in ovo on d 12 of incubation on the feed intake and on the FCE (the efficiency of feed conversion) of broiler chickens

| Item       | Day  | 1-10 | 1-41 |
|------------|------|------|------|
| Feed intake, g |     |      |      |
| Control     |     | 247b | 4930 |
| SYN1        |     | 254  | 4940 |
| SYN2        |     | 258  | 4989 |
| SEM         |     | 1.3291| 24.4097 |
| FCE, g/g    |     |      |      |
| Control     |     | 1.21 | 1.60 |
| SYN1        |     | 1.24 | 1.59 |
| SYN2        |     | 1.29 | 1.60 |
| SEM         |     | 0.0146| 0.0067 |

**Table 4** Effect of synbiotics treatment injected in ovo on d 12 of incubation on the length and on the weight of intestine of broiler chickens

| Item       | Day | 1 | 42 |
|------------|-----|---|----|
| Length of intestine, cm |     |   |    |
| Duodenum Control |     | 9.60 ± 0.24| 28.67 ± 0.73 |
| SYN1 |     | 9.03 ± 0.24| 31.37 ± 0.59 |
| SYN2 |     | 8.28 ± 0.19| 30.10 ± 0.96 |
| Jejunum Control |     | 17.20 ± 0.65| 64.00 ± 1.93 |
| SYN1 |     | 16.60 ± 0.65| 70.20 ± 2.34 |
| SYN2 |     | 17.27 ± 0.49| 68.50 ± 1.86 |
| Ileum Control |     | 15.60 ± 0.41| 60.80 ± 1.73 |
| SYN1 |     | 14.77 ± 0.55| 68.30 ± 2.03 |
| SYN2 |     | 14.54 ± 0.47| 66.47 ± 2.15 |
| Weight of intestine, g |     |   |    |
| Duodenum Control |     | 0.44 ± 0.01| 17.44 ± 0.36 |
| SYN1 |     | 0.41 ± 0.01| 19.38 ± 0.39 |
| SYN2 |     | 0.38 ± 0.02| 17.22 ± 0.45 |
| Jejunum Control |     | 0.47 ± 0.03| 45.31 ± 1.39 |
| SYN1 |     | 0.43 ± 0.02| 48.12 ± 2.19 |
| SYN2 |     | 0.44 ± 0.01| 40.61 ± 1.72 |
| Ileum Control |     | 0.36 ± 0.02| 39.25 ± 1.37 |
| SYN1 |     | 0.32 ± 0.02| 41.45 ± 2.65 |
| SYN2 |     | 0.32 ± 0.01| 35.98 ± 1.86 |

**Table 5** Effect of synbiotics treatment injected in ovo on d 12 of incubation on the small intestinal length, weight, and weight of neutral goblet cells of broiler chickens

| Item       | Day | 1   | 42  |
|------------|-----|-----|-----|
| Length of |     |     |     |
| duodenum   |     |     |     |
| Control    |     | 9.60 ± 0.24| 28.67 ± 0.73 |
| SYN1       |     | 9.03 ± 0.24| 31.37 ± 0.59 |
| SYN2       |     | 8.28 ± 0.19| 30.10 ± 0.96 |
| Jejunum    |     | 17.20 ± 0.65| 64.00 ± 1.93 |
| SYN1       |     | 16.60 ± 0.65| 70.20 ± 2.34 |
| SYN2       |     | 17.27 ± 0.49| 68.50 ± 1.86 |
| Ileum      |     | 15.60 ± 0.41| 60.80 ± 1.73 |
| SYN1       |     | 14.77 ± 0.55| 68.30 ± 2.03 |
| SYN2       |     | 14.54 ± 0.47| 66.47 ± 2.15 |
| Weight of |     |     |     |
| duodenum   |     |     |     |
| Control    |     | 0.44 ± 0.01| 17.44 ± 0.36 |
| SYN1       |     | 0.41 ± 0.01| 19.38 ± 0.39 |
| SYN2       |     | 0.38 ± 0.02| 17.22 ± 0.45 |
| Jejunum    |     | 0.47 ± 0.03| 45.31 ± 1.39 |
| SYN1       |     | 0.43 ± 0.02| 48.12 ± 2.19 |
| SYN2       |     | 0.44 ± 0.01| 40.61 ± 1.72 |
| Ileum      |     | 0.36 ± 0.02| 39.25 ± 1.37 |
| SYN1       |     | 0.32 ± 0.02| 41.45 ± 2.65 |
| SYN2       |     | 0.32 ± 0.01| 35.98 ± 1.86 |
SYN1 group, a significant decrease in the depth of crypts relative to the SYN2 group was reported. In the same group, a reduction in the number of goblet cells on the surface of 1 mm² of the villi was observed, while the villi were significantly larger compared to the Control group (P ≤ 0.05) (Table 5). In turn, on the 42nd day of rearing after treatment of symbiotic 1 (SYN 1 group), a greater weight of the duodenum (P ≤ 0.01) than in the other groups and an increased length of this section (P ≤ 0.05) compare to the Control group were observed (Table 4). This beneficial effect was reflected by the numerically higher villi (no statistical differences) with a larger surface (P ≤ 0.01) in the duodenum in the SYN1 group compare to the Control group (Table 5).

In the jejunum on the 1st day of life, similar to the duodenum, in the SYN1 group, significantly higher villi than in the Control group, with a simultaneous decrease in the depth of crypts (P ≤ 0.01), and also the largest width of villi and their absorbent area (P ≤ 0.01) in comparison to the other groups were found (Table 6). On the 42nd day of life, in the jejunum, an increase of the villi height whilst reducing the crypt depth in the SYN2 group was found (P ≤ 0.01). In turn, in the SYN1 group, there were significantly more neutral goblet cells observed compared with the control group (P ≤ 0.05), (Table 6).

In the ileum of 1-day-old chickens, the widest villi (P ≤ 0.05) and the deepest crypts (P ≤ 0.01) were found in the SYN2 group. In the same group, there was also the least amount of neutral goblet cells in comparison to the other groups (P ≤ 0.05) (Table 7). Despite the lack of a significant effect of the applied synbiotics on the length and weight of the analysed sections of the small intestine on the 1st day of life, on the 42nd day of rearing, the length of the ileum (P ≤ 0.05) was significantly greater in both treatment groups compared to the Control group (Table 4). In both experimental groups, a deepening crypt depth was observed (P ≤ 0.01) (Table 7). Other parameters, such as villus height, villus width and villus surface area, were similar in all the groups.

**Discussion**

Studying the impact of injected synbiotics on the body weight of chickens throughout their rearing, we found a positive effect of symbiotic 2 on this parameter just in 1-day-old chickens. Opposite, in the research conducted by Bogucka et al. [11], no significant effect of any bioactive
substance injected in ovo on the 12th day of incubation on the body weight of 1-day-old chickens was shown.

Our study showed a differential effect of the applied synbiotics (synbiotic 1 - *L. salivarius* IBB3154 + galacto-oligosaccharides and synbiotic 2 - *L. plantarum* IBB3036 + Raffinose Family Oligosaccharides) on the microstructure of individual sections of a broiler chicken small intestine. We found a positive effect for both synbiotics given in ovo on the height, width and the absorptive surface of duodenum villi in comparison to the Control group on the 1st day of life of the chickens. In turn, on the 42nd day of the life of the chickens, only synbiotic 1 demonstrated a positive impact in comparison to the Control group on the villi width and villi surface area. A similar effect of synbiotics on the width of the villi was observed by Bogucka et al. [11], however, it did not reflect on the absorptive surface of the intestine. Additionally, in birds from the same group at the end of rearing, the deepest crypts were found. The positive effect of in ovo injection of the synbiotic composed of Bi2tos and *Lactobacillus lactis* subsp. *cremoris* IBB SC1 on the height of duodenum villi on the 1st day of life of chickens was also demonstrated in our previous studies [11]. According to Pluske et al. [17], longer villi and their greater absorptive surface area translate into better utilisation of feed, and thereby improve the health of the birds. Deeper crypts, in turn, indicate rapid tissue regeneration processes to permit the renewal of villi to normal sloughing or inflammation due to the presence of pathogens or their toxins [18]. Awad et al. [19], studying the effect of synbiotic supplementation, which is a combination of *Enterococcus faecium* probiotic and a prebiotic derived from chicory rich in inulin and immunomodulatory substances derived from sea algae, did not demonstrate significantly higher villi and significantly deeper crypts in the duodenum of 35-day-old broiler chickens. Similar results were obtained by Awad et al. in their further study in 2009 [20].

In the jejunum of 1-day-old chickens, a beneficial impact of synbiotic 1 on the microstructure was demonstrated, but this wasn’t maintained for 42 d. Similar results in relation to the heights of villi after in ovo administration of bioactive substances (prebiotic: inulin, synbiotics: inulin + *Lactobacillus lactis* spp. *lactis*, Bi2tos + *Lactobacillus lactis* spp. *cremoris*) in 1-day-old chickens were obtained by Bogucka et al. [11]. At the end of rearing in the group, in which was applied synbiotic 2, significantly higher intestinal villi were stated in comparison to the SYN1 group and the biggest surface area of these villi (in this case no significant differences) which may have an impact on better absorption of nutrients. In our previous studies [12], there were no significant effects of both prebiotics and synbiotics on the height of intestinal villi in 35-day-old broiler chickens.

Analysing the crypts depth of the jejunum in examined groups of birds their significant shortening was stated in both experimental groups (SYN1 and SYN2). The crypt depth is one of the indicators of the health and functional status of the intestine in chickens, and their size can be a measure of the intensity of intestinal epithelial cell renewal processes [21, 22]. Xu et al. [23] indicate that “The crypt can be regarded as the villus factory, and a large crypt indicates fast tissue turnover and a high demand for new tissue” so in our study the decreased crypt depth may indicates an efficient tissue turnover and good condition of the gut. Different results were obtained after in ovo injection of a synbiotic containing the prebiotics inulin and Bi2tos. Bioactives had a significant impact on the deepening intestinal crypts of 35-day-old broiler chickens [12]. Rehman et al. [18] examining the effect of the bioactive substance - inulin - on the jejunum histomorphology on the 35th day of the rearing of chickens and found significantly longer villi (*P* ≤ 0.05) and significantly deeper crypts (*P* ≤ 0.01) as a result of this prebiotic supplementation compared to the Control group.

The ileum is characterised by much shorter villi and less absorptive surface of these structures compared to the duodenum and jejunum, which may explain the reduction of

### Table 7 Effect of synbiotics injected in ovo on the ileum morphology of chickens at 1st and 42nd d of age

| Item                        | Day |
|-----------------------------|-----|
|                             | 1   | 42                             |
| **Villus height, μm**       |     |                                |
| Control                     | 295.04 ± 9.16 | 933.31 ± 43.10                |
| SYN1                        | 298.67 ± 10.86 | 847.78 ± 50.01                |
| SYN2                        | 289.81 ± 9.09 | 949.05 ± 38.70                |
| **Villus width, μm**        |     |                                |
| Control                     | 46.42 ± 1.29 | 162.69 ± 5.58                  |
| SYN1                        | 46.62 ± 1.34 | 161.18 ± 4.45                  |
| SYN2                        | 50.85 ± 1.66 | 161.32 ± 5.30                  |
| **Villus surface area, μm²**|     |                                |
| Control                     | 43,547.4 ± 2124.7 | 471,197.5 ± 20,933.6          |
| SYN1                        | 44,138.3 ± 2318.3 | 445,293.0 ± 27,184.3          |
| SYN2                        | 45,834.9 ± 1735.9 | 484,522.9 ± 26,619.7          |
| **Crypt depth, μm**         |     |                                |
| Control                     | 46.14 ± 0.77 | 91.28 ± 2.59                   |
| SYN1                        | 38.55 ± 0.85 | 116.81 ± 3.18                   |
| SYN2                        | 50.14 ± 0.98 | 117.39 ± 5.53                   |
| **No. of neutral goblet cells/1mm²** |     |                                |
| Control                     | 974 ± 50.59 | 263 ± 26.05                    |
| SYN1                        | 1129 ± 61.42 | 358 ± 40.66                    |
| SYN2                        | 678 ± 19.60 | 285 ± 37.21                    |

*Diff. *P* ≤ 0.05, *A-C* diff. *P* ≤ 0.01 between treatments (vertical), Means ± SEM representing 15 birds. SYN 1 - *L. salivarius* IBB3154 + Bi2tos, SYN 2 - *L. plantarum* IBB3036 + lupin RFOs.
the absorption of nutrients at this stage. Evidence of this was the fact that most of the digested food substances had already been absorbed in the upper parts of the small intestine, i.e. the duodenum and jejunum [24]. Injection of the applied synbiotics slightly affected the microstructure of ileum differently compared to the duodenum and jejunum. In 1-day-old chickens, synbiotic *L. plantarum* IBB3036 + lupin RFOs (SYN2 group) significantly increased villi width and crypt depth relative to the other groups. In the studies of Bogucka et al. [11], a synbiotic containing prebiotic Bi2tos significantly contributed to the shortening of the villi at the end of rearing, a synbiotic (SYN2 group) significantly increased villi width in 1-day-old chicken small intestine, i.e. the duodenum and jejunum [24]. Injection of the synbiotics – in ovo with a synbiotic containing prebiotic Bi2tos and probiotic bacteria *Lactococcus lactis* spp. *Cremoritis* in the study of Bogucka et al. [12]. Different results were obtained by Awad et al. [19, 20], which showed a significant extension of the villi and decreased crypt depth in the ileum after application of the synbiotic, although the substance was administered as a feed additive.

The mucus layer in the small intestine is secreted by goblet cells, which permits the excretion of gastric contents and form a protective barrier against mechanical and chemical injuries (ingested food, microorganisms, pathogens) [25, 26]. The percentage of goblet cells in the duodenum is the lowest and increases towards the large intestine throughout the rearing period. This is because a greater number of microbial organisms is present in the proximal colon [27]. This was also confirmed by our findings. On the 1st day after hatching - in groups injected with synbiotics – a significantly lower number of neutral goblet cells on the surface of 1 mm² of duodenum villi was found. At the end of rearing, in the SYN1 group, there were significantly more neutral goblet cells in the jejunum compared to the other groups. In our previous studies [12], the significant effect of the symbiotic (galactooligosaccharides + *L. lactis* subsp. *cremoris* 477) on an increase in the number of goblet cells in the same segment of the intestine was also shown. However, only a several-fold increase of goblet cells may indicate an infection of the intestinal pathogens [28]. According to Langhout et al. [29] and Sharma et al. [30], the effect for increasing the number of neutral goblet cells both in the jejunum and in the ileum may be due to delayed feed intake by the animals. This results in a reduction in the surface of intestinal absorption and an increase in the number of these cells, which was also confirmed in our results.

The above mentioned research and the results of our study present the different effects of synbiotics, depending on their composition and depending on the anatomical structure of GI tract. These two synbiotics: synbiotic 1 (*L. salivarius* IBB3154 + Bi2tos, Clasado Ltd.) and synbiotic 2 (*L. plantarum* IBB3036 + lupin RFOs) designed based on our in vitro results [16] presented the two types of synergism, i.e., between the prebiotic and probiotic components (synbiotic 1) and synergism between the two independent bioactive compounds and the host (synbiotic 2). In this last case RFO is less efficiently used by the probiotic bacteria, and it remains available to other indigenous stains of intestinal microbiota. In this situation, the prebiotic has a positive influence on the host organism through improvement of microbial balance in the intestines.

However, the positive effect of injected synbiotics was mainly indicated in the duodenum and jejunum.

**Conclusions/implications**

Summarising our research focused on the evaluation of the impact of synbiotics given in ovo on the production traits and histomorphology of a broiler chicken small intestine, we observed that synbiotic *L. salivarius* IBB3154 + Bi2tos, Clasado Ltd. and synbiotic *L. plantarum* IBB3036 + lupin RFOs beneficially affected the examined characteristics on the 1st and 42nd day of life. The obtained results allow us to conclude that the use of synbiotics significantly affect gut structure which should contribute to improvement in nutrient absorption by the gut.

**Abbreviations**

FCR: Feed conversion ratio; PAS: Periodic acid–Schiff; RFO: Raffinose family of oligosaccharides; SEM: The standard error of the mean

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**Availability of data and materials**

Authors do not wish to share the data due to the propriety nature of the data.
Authors’ contributions
AS, JB, AD data and results interpretation, performed the statistical analysis, histological analysis, drafted the manuscript. JB histological study coordination, aided in data interpretation. GEW data and results interpretation. KS participated in study design and coordination. MB designed research. All authors read and approved the final manuscript.
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The use of animals reviewed and approved by the local ethics committee for the animal experiments in Bydgoszcz.
Consent for publication
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The authors declare that they have no competing interests.

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References
1. Yang Y, Ji PA, Choc M. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. Worlds Poult Sci J. 2009;65:97–114.
2. Adl S, Magray SN. Impact and manipulation of gut microflora in poultry: a review. J Anim Vet Adv. 2012;11:873–7.
3. Sławińska A, Siwek M, Zylinska J, Bardowski J, Brazinska J, Guzlewicz KA, et al. Influence of synbiotics delivered in ovo on immune organ development and structure. Folia Biol (Krakow). 2014;62:277–85.
4. Ciesioka D, Guzlewicz P, Martínez-Villaluenga C, Pilarski R, Bednarczyk M, Guzlewicz K. Products and biopreparations from alkaloid-rich lupin in animal nutrition and ecological agriculture. Folia Biol (Krakow). 2005;53:59–66.
5. Johnston FA, Liu H, O’Connell T, Phelps P, Bland M, Tyczkowski J, et al. Applications in ovo technology. Poult Sci. 1997;76:165–78.
6. Williams C. In ovo vaccination for disease prevention. Int Poult Prod. 2007;15(8):7–9.
7. Williams C J. In ovo vaccination and chick quality. Int Hatch Prac. 2011;19(2):7–13.
8. Villaluenga CM, Wadersinski M, Pilarski R, Bednarczyk M, Guzlewicz K. Utilization of the chicken embryo model for assessment of biological activity of different oligosaccharides. Folia Biol ( Krakow). 2004;52:135–42.
9. Pilarski R, Bednarczyk M, Lisowski M, Rutkowski A, Beracki Z, Wadersinski M, et al. Assessment of the effect of α-galactosides injected during embryogenesis on selected chicken traits. Folia Biol (Krakow). 2005;53:13–20.
10. Jankowski J, Hodorwałd W, Sawicki M. Intestinal villus and crypt depth and maintenance of villus height and crypt depth, and enhancement of disaccharide digestion and monosaccharide absorption, in pigs fed on cows’ whole milk after weaning. Br J Nutr. 1996;76:409–22.
11. Rehmann H, Rosenkranz C, Böhm J, Zentek J. Dietary inulin affects the morphology but not the sodium-dependent glucose and glutamine transport in the jejunum of broilers. Poult Sci. 2007;86:118–22.
12. Awad W, Ghareeb K, Böhm J. Intestinal structure and function of broiler chickens on diets supplemented with a symbiotic containing Enterococcus faecium and oligosaccharides. Int J Mol Sci. 2008;9:2025–16.
13. Awad WA, Ghareeb K, Abdel – Raheman S, Böhm J. Effects of dietary inclusion of probiotic and symbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult Sci. 2009;88:49–56.
14. Fan Y, Croom J, Christiansen V, Black B, Bird A, Daniel L, et al. Jejunal glucose uptake and oxygen consumption in turkey pouls selected for rapid growth. Poult Sci. 1997;76:1738–45.
15. Samanya M, Yamauchi KE. Histological alterations of intestinal villi in chickens fed dried Bacillus subtilis Var. natto. Comp Biochem Physiol A Mol Integr Physiol. 2002;135:104–106.
16. Xu ZR, Hu CH, Xiao MS, Zhan KA, Wang MQ. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poult Sci. 2003;82(6):1030–6.
17. Yamauchi K, Incharoen T, Yamauchi K. The relationship between intestinal histology and function as shown by compensatory enlargement of remnant villi after midgut resection in chickens. Anat Rec. 2010;293:701–79.
18. Kim JJ, Khan W. Goblet cells and mucins: role in innate defense in enteric infections. Pathogens. 2013;2:55–70.
19. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. Nat Rev Cancer. 2004;4:45–60.
20. Karam SM. Lineage commitment and maturation of epithelial cells in the gut. Front Biosci. 1999;4:D286–98.
21. Kim YS, Ho SB. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep. 2010;12:319–30.
22. Langhout DJ, Schatte JB, van Leeuwen PV, Wielenga J, Tammenga S. Effect of dietary high- and low- methylated citrus pectin on the activity of the ileal microflora and morphology of the small intestinal wall of broiler chicks. Br Poult Sci. 1999;40:340–7.
23. Sharma R, Schumacher U. Morphometric analysis of intestinal mucins under different dietary conditions and gut flora in rats. Dig Dis Sci. 1995;40:2532–9.