Effect of synbiotics administered in ovo on microvascularization and histopathological changes in pectoral muscle and the biochemical profile of broiler chicken blood

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ABSTRACT The aim of this study was to investigate whether injecting synbiotics to the egg air chamber on d 12 of embryo incubation will affect the processes of angiogenesis, and thus the share of histopathological changes in superficial pectoral muscle, as well as Ca and P in blood of 42-day-old broiler chickens. The eggs containing viable embryos were injected with 0.2 mL suspension of 1/physiological saline, 2/SYN1 composed of galactooligosaccharide (**GOS**) (trade name: Bi^{2}tos®, Clasado Biosciences Ltd, UK) and *L. salivarius* or 3/SYN2 composed of RFO and *L. plantarum*. All birds were fed *ad libitum* the standard commercial feed mixtures: starter, grower, and finisher, with a constant access to water and feed. Injecting synbiotics *in ovo* on d 12 of the embryonal development significantly affected the blood supply to superficial breast muscle in broiler chickens. The highest density of capillaries in the muscle area under study and per muscle fiber were identified in the group of birds the egg air chamber of which was provided with synbiotic GOS+*L. salivarius*. Consequently, for the muscles of the birds injected with the same synbiotic there was found the highest share of normal fibers and least necrosis and splitting, as compared with the control. The conducted research confirms the relationship between the blood supply to the muscle and the occurrence of pathological changes. We have observed a positive effect of synbiotics on the microvascularization and the size of histopathological changes in the chicken muscle, which, from a practical perspective, can affect the health status and the meat quality. Blood biochemical analyses showed that the *in ovo* injection of synbiotics did not significantly affect the level of parameters, except for Ca and P. A significant increase in the concentration of these minerals in the blood of chickens injected with SYN1 could have a positive effect on the angiogenesis process.

Key words: broiler chickens, synbiotics, pectoral muscle microvascularization, histopathological changes, blood

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INTRODUCTION

Recently, the poultry industry has made significant progress in the breeding of broiler chickens, which results in shorter fattening and faster weight gain (2−3 times). However, the rapid growth of birds results in histological and biochemical modifications of the muscle tissue, the appearance of myopathy, ischemic, and degenerative muscle changes (capillaries not to catch up with the muscle fiber hypertrophy) (Bogucka et al., 2018), as well as high weight of chickens, increased density and all activities related to animal trade and extreme stress. The histopathological changes which occur in myopathies include, for example, fiber necrosis, giant fibers, splitting, connective, and fat tissue hypertrophy, due to considerable distances between fasciculi, degeneration, size heterogeneity, and leukocyte infiltrations (Aviagen, 2019). Those abnormalities are most often found in pectoral muscle, as the most valuable carcass part, and those are mostly white striping (**WS**), wooden breast (**WB**), and spaghetti meat (**SP**) (Petracci et al., 2015; Kuttappan et al., 2016). The occurrence of the DPM (deep pectoral myopathy), the ischaemic necrosis which develops in minor breast muscles, is less extensively reported (Barbut, 2019). Definitely an important factor affecting the normal muscle development is blood supply. Capillaries are little vessels

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which, surrounding the muscle fibers, ensure continuous supply of nutrients and oxygen. The number of these vessels depends on the function and structure of the muscle and its oxygen demand (the more active the muscle, the greater number of capillaries, and the larger diameter fibers are less supplied with blood) (Hudlicka, 1985). The aim of this study was to investigate whether injecting synbiotics to the egg air chamber on d 12 of embryo incubation will affect the processes of angiogenesis, and thus the share of histopathological changes in superficial pectoral muscle, as well as Ca and P in blood.

MATERIALS AND METHODS

Animals and In Ovo *Injections*

Cobb 500FF eggs (n = 5850) were randomly distributed to Petersime incubators (Drobex - Agro Ltd., Solec Kujawski, Poland) and allocated into 3 experimental groups: the control group, and 2 synbiotic groups: SYN1 and SYN2. On d 12 of egg incubation, the eggs containing viable embryos were injected into the egg air chamber with 0.2 mL suspension of 1/ physiological saline, 2/ SYN1 composed of prebiotic galactooligosaccharide GOS (trade name: Bi^{2}tos®, Clasado Biosciences Ltd, UK) and probiotic in a form of *Lactobacillus salivarius* - IBB 3154 or 3/ SYN2 composed of RFO (lupin-based oligosaccharides of the raffinose family) and probiotic in a form *Lactobacillus plantarum* - IBB 3036. Combining the prebiotic with probiotic resulted in 2 components of synbiotics: 10^5 cfu of probiotic with 2 mg of prebiotic. Briefly, a total of 2,040 roosters were moved for rearing. Animals from each experimental group (n = 80 per group) were randomly chosen and split into 8 replicate pens, subjected to sampling. The remaining experimental males (n = 600 per group) were split into other 8 replicate pens (75 individuals per pen) to evaluate the effects on growth performance. The animals were reared compliant with the guidelines of the Polish Local Ethics Commission (No 22/2012. 21.06.2012) and with the animal welfare recommendations provided for in Directive 2010/63/EU. Birds were reared in pens (3.75 m^2) on a litter with a stocking density of 17.33 birds/m^2. All birds were fed *ad libitum* the standard commercial feed mixtures: starter (d 1−14), grower (d 15−30), finisher (d 31 to 41). The birds had free access to water and feed. Here, the study material was collected from 15 randomly selected cockerels per group on d 42 of rearing, directly after the slaughter survey.

Slaughter Surveys

On d 42 of age, 15 birds from each group (n = 45) were randomly selected individually weighed (after a fasting period of 12 h) and transported within 1 h (including careful catching and loading) to a commercial poultry slaughterhouse. After careful unloading and hanging in randomized order, all the birds were electrically stunned and slaughtered.

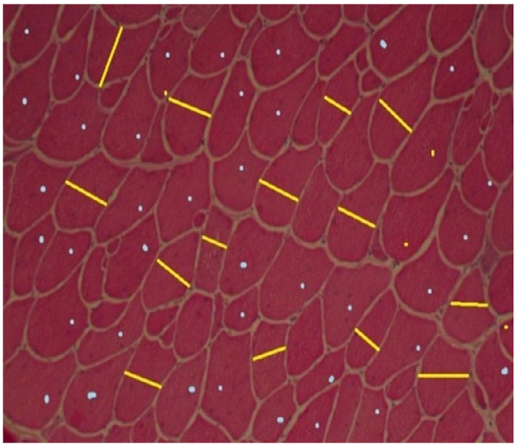

Figure 1. Image of the cross-section of the superficial pectoral muscle in 42-day-old Cobb 500 FF broiler chickens. H+E staining. Method of assaying the number and diameter of muscle fibers (mag. 100×).

Breast Muscle Microstructure Evaluation

Directly after slaughter of birds, samples of the breast muscle were taken from the left side of the breast, following the orientation of the muscle fibers. The histological slides used to evaluate the breast muscle microstructure of broiler chickens were developed using the frozen technique. The superficial breast muscle samples were cut in the cryostat (Thermo Shandon/Thermo Fisher Scientific, UK), into 10-μm-thick sections. The breast muscle microstructure was evaluated with 2 staining techniques with haematoxylin and eosin staining (H+E) and alkaline phosphatase staining. H+E staining was applied to measure the diameter and number of muscle fibers (Figure 1) and to specify the percentage share of normal fibers and the histopathological changes found in breast muscle of broiler chickens. The alkaline phosphatase staining was used to counting total number of capillaries and number of capillaries per muscle fiber (Figure 2). The histopathological analysis covered the quantitative determination of fiber necrosis (Figure 3), giant fiber

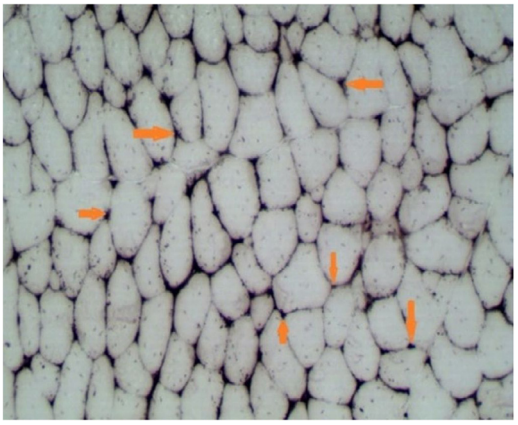

Figure 2. Image of the cross-section of the superficial pectoral muscle in 42-day-old Cobb 500 FF broiler chickens. Alkaline phosphatase staining to identify the presence of capillaries (mag. 100×).

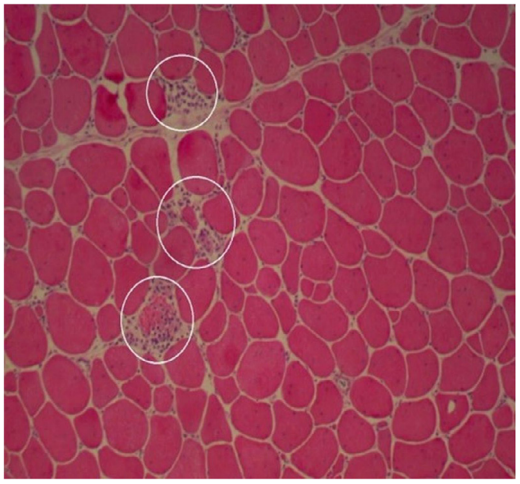

Figure 3. Image of the cross-section of the superficial pectoral muscle in 42-day-old Cobb 500 FF broiler chickens. H+E staining. Necrosis with phagocytosis (mag. 100×).

(Figure 4), and fiber splitting (Figure 5). The normal and pathological fibers were counted and the result was given as a percentage of the total number of fibers.

Microscopic Image Analysis

The microscopic analysis of microstructure of breast muscles was made on 1.5 mm^2 of the muscle surface area. From each muscle, two microscopic images from both staining on 100× magnification were randomly selected and saved on a computer disk. The microscopic images evaluation was done using MultiScan Base v. 18.03 software *(Computer Scanning System II, Warsaw, Poland)* and the Delta Optical Evolution 300 microscope equipped with a ToupCam^{TM} camera.

Biochemical Analyses

Blood samples (n = 45) were collected from 15 birds per group on d 42 of rearing. The blood was sampled into sterile glass tubes without any anticoagulant and centrifuged after clot formation in order to separate the serum. Ca, P levels in serum were assayed using a MINDRAY BS-120 biochemical analyzer and reference reagents (STAMAR, Dabrowa Gornicza, Poland).

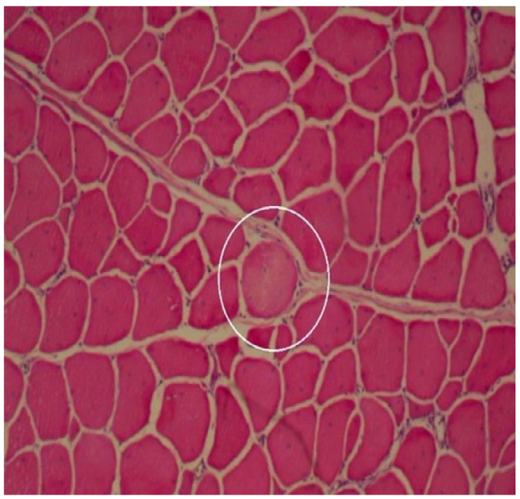

Figure 4. Image of the cross-section of the superficial pectoral muscle in 42-day-old Cobb 500 FF broiler chickens. H+E staining. Giant fibre (mag. 100×).

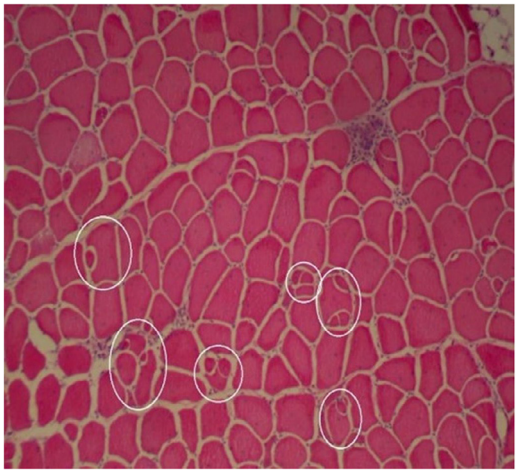

Figure 5. Image of the cross-section of the superficial pectoral muscle in 42-day-old Cobb 500 FF broiler chickens. H+E staining. Splitting (mag. 100×).

Statistical Analysis

The results were exposed to one-way analysis of variance (**ANOVA**), with STATISTICA AXAP v.13.0 MR.1. With that program, the arithmetic means and the standard error means (**SEM**) were calculated. The significance of differences across the bird groups was verified with the Tukey test (HSD).

RESULTS AND DISCUSSION

Body Weight of Birds and Histology Assays

In the present study the mean body weight in respective groups of 42-day-old chickens ranged from 2,986.3 g to 3,040.7 g (Table 1). No significant differences between the groups were found. Bogucka et al. (2018) also reported the higher breast muscle weight in the experimental group treated with synbiotic containing RFO prebiotic and Lavipan probiotic. The muscle weight of mammals and birds is mostly determined by the number and the diameter of muscle fibers. During postnatal growth, the number of muscle fibers remains almost unchanged and the gain in muscle mass during that time is caused by increases in muscle fiber length and thickness. In the present study the injection with bioactive substances did not have a significant effect on the number and the diameter of muscle fibers in the superficial pectoral muscle of birds (Table 1).

Bird muscles include 2 types of muscle fibers: white and red. They differ from each other not only in their structure and enzymatic activity, but also with the level of blood supply. The turkey study demonstrated that the number of capillaries per mm^2 per muscle area and per muscle fiber, respectively, is higher in *biceps femoris*

Table 1. Body weight, traits of the microstructure of the superficial pectoral muscle and biochemical blood indices in the chickens injected *in ovo* with synbiotics.

Trait	Group		
	C $\overline{X} \pm SEM$	SYN 1 $\overline{X} \pm SEM$	SYN 2 $\overline{X} \pm SEM$
Bird body weight [g]	3,033.0 ± 110.6	3,040.7 ± 130.9	2,986.3 ± 99.6
Number of muscle fibers /1.5 mm^2	300.13 ± 26.71	294.13 ± 26.17	317.33 ± 28.24
Muscle fiber diameter [um]	47.96 ± 1.42	50.48 ± 1.50	49.13 ± 1.46
Number of capillaries/1.5 mm^2	427^{b} ± 28.22	531^{a} ± 24.54	496^{ab} ± 18.81
Number of capillaries per muscle fiber	1.14^{b} ± 0.07	1.47^{a} ± 0.04	1.34^{ab} ± 0.03
Normal fibers (%)	95.53^{b} ± 0.52	97.32^{a} ± 0.43	96.08^{ab} ± 0.49
Fibre necrosis (%)	1.50^{a} ± 0.30	0.62^{b} ± 0.04	1.21^{ab} ± 0.32
Giant fibers (%)	0.07 ± 0.03	0.06 ± 0.03	0.05 ± 0.02
Splitting (%)	2.90^{a} ± 0.34	2.00^{b} ± 0.44	2.67^{ab} ± 0.33
Ca (mg/dL)	10.88^{b} ± 0.18	11.86^{a} ± 0.41	11.75^{ab} ± 0.16
P (mg/dL)	8.08^{b} ± 0.22	9.43^{a} ± 0.38	8.40^{b} ± 0.23

C – control group; SYN1 – group injected with synbiotic GOS (Bi^{2}tos®, Clasado Ltd + *Lactobacillus salivarius* - IBB 3154), SYN2 – group injected with synbiotic (RFO + *Lactobacillus plantarum* – IBB 3036).

Number of individuals in each group n = 15.

^{ab}Statistically significant differences for P < 0.05

muscle, where red fibers with oxidative metabolism prevail than in the breast muscle where white fibers with glycolytic metabolism dominate (Elminowska - Wenda et al., 2005). However, oxidative capacity is not only factor that determines capillarity. Capillary density is not only related to the type of muscle fibers, but also to the size of the fiber. As reported Hudlicka (1985), if the chicken growth and development is normal, the number of capillaries surrounding single muscle fibers increases. An intensive broiler chicken production makes the number of capillaries supplying the muscle with nutrients insufficient, which can lead to degenerative and ischemic changes in muscles (Sośnicki and Wilson, 1991). A significantly higher ($P < 0.05$) number of capillaries was found in group SYN1, as compared with the control group (Table 1). Similarly, in group SYN1, significantly more capillaries per muscle fiber were recorded, as compared with the control group. Similar results were reported by Bogucka et al. (2018). The synbiotic (RFO + Lavipan) was added to the feed, which significantly ($P < 0.05$) increased the blood supply to muscles, both per muscle fiber and for the specific area. According to Sośnicki and Wilson (1991), the quantitative capillary disorders result in some changes, for example, atrophy, degeneration, or necrosis. The decrease in the number of capillaries can be due to a decreased blood or oxygen flow rate in chicken muscles (Hudlicka, 1985).

The percentage of normal fibers and histopathological changes in breast muscle in chickens are provided in Table 1. In this study, bioactive substances had a positive effect on the percentage of normal muscle fibers of the broiler chickens. The most of normal fibers were noted in group SYN1 in which were observed the largest capillarity. Similarly, Bogucka et al. (2018) have demonstrated that synbiotics had a positive effect on the share of normal fibers in breast muscle.

Muscle tissue is very sensitive to damaging factors. Histopathological changes can be due to hypoxia, inflammations or to electrolyte disorders as well as calcium excess inside the cells or accumulation of connective tissue (Bogucka et al., 2018). Muscle fiber necrosis often accompanies green muscle disease due to a high stress in birds prior to slaughter. The intense fluttering with wings increases the blood pressure, which, in turn, limits blood supply to muscles and may lead to the occurrence of fiber destruction. In the present study, it was observed that SYN1 resulted in a significant ($P < 0.05$) decrease in the share of fiber necrosis in breast muscle as compared with the control group. An advantage of poultry production is providing a high slaughter yield within a very short period. The mostly desired carcass part is the breast muscle, which can account for about 30% of carcass weight. A fast growth and development of the muscle is essential from the economic perspective. However, it can lead to a pathological hypertrophy of muscle fibers; hence, possible giant fibers with the area of even 5-fold greater than the normal fibers. After getting the final product, the meat shows a low pH value, with observable PSE change symptoms (Bogucka et al., 2018). In the present study, in the muscles of all the chicken groups, giant fibers were noted, however, their share was low. Besides, in the present study the least percentage of split fibers was found in the SYN1 group. Similar results have been reported by Bogucka et al. (2018). Splitting is a change to be especially considered in the muscles of fast-growing chickens. The longitudinal splitting of muscle fibers is one of the degenerative changes which can be identified in transverse sections of the muscle tissue. Usually, in a split fiber it is possible to determine 2 to 5 daughter fibers. It seems that one of the reasons for splitting is an elevated stress or "a functional overload" of some overgrown fibers (personal communication).

Biochemistry

Our research showed a significantly higher level of Ca in the SYN1 group compared to the control. Similar results have been reported by Śliżewska et al. (2019) –after the synbiotic application. Our study has recorded a significantly higher P content in group SYN1, as compared with group SYN2 and the control. The increase in the concentration of Ca and P in the blood serum may be the result of a decrease in intestinal pH caused by the presence of lactic, acetic, or propionic acid due to the used synbiotic, leading to an increased degree of absorption of minerals (Śliżewska et al., 2019).

CONCLUSIONS

In our study, we observed that the *in ovo* injection of synbiotics had a positive effect on the capillarity of the pectoral muscles of chickens, and due to their better

nutrition and oxygenation, we noted a lower share of degenerative changes, such as muscle fiber necrosis or splitting. Adequate microvascularization is a fundamental factor in the growth and working of skeletal muscle and also their regeneration. A significant increase in the concentration of Ca and P in the blood of chickens injected with SYN1 could have a positive effect on the level of microvascularization, because these minerals support angiogenesis processes (Saghiri et al., 2015). Ca has a positive effect on endothelial cells (**EC**) proliferation of capillaries and is the only element whose level increases in response to pro-angiogenic factors. P affects genes with pro-angiogentic activity. However, research is needed to elucidate the mechanisms of action synbiotics, as these are complex processes based on the interaction of many types of cells.

DISCLOSURES

The authors declare that we have no conflict of interest. Research project financed from the National Science Centre in Poland, grant no. 2019/35/B/NZ9/03186 and research topic of the Faculty of Animal Breeding and Biology, University of Science and Technology (PBS) in Bydgoszcz fund.

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