Effect of *in ovo* administration of different synbiotics on carcass and meat quality traits in broiler chickens

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ABSTRACT The aim of this study was to examine the effect of *in ovo* synbiotic administration on slaughter performance and meat quality traits of broiler chickens. On day 12 of incubation, 5,850 eggs (Cobb 500 FF) were randomly divided into 3 experimental groups and automatically injected *in ovo* with physiological saline (control, C) and 2 different synbiotic formulations (SYN1: *Lactobacillus salivarius* + galactooligosaccharides; SYN2: *Lactobacillus plantarum* + raffinose family oligosaccharides). After hatching, 240 males were randomly chosen (80 chicks per group) and split into 8 replicate pens (10 birds per pen). At 42 d of age, 15 birds per treatment were weighed and slaughtered. *In ovo* synbiotic administration had a low effect on investigated traits, but depends on the kind of synbiotic administered. Both synbiotic formulations did not affect final BW, weight, and yield of carcass and pectoral muscle (PM); likewise, physicochemical properties (pH, color, water holding capacity), intramuscular collagen properties, and cholesterol content of PM were not affected by treatment. Synbiotic administration reduced ($P = 0.061$) the lipid content compared with C group, markedly ($P < 0.05$) with synbiotic SYN2. Meat from SYN1 birds displayed a higher ($P < 0.01$) content of saturated fatty acids (SFA), lower monounsaturated fatty acids ($P < 0.05$ compared only to SYN2), and lower ($P < 0.01$ and $P < 0.05$) polyunsaturated fatty acids (PUFA), n-6 PUFA and n-3 PUFA compared to C and SYN2 groups. The ratio of n-6 to n-3 PUFA was affected by the synbiotic administration ($P = 0.039$). Meat from C and SYN2 groups displayed a higher ($P < 0.01$) ratio of PUFA to SFA and lower ($P < 0.01$) atherogenic and thrombogenic indices compared to SYN1. In conclusion, this study has shown that *in ovo* administration of synbiotics did not negatively affect slaughter performance and physicochemical properties of meat. However, meat from C and SYN2 birds showed a preferable fatty acid profile, with a positive effect on nutritional properties of chicken meat.

Key words: synbiotics, *in ovo*, broiler chicken, meat quality, fatty acids

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INTRODUCTION

It is imperative that today's poultry industries be proactive in improving animal health and growth, and the safety of poultry products in a sustainable way. Probiotics, prebiotics, and synbiotics are proposed as natural and safe alternatives to antibiotic growth promoters in order to solve the intestinal problems of birds through the modulation of the composition and function of the intestinal microbiota, therefore improving health and performance of birds (Yang et al., 2009; Ducatelle et al., 2015). The synbiotics, developed to overcome possible survival difficulties for probiotics, are the synergistic combination of probiotics and prebiotics (de Vrese and Schrezenmeir, 2008). Synbiotics tend to act by 2 divergent mechanisms of synergism: synergistic to each other or synergistic to the host (reviewed in Dunislawska et al., 2017). For an effective microbiome stimulation, pre-, pro-, and synbiotics need to be delivered as early as possible, since the microflora of the hatched chicks is very poorly developed. To date, the main production practice in poultry is to provide in-feed or in-water doses of bioactives as soon as possible after hatch, to help newly hatched chicks to rapidly establish a healthy gut microbiome. However, implementation of *in ovo* technology for bioactives delivery allows to provide the growing embryo with the potent microbiome stimulant as early as on 12th day of embryonic incubation. Such procedure results in improvement of beneficial bacteria count in gut with a life lasting effects, and positively influences growth and development of the adult chickens (Villaluenga et al., 2004; Bednarczyk et al., 2011, 2016; Maiorano et al., 2012, 2017; Tavaniello et al., 2018). Furthermore, the *in ovo* synbiotics administration is a promising approach

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in chicken immune system enhancement, as it combines advantages of the synergism between prebiotics and probiotics and by early administration into the embryo; it supports development of immune organs (Slawinska et al., 2014; Madej et al., 2015; Madej and Bednarczyk, 2016), influencing also the immunomodulatory gene expression in gut-associated lymphatic tissue (Dunislawska et al., 2017). It has also been shown that the *in ovo* delivery of pre- or synbiotics significantly increases the total activity of pancreatic enzymes (amylase, lipase, and trypsin) (Pruszynska-Oszmalek et al., 2015) and influences the histological structure of chicken intestinal tissue (Bogucka et al., 2016; Sobolewska et al., 2017). It is clear that the intrinsic crosstalk between the microbiome and its host is not limited only to the gastrointestinal tract but it involves all the organisms, affecting also growth performance and meat quality traits. Regarding the effect of synbiotics (delivered *in ovo* or in feed) on growth performance and meat quality different are the studies yielding sometimes contradictory results. Some studies (Awad et al., 2009; Mookiah et al., 2014; Ghasemi et al., 2016; Cheng et al., 2017), using different kind of synbiotics supplemented in feed, reported beneficial effects on growth performance, feed efficiency, carcass, and some meat quality traits. Differently, other authors found minimal (Maiorano et al., 2012) or none (Jung et al., 2008; Midilli et al., 2008) effect of synbiotics, delivered *in ovo* or in feed, on growth performance and on meat quality traits. These results reveal the complexity of the interaction taking place in the gastrointestinal tract, also related to the kind of bioactive administered. Very few information exists regarding the effects of synbiotics on fatty acid composition in broiler chicken meat (Ghasemi et al., 2016). Animal studies showed that probiotics and prebiotics can positively act on lipid metabolism (Fukushima and Nakano, 1996; Trautwein et al., 1998). Synbiotics can improve (increase) the ratio of polyunsaturated to saturated fatty acids in chicken breast meat (Ghasemi et al., 2016); therefore, we expected that synbiotics could modify the fatty acid profile with relevant effect in terms of human health. There is need to fill the existing gap in the knowledge on the impact of *in ovo* treatments on meat quality traits. Therefore, this study was designed to evaluate the effect of *in ovo* administration of 2 different synbiotic formulations (previously selected based on in vitro and in vivo results by Dunislawska et al., 2017) on carcass and meat quality traits (physico-chemical characteristics, intramuscular collagen properties, total lipids and cholesterol contents, fatty acid composition) in broiler chickens.

MATERIALS AND METHODS

Birds and Experimental Design

A total of 5,850 Cobb 500 FF eggs were incubated in a commercial hatchery (Drobex-Agro Sp. z o.o., Solec Kujawski, Poland) a Petersime incubator (vision version, Petersime NV, Zulte, Belgium) for the *in ovo* protocol. On day 12 of incubation, after candling, the fertile eggs were randomly divided into 3 experimental groups. The eggs were injected with bioactive solution directly into the egg air chamber with the aid of a dedicated automatic system (Bednarczyk et al., 2011). The injected formulations were either 0.2 mL of a physiological saline solution (Control, **C**), 0.2 mL of a synbiotic formulation containing 2 mg/embryo of Bi^{2}tos (Clasado Biosciences Ltd., Jersey, UK), a nondigestive *trans*-galactooligosaccharides (**GOS**) from milk lactose digested with *Bifidobacterium bifidum* NCIMB 41171, enriched with 10^5 bacteria cfu/embryo of *Lactobacillus salivarius* IBB3154 (**SYN1**), or 0.2 mL of a synbiotic formulation containing 2 mg/embryo of raffinose family oligosaccharides (**RFO**), isolated and purified from seeds of lupin *Lupinus luteus* L. *cv.* Lord, (Ciesiolka et al., 2005), enriched with 10^5 bacteria cfu/embryo of *Lactobacillus plantarum* IBB3036 (**SYN2**). The 2 synbiotic formulations and doses were optimized based on in vitro results (hatchability and growth curve) as described in detail by Dunislawska et al. (2017). After the injection, each hole was sealed with a natural glue and the incubation was continued until hatching. Post hatching, 240 males were randomly chosen (80 chicks per group) and split into 8 replicate pens (10 birds per pen). In the same trial, separate pens (n = 75 chickens per pen) with 8 replications per each experimental group were reared in the chicken house to evaluate the effects on growth performance, as reported by Dunislawska et al. (2017). Birds were reared in a commercial farm (Piast, Olszowa Experimental Unit 0161, Poland) according to the regulations and permission of the local Ethical Commission (decision No.22/2012 21.06.2012) and in accordance with the animal welfare recommendations of European Union directive 86/609/EEC. Animals were fed ad libitum with the standard commercial feed mixtures (Table 1).

Slaughter Surveys and Physico-Chemical Analyses

At 42 d of age, 15 randomly chosen birds per group were individually weighed (after a fasting period of 12 h) and transported within 1 h (including careful catching and loading) to a commercial poultry slaughterhouse. Birds were electrically stunned and slaughtered. After evisceration, the hot carcass weight (without head and feet) was recorded, and carcass yield was calculated. In addition, the breast muscle was removed from all carcasses and its percentage was calculated based on hot carcass weight. On the right pectoral muscle (PM), pH and color were recorded at 45 min ($\mathbf{pH_{45}}$) and 24 h ($\mathbf{pH_{24}}$) post mortem. pH was measured using a portable pH meter (FiveGo, Mettler-Toledo, Switzerland) equipped with a penetrating glass electrode. Tri-stimulus color coordinates (lightness, $\mathbf{L^*}$;

Table 1. Composition and nutritive value of the diets.

Item (% unless noted)	Period		
	1 to 10 d	11 to 21 d	22 to 41 d
Ingredients			
Maize (7.75% CP)	61.16	65.99	67.93
Soybean meal (47.75% CP)	33.09	28.16	26.03
Soybean oil	1.75	2.06	2.77
Limestone flour	1.10	0.98	0.70
NaCl	0.20	0.20	0.23
Dicalcium phosphate	1.605	1.504	1.337
Vitamin-mineral premix[1]	1.10	-	-
Vitamin-mineral premix[2]	-	1.10	-
Vitamin-mineral premix[3]	-	-	1.10
Chemical analysis, %			
DM	88.87	88.94	88.91
CP	21.00	19.00	18.00
Lipid	4.61	4.99	5.72
Crude fiber	2.69	2.63	2.59
Ash	5.82	5.40	5.02
Calculated analysis			
ME, MJ/kg of diet	12.72	13.00	13.30
Lysine, %	1.32	1.19	1.05
Methionine, %	0.65	0.58	0.52
Methionine+cysteine, %	0.98	0.89	0.82
Threonine, %	0.86	0.78	0.71
Tyrosine, %	0.25	0.22	0.21
Calcium, %	0.90	0.84	0.76
Available P, %	0.71	0.68	0.63
Sodium, %	0.16	0.16	0.15
Salt, %	0.35	0.35	0.35
Potassium, %	0.93	0.83	0.79

[1]Supplied per kilogram of diet: vitamin A, 13,000 IU; vitamin D3, 5,000 IU; vitamin E, 80 mg; vitamin B1, 3 mg; vitamin B2, 9 mg; vitamin B6, 4 mg; vitamin B12, 20 μg; vitamin K, 3 mg; biotin, 0.15 mg; Ca pantothenate, 15 mg; nicotinic acid, 60 mg; folic acid, 2 mg; cholinechloride, 0.50 mg; lysine, 2812 mg; methionine, 3405 mg; threonine, 745 mg; Ca iodate, 1 mg; Se, 0.35 mg; Fe, 40 mg; Mo, 0.50 mg; Mn, 100 mg; Cu, 15 mg; Zn, 100 mg.

[2]Supplied per kilogram of diet: vitamin A, 10,000 IU; vitamin D3, 5,000 IU; vitamin E, 50 mg; vitamin B1, 2 mg; vitamin B2, 8 mg; vitamin B6, 3 mg; vitamin B12, 15 μg; vitamin K, 3 mg; biotin, 0.12 mg; Ca pantothenate, 12 mg; nicotinic acid, 50 mg; folic acid, 2 mg; cholinechloride, 0.40 mg; lysine, 2831 mg; methionine, 3018 mg; threonine, 726 mg; Ca iodate, 1 mg; Se, 0.35 mg; Fe, 40 mg; Mo, 0.50 mg; Mn, 100 mg; Cu, 15 mg; Zn, 100 mg.

[3]Supplied per kilogram of diet: vitamin A, 10,000 IU; vitamin D3, 5,000 IU; vitamin E, 50 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B6, 3 mg; vitamin B12, 15 μg; vitamin K, 3 mg; biotin, 0.12 mg; Ca pantothenate, 10 mg; nicotinic acid, 50 mg; folic acid, 1.5 mg; cholinechloride, 0.35 mg; lysine, 1779 mg; methionine, 2514 mg; threonine, 361 mg; Ca iodate, 1 mg; Se, 0.35 mg; Fe, 40 mg; Mo, 0.50 mg; Mn, 100 mg; Cu, 15 mg; Zn, 100 mg.

redness, $\mathbf{a}^*$; yellowness, $\mathbf{b}^*$) were detected on the right PM muscle using a Chroma Meter CR-300 (Italia s.r.l., Milano). Reflectance measurements were performed after the samples had oxygenated in air for at least 30 min by which time measurements were stable (Skrlep and Candek-Potokar, 2007), taking 3 readings for each sample. Water holding capacity (**WHC**) was measured on the right PM 24 h after chilling using filter paper (Whatman No.1) press method (Grau and Hamm, 1953) and was expressed as free water in meat. The left PM was vacuum packaged and stored frozen (–20°C) until chemical analysis for intramuscular collagen properties, total lipids, fatty acid composition, and cholesterol content.

Intramuscular Collagen Properties

Approximately 100 g of PM (wet weight) was thawed at room temperature, trimmed of fat and epimysium, lyophilized for 48 h, weighed, and hydrolyzed in Duran tubes (Schott AG, Mainz, Germany) in 5 mL of 6 N HCl at 110°C for 18 to 20 h (Etherington and Sims, 1981) for determination of hydroxyproline (Woessner, 1961) and crosslinking. The analyses were carried out in duplicate. Intramuscular collagen concentration was calculated, assuming that collagen weighed 7.25 times the measured hydroxyproline weight (Eastoe and Leach, 1958) and expressed as micrograms of hydroxyproline per milligram of lyophilized tissue. Hydroxylysylpyridinoline (**HLP**) concentration, the principal nonreducible crosslink of muscle collagen and highly correlated with the thermal stability of collagen (McCormick, 1999), was determined using the procedure described by Eyre et al. (1984). A Kontron HPLC (Kontron Instruments, Milan, Italy) model 535, equipped with a Luna C18 column (250 × 4.6 mm × 5 μm; Phenomenex, Torrance, CA), was used. The concentration of HLP residues in the samples was calculated based on the concentration of collagen in each hydrolyzate, assuming that the molecular weight of collagen was 300,000 and the molar fluorescence yield of pyridoxamine (internal standard) was 3.1 times that of HLP (Eyre et al., 1984). Crosslink concentration was expressed as moles of HLP per mole of collagen and also as μg HLP/mg of lyophilized tissue.

Measurement of Muscle Cholesterol

Cholesterol was extracted using the method of Maraschiello et al. (1996) and then quantified by HPLC. A Kontron HPLC (Kontron Instruments, Milan, Italy) model 535, equipped with a Kinetex C18 reverse-phase column (150 × 4.6 mm × 5 μm; Phenomenex, Torrance, CA), was used. The HPLC mobile phase consisted of acetonitrile: 2-propanol (55:45, vol/vol) at a flow rate of 1.0 mL/min. The detection wavelength was 210 nm. The quantitation of muscle cholesterol content was based on the external standard method using a pure cholesterol standard (Sigma, St. Louis, MO).

Total Lipids and Fatty Acid Composition

Lipid extraction from breast muscle was performed using the method described by Folch et al. (1957). Fatty acids (**FA**) were quantified as methyl esters (**FAME**) using a gas chromatograph GC Trace 2000 (ThermoQuest EC Instruments) equipped with a flame ionization detector (260°C) and a fused silica capillary Column (Omegawax 320, Phenomenex, Torrance, CA) 30 m × 0.32 mm × 0.25 μm film thickness. Helium was used as carrier gas. The oven temperature program was 120°C for 1 min then increasing at 5°C/min up to 230°C where it was maintained for 20 min. The individual FA

peaks were identified by comparison of retention times with those of FAME authentic standards run under the same operating conditions. Results were expressed as percentage of the total FA identified. To assess the nutritional implications, the ratio of n-6 to n-3 FA (**n-6/n-3**) and the ratio of PUFA to SFA (**P/S**) were calculated. Moreover, in order to evaluate the risk of atherosclerosis and the potential aggregation of blood platelets, the atherogenic index (**AI**) and the thrombogenic index (**TI**) were calculated, according to the formulas suggested by Ulbricht and Southgate (1991).

Statistical Analyses

Data were analyzed by one way analysis of variance (SPSS Inc., 2010). Scheffé's test was applied to compare the differences among means.

RESULTS AND DISCUSSION

Productive Performance and Carcass Traits

Growth performance results (BW, feed intake, and feed conversion ratio) regarding all reared chickens (2040) are reported by Dunislawska et al. (2017). Final BW and carcass traits of slaughtered chickens (15 per group) are reported in Table 2. According to the overall results reported by Dunislawska et al. (2017), also for the slaughtered chickens both synbiotic formulations did not influence ($P > 0.05$) final BW (ranging from 2,986 to 3,040 g). Similar results were found by Maiorano et al. (2012) using a homemade synbiotics injected *in ovo* (RFO + *Lactococcus lactis* ssp. *lactis* SL1 or RFO + *Lactococcus lactis* ssp. *Cremoris* IBB SC1) or a commercial synbiotic (Duolac, containing *Lactococcus acidophilus* + *Streptococcus faecium* with lactose). On the contrary, Madej et al. (2015), found that *in ovo* delivery of 2 synbiotics (inulin + *Lactococcus lactis* subsp. *lactis* and GOS + *Lactococcus lactis* subsp. *Cremoris)*, significantly improved final BW gain. In a feeding trial, Cheng et al. (2017) found that supplementation with a synbiotic increased ADG in broilers from 1 to 42 d of age. Similarly, Awad et al. (2009) observed an increased BW with the dietary inclusion of synbiotic compared to control and probiotic-fed broilers. The variable effects of synbiotics (delivered *in ovo* or in-feed) on growth performance, obtained in different trials and experimental conditions, reveal not only the existence of several variables (endogenous factors related to animals, type and doses of synbiotic, environmental factors, etc.), but also the complexity of the interactions taking place in the gastrointestinal tract. In addition, these effects are more complex for synbiotics containing at least 2 bioactives, which exert different effects on the organism when they are delivered alone than when they are delivered together (Dunislawska et al., 2017). Regarding carcass traits, no effects ($P > 0.05$) of *in ovo* injection of synbiotics were found for values of carcass weight, carcass yield (ranging from 63.49 to 64.32%), and PM weight and yield (ranging from 30.46 to 31.31%). These results are partially in line with findings of Maiorano et al. (2012), who observed lower carcass yield and higher PM yield in the group, which was injected *in ovo* with commercial synbiotic. In contrast, taking into account the effect of seaweed and GOS prebiotics injected *in ovo*, Maiorano et al. (2017) recorded an increasing effect of these bioactives on carcass weight and yield, breast weight, and lack of marked impact on breast yield. Awad et al. (2009) found that BW, carcass yield percentage, and feed conversion rate were significantly increased by the dietary inclusion of the synbiotic (Biomin IMBO) compared with the control and probiotic (*Lactobacillus* sp.)-fed chickens. The contradictory responses of broilers to pre-, pro-, or synbiotics (*in ovo* or in feed) could be due to different strains and amount of microorganism, as well as their survivability in the feed, type of prebiotic administered, inclusion level, and dietary nutrients levels.

Table 2. Effect of *in ovo* injection of different synbiotic preparations on final BW and slaughter traits of Cobb broiler chickens.

Groups[1]	C	SYN 1	SYN 2	SEM	*P*-value
Final BW, g	3033	3040	2986	17.70	0.380
Carcass weight, g	1951	1930	1916	14.87	0.632
Carcass yield, %	64.32	63.49	64.15	0.31	0.527
Pectoral muscle weight, g	610.4	588.9	587.9	8.12	0.451
Pectoral muscle yield, %	31.31	30.46	30.65	0.31	0.520

[1]Groups: C = Control, *in ovo* injection of physiological saline; SYN 1 = *Lactobacillus salivarius* + Bi^{2}tos; SYN2 = *Lactobacillus plantarum* + Lupin RFO.
SEM = standard error mean.

Physico-Chemical Characteristics and Intramuscular Collagen

Results of the effects of *in ovo* administration of synbiotics on pH, color, and WHC are presented in Table 3. pH_{45} (ranging from 6.36 to 6.50) and pH_{24}

Table 3. Effect of *in ovo* injection of different synbiotic preparations on physico-chemical properties of breast muscle from Cobb broiler chickens.

Groups[1]	C	SYN1	SYN2	SEM	*P*-value
pH_{45}	6.36	6.50	6.36	0.04	0.228
pH_{24}	5.85	5.91	5.91	0.02	0.256
Color 45 min					
L*	44.82^{AB}	46.76^{A}	44.00^{B}	0.35	0.003
a*	4.58	3.58	4.02	0.28	0.348
b*	6.91	7.72	6.02	0.36	0.182
Color 24 h					
L*	50.81	49.52	49.95	0.42	0.449
a*	5.08	4.46	4.77	0.31	0.737
b*	11.39	10.85	11.33	0.39	0.839
WHC, %	13.35	13.16	13.22	0.28	0.965

[1]Groups: C = Control, *in ovo* injection of physiological saline; SYN 1 = *Lactobacillus salivarius*+ Bi^{2}tos; SYN2 = *Lactobacillus plantarum* + Lupin RFO.
SEM = standard error mean.
[A,B]Means within a row lacking a common superscript differ ($P < 0.01$).

(ranging from 5.85 to 5.91) were not significantly affected ($P > 0.05$) by both synbiotic treatments. The pH_{24} values observed in our study are within the acceptable range for commercial poultry meats, with no evidence of pre-slaughter stress. Our results are in agreement with Maiorano et al. (2012), who reported that *in ovo* synbiotics administration did not significantly affect pH. On the contrary, Cheng et al. (2017) found that the dietary supplementation with synbiotic, prepared from probiotics (*Bacillus subtilis, Bacillus licheniformis, and Clostridium butyricum*) and prebiotics (yeast cell wall and xylooligosaccharides), increased significantly the pH_{24} of breast muscle in Arbor Acres Plus broiler chickens; the value found (6.08) was elevated and higher compared with the pH_{24} values observed in the present study. Considering the fact that the synbiotic concept combines efficacious probiotic strains with specific prebiotic compounds, it is justified to compare our results with studies on effects of pro- and prebiotics on meat quality. In case of probiotics, in the study described by Zheng et al. (2015) the influence of *Enterococcus faecium* on meat pH values (at 45 min and 24 h post mortem) was observed; both values were higher in treated groups ($P < 0.05$) compared with control. Although taking into account influence of prebiotics on meat pH, Park and Park (2011) noted that dietary inulo-prebiotic reduced ($P < 0.05$) pH of chicken meat. The pH influences important meat characteristics, e.g., WHC and color (Puolanne et al., 2002). Regarding the color parameters, only the lightness of meat (L^*) measured at 45 min post mortem was affected by the type of synbiotics injected: meat from chickens of SYN1 group was lighter ($P < 0.01$) compared with that of SYN2 group. No differences ($P = 0.449$) were detected for L^* measured at 24 h among experimental groups. Also, redness (a^*) and yellowness (b^*) of meat measured at 45 min and 24 h post mortem were found to be similar ($P > 0.05$) among groups. Similarly, Cheng et al. (2017) did not find any effect of synbiotic dietary administration on meat color of breast muscle from Arbor Acres Plus broiler chickens. The L^*, a^*, and b^*values observed at 24 h, when the color is stabilized, are within the acceptable range for commercial meats and were not affected by *in ovo* synbiotic treatments. The WHC of meat products is a very important quality attribute which has an influence on product yield, which in turn has economic implications, but is also relevant in terms of eating quality (Cheng and Sun, 2008). It is important to note that water loss reduces the meat nutritional value because some nutrients may be lost in the exudate, resulting in a less tender meat, which is worse in flavor (Pelicano et al., 2003). This adverse effect was not the case observed in our study, because the percentage of free water was low (approximately 13%). *In ovo* synbiotic treatment did not affect ($P > 0.05$) WHC of meat. Similarly, Cheng et al. (2017) did not find any effect of synbiotic dietary administration on WHC of breast muscle from Arbor Acres Plus broiler chickens.

Collagen is a protein abundant in connective tissue and is a factor contributing to the variation in meat tenderness and texture (Purslow, 2005) of different species, including birds (Baeza et al., 1998; Maiorano et al., 2011, 2012; Tavaniello et al., 2014; Sirri et al., 2016). Collagen molecules are bound together through intermolecular crosslinks that help to provide structure and strength. These crosslinks are initially reducible, but over time are replaced by mature, thermally stable, and less soluble crosslinks (McCormick, 1999; Maiorano et al., 2015). In the current study, IMC concentration (ranging from 15.62 to 15.98 μg/mg of lyophilized muscular tissue), collagen maturity (ranging from 0.103 to 0.139 mol HLP/mol of collagen), and muscle HLP concentration (ranging from 2.26 to 2.85 μg/mg of lyophilized muscular tissue) were not significantly ($P > 0.05$) influenced by *in ovo* synbiotic administration. Of particular interest in the present study are the overall low values for HLP in the PM, quite similar with those reported by Maiorano et al. (2012), indicating the immaturity of the newly deposited collagen. Nowadays, this condition occurs very often in the modern fast-growing lines due to the drastic increase of PM myofiber diameters with the progressive decrease in connective tissue spacing between myofibers (endomysium) and muscle fiber bundles (perimysium) (reviewed in Velleman et al., 2017). In severe condition this altered structure integrity lead to a new muscular abnormality termed as spaghetti meat (Baldi et al., 2018). Anyway, in the present study no evidence of muscle defects was detected.

Total Lipids, Cholesterol Content and Fatty Acid Composition

Total lipids, cholesterol content, and fatty acid composition are reported in Table 4. Lipid content in meat is one of the most important factors for consumer acceptance and may impose meat buying decisions. In this study, treatment with synbiotics reduced ($P = 0.061$) the lipid content compared with C group, markedly ($P < 0.05$) with synbiotic SYN2. Reduction of total lipid level by the use of synbiotics can have positive effects in terms of human health. Different studies evidenced the hypolipidemic and hypocholesterolemic properties of synbiotics in different species, broilers (Ghasemi et al., 2014), quails (Sharifi et al., 2011), and pigs (Liong et al., 2007). Mista et al. (2017) suggested that probiotics beneficially regulate lipid metabolism. Some studies reported that probiotics added to broiler diets can reduce fat contents in carcass and muscles (Santoso et al., 1995; Homma and Shinohara, 2004; Kalavathy et al., 2006). In the present study, cholesterol content, ranging from 39.99 to 41.88 mg/100 g, was similar ($P > 0.05$) among the experimental groups. Similarly, Maiorano et al. (2012) did not find any significant effect of *in ovo* injected synbiotics and prebiotic on cholesterol content of breast muscle in Ross broiler

Table 4. Effect of *in ovo* injection of different synbiotic preparations on total lipids and cholesterol contents, fatty acid composition (% of total fatty acids), and nutritional ratios in breast muscle from Cobb broiler chickens.

	Groups[1]				
Item[2]	C	SYN1	SYN2	SEM	*P*-value
Total lipids (g/100 g)	1.51^{a}	1.38^{ab}	1.18^{b}	0.06	0.061
Cholesterol (mg/100 g)	41.43	41.88	39.99	1.70	0.439
Fatty acids					
C 14:0	0.79^{A}	0.54^{B}	0.83^{A}	0.03	0.001
C 14:1	0.58^{Ab}	0.33^{Bb}	0.46^{a}	0.02	0.001
C 16:0	18.94^{B}	23.30^{A}	18.84^{B}	0.38	0.001
C 16:1	2.54^{B}	1.99^{C}	3.13^{A}	0.09	0.001
C 18:0	7.95^{B}	9.19^{A}	7.31^{B}	0.16	0.001
C 18:1	33.75	34.00	33.96	0.17	0.815
C 18:2 n-6	24.19^{A}	21.95^{B}	23.88^{A}	0.21	0.001
C 18:3 n-3	2.25^{Aa}	1.47^{B}	2.05^{Ab}	0.06	0.001
C 18:3 n-6	0.34^{A}	0.23^{B}	0.21^{B}	0.01	0.001
C 20:0	0.73^{A}	0.26^{B}	0.84^{A}	0.05	0.001
C 20:1	0.67^{a}	0.55	0.51^{b}	0.02	0.009
C 20:2 n-6	0.62	0.52^{B}	0.67^{A}	0.02	0.002
C 20:3 n-6	0.60	0.48^{b}	0.64^{a}	0.03	0.027
C 20:4 n-6	3.85^{B}	3.15^{C}	4.62^{A}	0.12	0.001
C 20:5 n-3	0.77^{A}	0.56^{Bb}	0.70^{a}	0.02	0.001
C 22:5 n-3	0.67	0.73	0.74	0.02	0.408
C 22:6 n-3	0.74	0.75^{a}	0.62^{b}	0.02	0.020
Partial sum					
ΣSFA	28.40^{B}	33.29^{A}	27.83^{B}	0.44	0.001
ΣMUFA	37.54^{ab}	36.87^{b}	38.06^{a}	0.20	0.052
ΣPUFA	34.05^{A}	29.84^{B}	34.12^{A}	0.35	0.001
Σn-6	29.61^{A}	26.33^{B}	30.02^{A}	0.29	0.001
Σn-3	4.44^{A}	3.51^{Bb}	4.10^{a}	0.09	0.001
Nutritional ratios					
n-6/n-3	6.75	7.58	7.44	0.14	0.039
P/S	1.21^{A}	0.90^{B}	1.23^{A}	0.03	0.001
Atherogenic index	0.31^{B}	0.38^{A}	0.31^{B}	0.01	0.001
Thrombogenic index	0.59^{B}	0.78^{A}	0.58^{B}	0.02	0.001

[1]C = Control, *in ovo* injection of physiological saline; SYN1 = *Lactobacillus salivarius* IBB3154 + Bi2tos; SYN2 = *Lactobacillus plantarum* IBB3036+ Lupin RFO.

[2]SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; P/S = PUFA/SFA ratio.

SEM = standard error mean.

[a,b]Means within a row lacking a common superscript differ ($P < 0.05$).

[A,B]Means within a row lacking a common superscript differ ($P < 0.01$).

chickens. Kalavathy et al. (2006) found a lower cholesterol content in carcass and liver but a similar content in breast muscle in probiotic-fed chickens compared to control ones. In different studies, it was speculated that *Lactobacillus* bacteria reduces serum cholesterol level by deconjugating bile salts in the intestine, thereby preventing them from acting as precursors in cholesterol synthesis (reviewed in Kalavathy et al., 2006).

In terms of human health, the FA composition of meat products is an important parameter of meat quality. In comparison with meat from ruminants and pork, poultry fat is characterized by a high degree of polyunsaturated fatty acids (**PUFA**), in particular n-3 PUFA (Valsta et al., 2005; D'Alessandro et al., 2012). Concerning the FA profile (Table 4), total monounsaturated fatty acids (**MUFA**) were the most abundant FA (ranging from 36.87 to 38.06%) followed in descending order by PUFA (ranging from 29.84 to 34.12%) and saturated fatty acids (**SFA**) (ranging from 27.83 to 33.29%). To our knowledge, limited information is available in literature on the effect of synbiotics on modification in the FA pattern of lipids in chicken meat. Total SFA content in breast muscle was markedly increased ($P < 0.01$) with SYN1 treatment in comparison with C and SYN2, due to the higher contents of palmitic (C16:0) and stearic (C18:0) acids; in addition, SYN1 group showed lower ($P < 0.01$) proportions of myristic (C14:0) and arachidic (C20:0) acids, even if the amount of each acid was less than 1%. Ghasemi et al. (2016) did not find any effect of dietary synbiotic (Biomin IMBO at different inclusion levels) on the FA profile of breast muscle of 42-day-old chickens. Similarly, Kalavathy et al. (2006) did not find any effect of feed supplementation with a mixture of *Lactobacillus* cultures on total FA composition of breast muscle of Avian-43 broiler chickens. The total MUFA content was influenced ($P = 0.052$) by *in ovo* synbiotic treatment. In particular, the administration of SYN1 reduced the amount of MUFA compared with the SYN2 administration (–1.2%; $P < 0.05$), but not with respect to C group ($P > 0.05$). This is essentially due to the lower ($P < 0.01$) content of palmitoleic acid (C16:1) in SYN1 group compared to C and SYN2 groups. No significant treatment effect was found for linoleic acid (C18:1), which is quantitatively the most abundant MUFA (ranging from 33.75 to 34.00%; $P > 0.05$). Kalavathy et al. (2006) found a reduction of oleic acid in probiotic-fed chickens, suggesting that supplementation of *Lactobacillus* cultures may reduce the synthesis or absorption of oleic acid. On the other hand, Yang et al. (2010) did not observe significant effects on the total MUFA content in breast muscle of chickens supplemented with *C. butyricum* in their diet. Differently, Zhou et al. (2009) detected a greater concentration of total MUFA in breast muscle of broiler chickens fed with a diet supplemented with chitooligosaccharide prebiotic (obtained by chemical and enzymatic hydrolysis of polychitosan). In the current experiment, the total PUFA content was markedly reduced ($P < 0.01$) by the administration of SYN1 compared to C and SYN2, whereas similar values ($P > 0.05$) were found between C and SYN2. Conflicting results are reported in literature regarding the effect of probiotics or prebiotic on PUFA content in breast meat from broiler chickens. Hossain et al. (2012) detected a lower PUFA content in breast meat from broiler chickens fed diets supplemented with herbal extract (*Alisma canaliculatum*) combined with different strains of probiotic microorganisms. On the contrary, Salma et al. (2007) found an increase in total PUFA concentration when chickens were fed 0.04% of *Rhodobacter capsulatus* for 6 wk. Differently, Zhou et al. (2009) did not find any effect for a diet supplemented with prebiotic. Similarly, Ghasemi et al. (2016) did not find any effect of dietary synbiotic (Biomin IMBO at different inclusion levels) on total PUFA content of breast muscle of 42-day-old chickens. These rather contradictory results may be related to differences in the type of bioactive used, related to their different modulation effect on lipid metabolism, through the different

kind and concentration of short-chain fatty acids produced by the microbial fermentation in the gut. In general, SYN1 treatment significantly reduced the content of both total n-6 and n-3 PUFA compared with C and SNY2 ($P < 0.05$ and $P < 0.01$). The precursor of the n-6 family, the linoleic acid (C18:2), quantitatively the most concentrated n-6 PUFA, was lower ($P < 0.01$) in SYN1 compared to SYN2 and C groups; also the arachidonic acid (C20:4) was detected in the lowest amount ($P < 0.01$) in SYN1 group compared to the other groups. Significant differences ($P < 0.01$) in the other n-6 long-chain PUFA were observed among groups. Hossain et al. (2012) found a reduction of n-6 PUFA in breast muscle of chickens supplemented with a combination of *Alisma canaliculatum* and multiprobiotic strains in their diet. On the contrary, they did not find significant differences in α-linolenic concentration; instead, they observed a decrease in arachidonic and docosahexaenoic acids, among total PUFA, and n-6 levels compared with the control group. The precursor of the n-3 family, α-linolenic acid (C18:3), decreased ($P < 0.01$ and $P < 0.05$) with synbiotic administration. Regarding the n-3 long-chain PUFA, eicosapentaenoic acid (**EPA**, C20:5 n-3) was reduced with SYN1 compared to C ($P < 0.01$) and SYN2 ($P < 0.05$) groups; docosahexaenoic acid (**DHA**, C22:6 n-3) was higher in SYN1 in comparison with SYN2 ($P < 0.05$); and, docosapentaenoic acid (**DPA**, C22:5 n-3) was not affected ($P > 0.05$) by both synbiotic treatments. *In ovo* treatment with synbiotics affected all nutritional ratios. In general, n-6/n-3 ratio was slightly higher ($P = 0.039$) in synbiotic groups in comparison with C group. In general, poultry meat is characterized by the highest n-6/n-3 ratio compared to other types of meat. Broiler fat contains a higher level of unsaturated FA compared to beef and lamb, and linoleic acid is the most predominant PUFA (Valsta et al., 2005). Nowadays, epidemiological studies suggested that the major risk factor for atherosclerosis and coronary heart diseases was found to be due to a high n-6/n-3 ratio rather than to a high intake of cholesterol and the consequent hypercholesterolemia (reviewed in Milicevic et al., 2014). It is thus recommended that n-6/n-3 ratio should be less than 4.0 (Department of Health, 1994). However, according to the nutritional changes described in the western diet, this ratio has now increased to be within the range of 10:1 to 20:1 (Patterson et al., 2012). The reduction of linoleic acid content with the *in ovo* injection of SYN1 reduced the levels of PUFA and consequently P/S ratio. In fact, meat from SYN1 had lower value of P/S ratio compared with C and SYN2 groups (0.90 vs. 1.21 and 1.23%, respectively; $P < 0.01$). The P/S ratio is known to be a measure of the propensity of the diet to influence the occurrence of coronary disease (Wood et al., 2003). A higher P/S ratio is recommended, since dietary intake of unsaturated fatty acids has been shown to reduce the risk of cardiovascular diseases and possibly the incidence of some cancers, asthma, and diabetes among other conditions (Milicevic et al., 2014). The recommended ratio of P/S should be above 0.4. Since some meats naturally have a P/S ratio of around 0.1, such a meat has been considered as causative for imbalanced intake of fatty acids by today's consumers (Wood et al., 2003). The values of P/S ratio obtained in the current research were higher compared to those described by Milicevic et al. (2014) (ranging between 0.39 and 0.97 in breast muscle).

The atherogenic and thrombogenic indices represent a criterion for evaluating the likelihood of FA to have atherogenic or thrombogenic properties, respectively. Administration of SYN1 resulted in an increase ($P < 0.01$) of both AI and TI compared with C and SYN2 (AI: 0.38 vs. 0.31 and 0.31, respectively; TI: 0.78 vs. 0.59 and 0.58, respectively) (Table 4). Nevertheless, the values found in the current study can be considered as low, which is in agreement with Ulbricht and Southgate (1991), and similar to other meat types poultry (Hubbard broiler chicks, AI: 0.56 ± 0.13, TI: 0.55 ± 0.14, Laudadio and Tufarelli, 2010; Graylag geese, AI: 0.43 ± 0.02, He et al., 2015; Japanese quails, AI: 0.33 ± 0.01, TI: 0.80 ± 0.013, Tavaniello et al., 2017). Low AI and TI indicate pro-health status of a meat in terms of fatty acid composition and the antioxidant capacity (Attia et al., 2017).

In conclusion, synbiotics had effects only on some traits considered, depending on the type of synbiotic formulation administered. However, neither final BW nor slaughter performance was affected negatively by synbiotics treatment, as well as physico-chemical properties of meat (pH, color at 24 h, WHC), intramuscular collagen properties, and cholesterol content. Even if it seems that the application of synbiotics does not have a great impact on performance traits, which are one of the main economic traits, their effect is especially linked to improving chickens' health, increasing their resilience thus reducing enteric diseases and mortality with a great economic impact (Dunislawska et al., 2017; Sobolewska et al., 2017). Moreover, the *in ovo* technology allows an accurate and precise delivery of bioactives at very low doses in the early embryonic stage, replacing a prolonged and costly in feed supplementation. Total lipids and FA composition of breast meat were affected by synbiotics treatment. In fact, synbiotics reduced the lipid content, markedly with synbiotic SYN2, which could have positive effects in terms of human health. The group treated with SYN1 displayed a meat with a higher amount of total SFA, lower amount of MUFA, PUFA, total n-6 and n-3 PUFA, P/S, and higher AI and TI, whereas SYN2 did not affect FA profile or nutritional properties of chicken meat, as compared to C group. It can be assumed that there is a crosstalk between gut microbiome and lipid and FA metabolism, even if it is difficult to identify the mechanism linked with the metabolic changes mentioned above. In light of this, it is necessary to deepen the research and to simplify biological models in order to better understand the effect of these compounds on animal metabolism (e.g., lipid and FA metabolic pathways). In addition,

further research is needed to improve knowledge concerning the effect of *in ovo* delivery of synbiotics on performance and meat quality of broiler chickens in both experimental and field conditions.

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