Interference of age and supplementation of direct-fed microbial and essential oil in the activity of digestive enzymes and expression of genes related to transport and digestion of carbohydrates and proteins in the small intestine of broilers

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ABSTRACT The objectives of this study were to describe alterations that age and dietary inclusion of direct-fed microbial (DFM) *Bacillus subtilis* (BS) and a specific essential oil (EO) blend (carvacrol, cinnamaldehyde, cineol, and pepper extract) causes in the activity of digestive enzymes (maltase: MALT; aminopeptidase-N: APN; intestinal alkaline phosphate: IAP) and expression patterns of genes related to transport (oligopeptide transporter gene: *SLC15A1*; Na^+-dependent glucose and galactose transporter gene: *SLC5A1*; Na^+-independent glucose, galactose, and fructose transporter gene: *SLC2A2*; ATPase, Na^+/K^+ transporting gene: *ATP1A1*) and digestion (aminopeptidase-N gene: *ANPEP*; maltase-glucoamylase gene: *MGAM*; Sucrase-isomaltase gene: *SI*) of carbohydrates and proteins in the small intestine of broilers. Also, the objective was to analyze if growth performance of broilers is affected by supplementation (BS and EO blend). Day-old male broiler chicks (n = 1,320) were assigned to 5 treatments. Diets included a basal diet (BD) as a negative control (CON); experimental diets were BD + BS; BD + BS + EO; BD + EO; BD + antibiotic growth promoter (AGP) avilamycin was the positive control. Performance was evaluated between 1 to 42 d. Transcript abundance of transport-related genes and digestion-related genes were assayed by RT-qPCR and determined at d 7, 21, and 42. MALT-, APN-, and IAP-specific activities were determined at d 7, 21, and 42. Broilers fed BS had greater *SLC15A1* mRNA abundance compared to CON, while EO and AGP were related to higher activities of IAP and APN. Analysis over time revealed higher abundance of *MGAM, SLC2A2*, *SLC15A1*, *SLC5A1* and *SI* mRNA at d 42 when compared to d 7. Activity of IAP decreased after d 7 and activity of MALT increased with age. The current study suggests that age had effect over carbohydrate and protein transport and carbohydrate digestion. The supplementation of BS DFM hade evident effect over protein transport and that the use of EO in the diet enhanced the activities of carbohydrate and protein digestion, reflecting improvement in digestive and transport physiology of birds. Changes performed by BS DFM and EO did not favor performance.

Key words: *Bacillus subtilis*, broiler chicken, feed additives, intestine, probiotics

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INTRODUCTION

Antibiotic growth promoters (**AGPs**) have been largely used in the poultry industry to improve performance through intestinal microbiota modulation (Dibner and Richards, 2005; Niewold, 2007). However, there is a global trend towards rearing broilers on AGP-free diets due to concerns about public, food, and environment safety (Diarra and Malouin, 2014). Alternatives, including direct-fed microbials (**DFMs**) and essential oils (**EOs**), have been reported to benefit the growth and increase nutrient digestibility of broilers (Jang et al., 2007; Basmacioğlu Malayoğlu et al., 2010; Wang and Gu, 2010).

DFM mediate their action in part via competitive exclusion, whereby beneficial bacteria prevents colonization of potentially harmful bacteria (Lee et al., 2003; Vilà et al., 2010). EOs have antimicrobial

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properties, and antioxidant and anti-inflammatory functions have also been described (Brenes and Roura, 2010). Both DFM and EOs have also been reported to increase digestive enzyme activity in broilers (Windisch et al., 2008; Murugesan et al., 2014a; Murugesan et al., 2014b). In contrast, others have not supported any positive effects of DFM or EOs on performance (Lee et al., 2003; Martin et al., 2012), gut morphometry (Windisch et al., 2008; Martin et al., 2012), or nutrient utilization (Lee et al., 2003; Martin et al., 2012).

Additionally, most experiments that assessed the development of digestive function in chickens concentrated their efforts in the first 2 wk of life (Uni et al., 2003; Gilbert et al., 2007). Consequently, assessing gene expression and enzyme activity profile until slaughter age could shed light on diet manipulation for optimizing nutrient utilization.

Therefore, the objectives of this study were to: 1) Describe the alterations that dietary inclusion of the DFM *Bacillus subtilis* (**BS**) and a specific EO blend (carvacrol, cinnamaldehyde, cineol, and pepper extract) causes in the activity of digestive enzyme maltase (**MALT**) and expression patterns of genes related to digestion (*MGAM* and *SI*) and transport (*SLC5A1*, *SLC2A2*, and *ATP1A1*) of carbohydrates in the small intestine mucosa of broilers; 2) describe the alterations that dietary inclusion of DFM BS and a specific EO blend causes in the activity of digestive enzyme aminopeptidase-N (**APN**) and expression patterns of genes related to digestion (*ANPEP*) and transport (*SLC15A1* and *ATP1A1*) of proteins in the small intestine mucosa of broilers; 3) describe the alterations that age causes in the activity of digestive enzyme MALT and expression patterns of genes related to digestion (*MGAM* and *SI*) and transport (*SLC5A1, SLC2A2* and *ATP1A1*) of carbohydrates in the small intestine mucosa of broilers; 4) describe the alterations that age causes in the activity of digestive enzyme APN and expression patterns of genes related to digestion (*ANPEP*) and transport (*SLC15A1* and *ATP1A1*) of proteins in the small intestine mucosa of broilers; 5) analyze if growth performance of broilers is affected by dietary inclusion of BS and a specific EO blend.

MATERIAL AND METHODS

Birds, Performance, and Sample Collection

This experiment was conducted according the rules of the Animal Ethics Committee (CEUA) of the School of Agriculture and Veterinary Science, São Paulo State University, SP, Brazil.

Day-old male Cobb 500^{TM} broiler chicken (n = 1320) were purchased from a commercial hatchery. At d 1, the birds were assigned in a completely randomized design to 5 dietary treatments. Diets included a basal diet as a negative control (**CON**); basal + BS; basal + BS + EO; basal + EO; basal; + avilamycin (an AGP) as positive control. Each treatment consisted of 6 pen replicates with 44 birds per pen replicate, totaling 30 experimental units.

Table 1. Ingredient and calculated nutrient composition of the basal starter and finisher diets.

Ingredients (%)	Starter (d 1 to 21)	Grower (d 22 to 42)
Corn	55.451	61.297
Soybean meal	37.216	30.809
Soybean oil	3.202	4.375
Dicalcium phosphate	1.669	1.235
$CaCO_3$	0.934	0.844
NaCl	0.490	0.452
DL- methionine	0.325	0.279
L-Lysine·HCl	0.243	0.240
Vitamin and mineral premix[1]	0.400	0.400
Antioxidant	0.010	0.010
Avilamycin[2]	.....	.....
Essential oil[3]	.....	.....
Bacillus subtilis[4]	.....	.....
Kaolin	0.060	0.060
Total	100	100
Calculated nutrient composition		
Metabolizable energy (kcal/kg)	3.020	3.174
Crude protein (%)	21.600	19.133
Lysine (%)	1.253	1.097
Methionine (%)	0.609	0.536
Methionine + cysteine (%)	0.902	0.801
Tryptophan (%)	0.244	0.209
Calcium (%)	0.867	0.713
Available phosphorus (%)	0.424	0.333
Sodium (%)	0.213	0.198

[1]Broiler/FOCUS vitamin-mineral premix provided by Agroceres Multimix. Guaranteed analysis (per kg of premix): Vitamin A, 2,000,000 IU; vitamin D3, 600,000 IU; vitamin E, 3,000 IU; vitamin K3, 500 mg; vitamin B1, 600 mg; vitamin B2, 1,500 mg; vitamin B6, 1,0000 mg; vitamin B12, 3,500 mcg; niacin, 10 g; pantothenic acid, 3,750 mg; folic acid, 250 mg; choline, 86.6 g; iron, 12.5 g; manganese, 17.5 g; zinc, 12.5 g; copper, 25 g; iodine, 300 mg; selenium, 50 mg.

[2]Surmax-200® (ELANCO, Division Eli Lilly Canada Inc., 150 Research Lane, Suite 120, Guelph, Ontario, N1G 4T2). The addition of Surmax-200 in replacement for inert (kaolin) provided 0.01 g avilamycin activity/kg diet.

[3]Activo® (GRASP, Curitiba, Brazil). Activo was added in replacement for inert (kaolin), in the proportion of 0.125 g/kg diet.

[4]Biotop® (FATEC, Arujá, São Paulo, Brazil). The addition of Biotop in replacement for inert (Koalin) provided 5×10^8 CFU/Kg diet.

The experimental diets (Table 1) were formulated using corn and soybean meal to be isoenergetic and meet all the minimum nutrient requirements of the birds (Rostagno et al., 2011). A 2-phase feeding program was used with a starter diet given from d 1 to d 21 and a grower diet given from d 22 to 42. BS, EO, and avilamycin were added to the basal diet in replacement for Kaolin and at 500, 125 and 50 g/ton, respectively. The BS was a commercial product that provided 5.0×10^8 CFU/Kg diet (Biotop®, FATEC S.A., Sao Paulo-SP, Brazil). The EO was a commercial preparation containing carvacrol, cinnamaldehyde, cineol and pepper extract (Activo®, GRASP Ltda., Curitiba-PR, Brazil). The avilamycin (Surmax-200®, Eli Lilly, Indianapolis, IN) activity/kg diet was 0.01 g. The birds were reared in pens of 1.5 width × 2.5 length (3.75 m^2) on floor with wood shavings, with a depth of 10 cm in an environmentally controlled room where ambient temperatures were maintained at thermoneutrality according to bird

Table 2. Primers used for real-time PCR.[1]

Gene	GenBank ID	Description or gene function	Real-time PCR, sense/antisense
ANPEP	NM_204861.1	Aminopeptidase N, digestive enzyme	TGGTTGAATGAGGGCTTTGC/ GGTCGCCATCACCGTGTAC
SI	XM_422811.4	Sucrase-isomaltase, digestive enzyme	GTGCTCCATGAGTTTGCACAAG/ CAGCGGGCATTGGGTAAGTA
MGAM	XM_423298.3	Maltase-glucoamylase, digestive enzyme	GGGACCAGATGACAAAATCCAT/ CGAGTCTGAGAAAAACCCACAGAT
SLC15A1	NM_204365.1	Oligopeptide transporter	GACCAGCAGGGATCGAGATG/ CTGAATCTGCATAGCTCCAAAGTC
SLC5A1	NM_001293240	Na^+ dependent glucose and galactose transporter	AGGAGGAGAAACCCGATGAAA/ TCTTTAGGCATCCTGGGTCTTC
SLC2A2	NM_207178.1	Na^+ independent glucose, galactose and fructose transporter	GGTCTGATGGGCATGTTAATCA/ GACATAACTCATCCAGGCGAACT
ATP1A1	NM_205521.1	ATPase, Na^+/K^+ transporting, alpha 1 polypeptide	AAGATATTGCTGCTCGACTCAACA/ AATCAGAGCCATGAACAACACATG
ACTB	NM_205518.1	Cytoplasmic beta-actin	GAGAGAGAAATTGTGCGTGACATC/ GCCATCTCCTGCTCGAAATC
GAPDH	NM_204305.1	Glyceraldehyde-3-phosphate dehydrogenase	GTGCTGGCATTGCACTGAAT/ TTGCTGTATCCAAACTCATTGTCA
HPRT1	NM_204848.1	Hypoxanthine phosphoribosyltransferase 1	GCAGTACAATCCAAAGATGGTGAA/ GCCGATATCCCACACTTCGA

[1]Primers designed by Primer Express software (Applied Biosystems)

age (Vantress, 2003). The birds were reared under a program of continuous light for 24 hours.

The birds were weighed on d 7, 21, and 42, and feed intake was recorded daily. On the same days, one bird from each replicate was fasted for 6 h and euthanized by CO_2 asphyxiation. The small intestine was rapidly removed and samples from the jejunum were opened longitudinally, and rinsed with phosphate buffered saline (pH 7.4). The mucosa was scraped with a sterile metal blade and the material obtained was mixed in a sterile, RNase-free petri plate, aliquoted, frozen in liquid nitrogen, and stored in a freezer at -70°C.

RNA Isolation, cDNA Synthesis, and Quantitative Reverse-Transcription PCR (RTq-PCR)

Total RNA was isolated from the jejunal mucosa using the Multisource Total RNA Kit AxyPrep Miniprep Kit® (Axygen). The manufacturer's protocol was modified by the addition of a DNase treatment between the 2 wash steps prior to elution of the RNA from the ceramic column. For this, the column was incubated for 30 minutes at 37°C with a mixture composed of 20 μL of RNase-free DNase (1U/μL) (Prodimol,), 8 μL of 10× "reaction buffer" and 52 μL of diethylpyrocarbonate (**DEPC**) water (0.1% diethylpyrocarbonate). The RNA quantity and quality were assessed with a Qubit fluorometer (Invitrogen) and an Agilent 2100 bioanalyzer (Agilent Technologies), respectively. Only samples with RNA integrity number (**RIN**) greater than 8.0 were used for cDNA synthesis.

The first-strand cDNA synthesis was carried out by reverse transcription (**RT**) from equal quantities (5.0 μg) of total RNA using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). The manufacturer's protocol was modified by the addition of a final step of digestion with RNase H (1U) (Invitrogen) for 22 minutes at 37°C. First-strand cDNAs were purified as described by Madsen et al. (2004). First-strand cDNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific), and working solutions were prepared and then stored in -20°C until use in the quantitative reverse-transcription polymerase chain reaction (**RTq-PCR**) reactions.

The set of primers used to determine the expression of enzymes (*ANPEP*, *MGAM* and *SI*), transporters (*SLC15A1*, *SLC5A1*, *SLC2A2*, and *ATP1A1*) and reference genes (cytoplasmic beta-actin: *ACTB*; hypoxanthine phosphoribosyltransferase: *HPRT1*; glyceraldehyde-3-phosphate dehydrogenase: *GAPDH*) were designed using the Primer Express 3.0 software (Applied Biosystems) (Table 2). The RT-qPCR reactions used SYBR Green reagent (Invitrogen) and were performed in a GeneAmp 7900 instrument (Applied Biosystems), following the protocol described by Madsen et al. (2004).

The results of RT-qPCR reactions were analyzed with RQ Manager 1.2.1 (Applied Biosystems) and the values of quantification cycle (Cq) were corrected for primer amplification efficiency and normalized for control gene. The amplification efficiency of all primers ranged between 90% and 110%. The stability of expression was calculated by geNorm and NormFinder that revealed the combination of *ACTB*, *GAPDH*, and *HPRT1* as the most accurate to normalize gene expression results in this study.

Activity of Intestinal Membrane Enzymes

Brush border membrane vesicles (**BBMV**) were prepared based on the method of Louvard et al. (1973). The enrichment factor of BBMV was calculated for IAP as the ratio between the final and

initial specific enzymatic activity. The enrichment factor obtained was 4.86. IAP (EC 3.1.3.1) was determined according to Pizauro et al. (1995), and APN (EC 3.4.11.2) and MALT (EC 3.2.1.20) activities according to Rueda et al. (2007). The substrates used were *p*-nitrophenylphosphate, maltose, and L-leucine-*p*-nitroanilide for IAP, MALT, and APN, respectively. Their specific activities were expressed as nanomoles of product formed per minute per milligram of protein, under assay conditions. All enzyme assays were performed in triplicate. Total protein content in the samples was determined using Qubit fluorometer (Invitrogen) according to the manufacturer's instructions.

Statistical Analysis

All analysis was performed using PROC MIXED option in SAS (SAS, 2002). For performance traits, a one-way analysis of variance (**ANOVA**) with fixed effect of treatment was used and for each age separately because we were not interested in the time course of these variables. For the mRNA abundance and enzyme activity, a 2-way mixed model was used the fixed effects of diet, age, and their interaction. All lab analyses for these traits where blocked, hence lab analysis block was included as a random effect.

In all cases, when diet × age interaction was significant ($P < 0.05$), single-effects contrasts of diet within ages were considered, but if the interaction was not significant, main effect comparisons of treatment or age were considered. Treatments were compared against controls (CON and AGP) using the Dunnett's test, and for all age comparisons we used protected least significant difference based on the ANOVA *t*-test.

RESULTS AND DISCUSSION

All results are shown in Table 3 and Figures 1 to 4. Enzyme activities data (IAP, APN, and MALT) are presented as log-10 and square-root values, and as a percentage of CON (Figures 1A, B, and 2A) or percentage of reference age, that was fixed on d 7 (Figures 3B, 4A, B). Gene expression data (*SLC15A1*, *SLC2A2*, *SLC5A1*, and *SI*) are presented as log2 fold-change and as a fold-change (Figures 2B, 3A, and 4C). Effect of probiotic and EO on average body weight gain, feed intake and feed conversion ratio of broiler from 1 to 7, 1 to 21 and 1 to 42 d are also presented (Table 3).

Table 3. Effect of probiotic and essential oil on average body weight gain, feed intake, and feed conversion ratio of broilers from 1 to 7, 1 to 21, and 1 to 42 d.

	Treatment					
Age	CON	BS	BS + EO	EO	AGP	SEM
1 to 7 d						
FI (g)	138.17	137.50	136.83	135.67	140.67	3.20
WG (g)	124.83	122.50	128.83	126.33	129.17	2.81
FCR	1.11	1.13	1.07	1.08	1.10	0.03
1 to 21 d						
FI (g)	1196.67	1216.33	1203.83	1197.83	1169.50	10.79
WG (g)	923.00	927.00	913.83	924.50	905.33	6.70
FCR	1.30	1.31	1.32	1.29	1.30	0.01
8 to 21 d						
FI (g)	1092.17	1113.67	1105.83	1099.50	1072.50	9.71
WG (g)	798.33	804.33	784.67	798.50	776.17	7.56
FCR	1.37	1.39	1.41	1.38	1.38	0.01
22 to 42 d						
FI (g)	3787.33	3863.67	3802.67	3866.50	3765.50	42.48
WG (g)	2276.00	2311.83	2266.00	2278.17	2268.50	28.86
FCR	1.66	1.67	1.68	1.70	1.66	0.01
1 to 42 d						
FI (g)	4893.17	4955.00	4921.50	4972.33	4895.00	42.58
WG (g)	3199.33	3239.00	3179.83	3202.50	3173.83	29.68
FCR	1.53	1.53	1.55	1.55	1.54	0.01

SEM: Standard Error of Mean

Diet Influence in Digestion and Transport of Carbohydrates in the Small Intestine mucosa

Expression of genes *SLC5A1*, *SLC2A2*, and *ATP1A1*, that encode carbohydrate transporters in the small intestine mucosa remained stable for all diets, indicating that control group (CON) as well as treatments did not have affect over carbohydrate transport in the small intestine mucosa.

The expression of the genes *MGAM* and *SI,* which encode enzymes responsible for carbohydrate brush border digestion, as well as MALT and IAP activities did not differ significantly in animals receiving BS diet as compared to CON (Figure 1A, B), indicating that the probiotic did not stimulate the maturation of the digestive system and consequently did not enhance the activities of these enzymes.

Although the expression of the genes *MGAM* and *SI* did not differ significantly in animals receiving EOs and AGPs diet as compared to CON, these groups presented higher IAP activities than CON, with MALT activity following the same trend (Figure 1A, B). Therefore, increasing MALT activity observed herein reflects improvement in digestive physiology of birds by the inclusion of AGP or EO.

The distribution of enzyme activities on crypt:villus axis depend on the rate of enzyme synthesis, cell migration and extrusion (Iji et al., 2001b). The discrepancy between *MGAM* and *SI* mRNA quantities and the respective enzyme activity levels (MALT) shows that the higher activities were not response to increased synthesis. Actually, the higher enzyme activities could lie on lower epithelial loss and alteration in cell renewal with AGP and EO supplementation. AGP and EOs are essentially antimicrobial substances, and reduced microbial activity in digesta or at the level of the brush border would reduce damage to enterocytes and change cell renewal in the gut (Hughes, 2003). Also, most enzymes are secreted as inactive precursors and become activated only after the enterocyte maturation (Iji et al., 2001a), so that mature villus cells are more actively

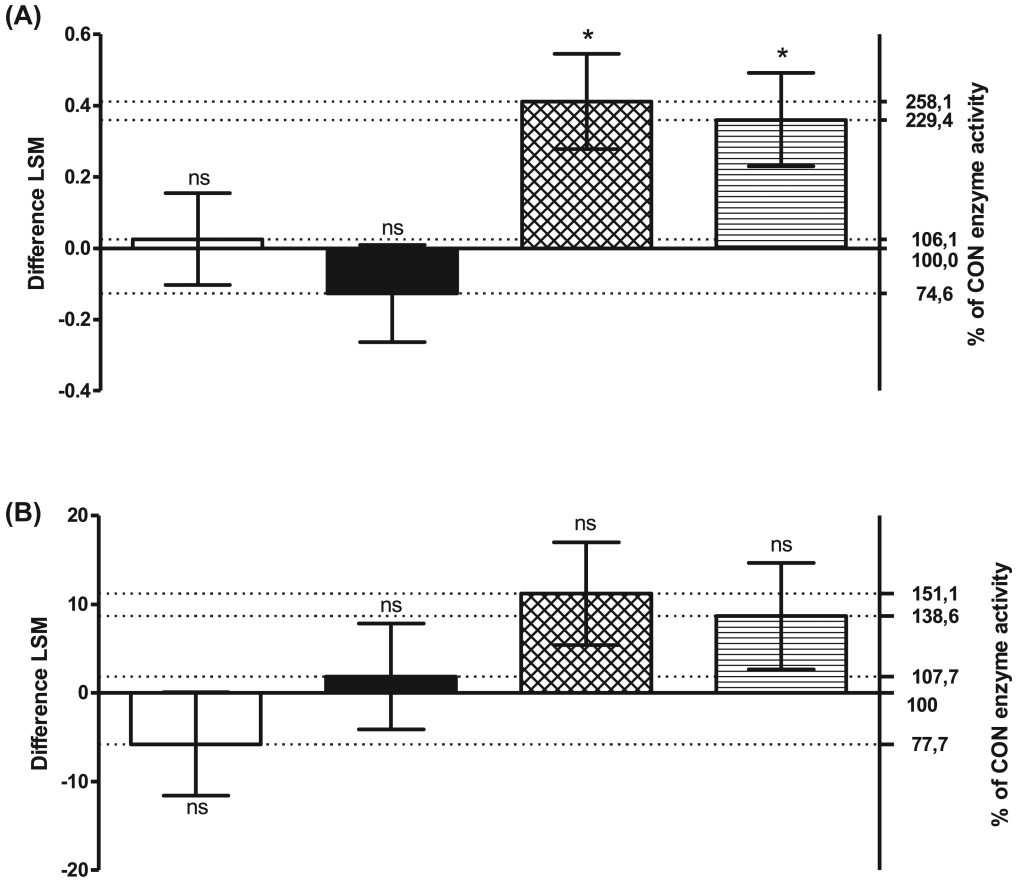

Figure 1. Enzyme activity estimated comparing treatments to CON for IAP **(Panel A)**, and MALT **(Panel B)**. The difference of LSM for: □ = BS, ■ = BS + EO, ▩ = EO, and ▤ = AGP. Percent of activity in relation to CON group is included on the right axis. There was a main effect of treatment ($P < 0.05$) as indicated by *. ns = not significant.

secreting enzymes and absorbing more than immature crypt cells (Uni et al., 1998; Ferraris, 2001). However, as enterocytes proceed up to villus tip they can become senescent, being extruded in the villus tip (Iji et al., 2001b). The higher MALT activities in AGP and EO birds should be linked to change in migration rate of enterocytes, so that synthesis and allocation of proteins in the cell membrane may be benefited.

Diet Influence in Digestion and Transport of Proteins in the Small Intestine Mucosa

The expression of *ANPEP* gene, encoding APN enzyme responsible for protein brush border digestion, as well as APN activities remained unchanged in animals receiving BS diet as compared to CON (Figure 2A), also indicating that the probiotic did not stimulate the maturation of the digestive system and consequently, did not enhance the activities of these enzymes.

In contrast, there was a main effect of treatment ($P = 0.01$) on protein transport related to gene SLC15A1 expression, with increased expression values in animals fed BS compared with CON (Figure 2B). Additionally, although the BS diet increased the *SLC15A1* mRNA quantities, it did not change significantly the expression of *ATP1A1*.

Bacillus spp. has recognized proteolytic activity (Pugsley and Schwartz, 1985), and increased protease activity and protein digestibility resulting from *Bacillus spp.* supplementation has been demonstrated (Wang and Gu, 2010; Ray et al., 2012). In the other hand, feeding *Enterococcus faecium* DFM did not markedly influence brush border enzyme expression or activity in piglets (Martin et al., 2012), indicating that different probiotic strains can have different capacity to produce enzymes and/or stimulate endogenous enzymes production.

Absorption of protein digestion products in the small intestine occurs primarily in the form of small peptides rather than free amino acids (Matthews, 1975) and peptide transporter (*SLC15A1*) activity can stimulate free amino acids uptake by the $b^{0,+}$ system (Wenzel et al., 2001). Therefore, the greater *SLC15A1* mRNA level in birds fed BS diet could mean that the birds treated with BS may have had a considerable advantage in the absorption of AA nitrogen compared with CON.

The most likely mechanism of action of EOs is based on its antimicrobial action, like the AGPs. Interestingly, although *ANPEP* gene expression remained constant, the AGP and EO groups presented higher APN (Figure 2A) and IAP (Figure 1A) activities than CON. Therefore, increasing APN and IAP activity observed

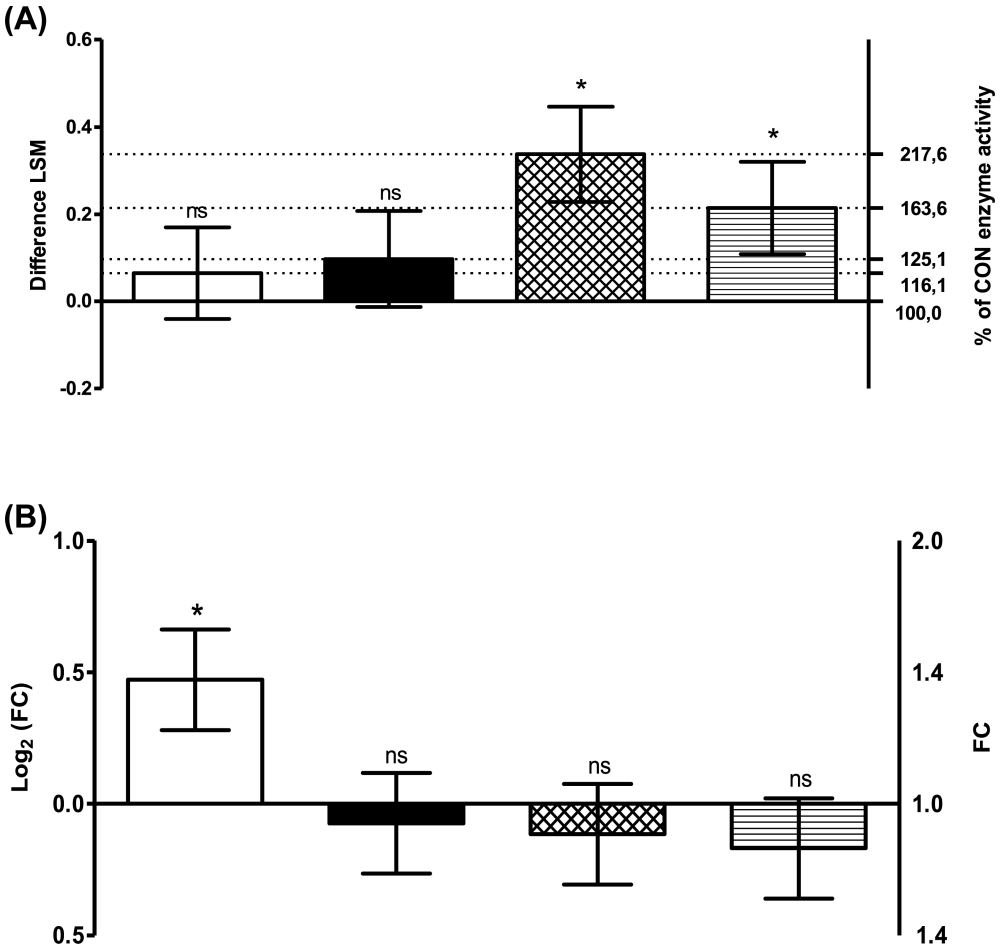

Figure 2. Panel A: Enzyme activity estimated comparing treatments to CON for APN for: □ = BS, ■ = BS + EO, ⊠ = EO, and ▤ = AGP. Percent of activity in relation to CON group is included on the right axis. **Panel B:** Gene expression fold changes estimated comparing treatments to CON for *SLC15A1*. The $\log_2$-fold changes for: □ = BS, ■ = BS + EO, ⊠ = EO and ▤ = AGP. Fold change (FC) scale is included on the right axis. There was a main effect of treatment ($P < 0.05$) as indicated by *. ns = not significant.

herein reflects improvement in digestive physiology of birds by the inclusion of AGP or EO.

As in this experiment, AGP and EOs were continuously supplied in the diet. In this sense, considering its antimicrobial actions it is possible that the higher similarity of APN and IAP activities in AGP and EO groups resides on their common influence on microbiota.

It is know that LPS binds to the tool like receptor 4 triggering intestinal inflammation and increasing IAP activity (Lalles, 2010). One homeostatic feedback loop between LPS and IAP has been shown to control the level of bowel inflammation (Bates et al., 2007). Thus, higher activity of IAP in response to EO or AGP may be a result of greater exposure to bacterial LPS, either by direct lysis of Gram negative bacteria, or by increased Gram negative/Gram positive bacteria ratio in the intestinal microbiota, since avilamycin and EO are more active against Gram + bacteria (Butaye et al., 2003; Giannenas et al., 2013). Higher IAP activity would be an advantage especially in case of high pathogenic load in the intestine, when sub-clinical infections can impair intestinal health and increase energy expenditure towards perpetual activation of acute phase immune response, resulting in reduced performance (Klasing and Johnstone, 1991).

The increase in APN activity, additionally to the trend of increasing MALT activity observed in our work, reflect improvement in digestive physiology of birds by the inclusion of AGP or EO. Also, the increase in APN activity, as well as MALT, can be explained by cell migration and extrusion, but not by increased rate of enzyme synthesis, since *ANPEP* mRNA quantities were not increased due to inclusion of AGP or EO and therefore could not be responsible for the increased enzyme activity levels of APN.

Age Influence in Digestion and Transport of Carbohydrates in the Small Intestine Mucosa

We observed that genes encoding enzymes responsible for carbohydrate digestion (*MGAM* and *SI*) presented higher expression at d 42 than at d 7 (2.50 fold for *MGAM* and 2.58 fold for *SI*) (Figure 3A). This was

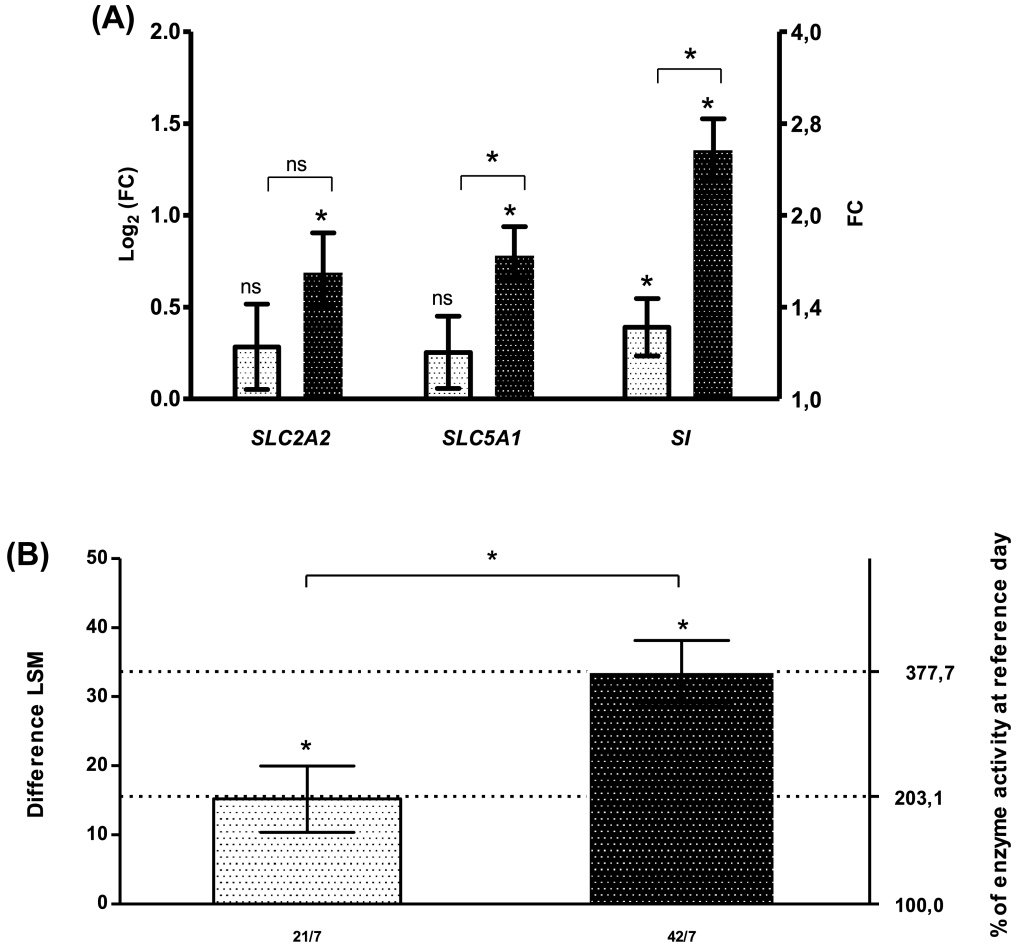

Figure 3. Panel A: Gene expression fold changes estimated comparing days for *SLC2A2*, *SLC5A1,* and *SI*. The $\log_2$ fold-changes: ▨ = 21 and ▩ = 42. The contrast 42/21 is indicated by the bracket. Fold-change (FC) scale is included on the right axis. **Panel B:** Enzyme activity estimated comparing days for MALT. The difference LSM for: ▨ = 21 and ▩ = 42. The contrast 42/21 is indicated by the bracket. Percent in relation to reference day is included on the right axis. There was a main effect of day ($P < 0.05$) as indicated by *. An asterisk above the bracket indicates significant difference ($P < 0.05$) between 21 and 42 d by t test. ns = not significant.

in accordance with MALT activity increase in the same period (3.78 fold) (Figure 3B). However, while the activity of MALT increased 2.03-fold from d 7 to d 21, the expression of *MGAM* and *SI* (although significant for SI) remained relatively stable in the same period (0.85-fold and 1.31-fold, respectively) (Figure 3A). The increase in MALT activity observed here is similar to that reported by Uni et al. (2003), who observed that mucosal sucrase and MALT activities gradually rose in jejunum from d 4 to d 11 posthatch in other strain of chickens.

The results of this study indicate that the increase in MALT activity with age occurred by two different processes, the initial related to post-translational modifications and the later involving cellular reprogramming. After 14 d of age, the rate of cell migration in broilers villus drops, leading to greater density of mature enterocytes (Iji et al., 2001a). Although the mature enterocyte is more functional, when the rate of enterocyte migration is reduced they can become senescent before being extruded in the villus tip (Iji et al., 2001b). In broilers with 21 d, the peak of α-glucosidase activity occurs at 300 μm from the crypt tip, above that enzyme secretion undergoes reduction through increases in enzymatic degradation (Iji et al., 2001b). Thus, increased activity of MALT between 7 and 21 d in this study suggests increased protein stability in the line studied.

Moreover, the increase in MALT activity between 21 and 42 d involved increased levels of mRNA, which could be a response to higher carbohydrate levels in the diet in the same period. Indeed, regulation by the substrate as well as control at transcriptional level had already been demonstrated for both MALT and sucrase (Uni et al., 2003; Karasov et al., 2010).

The increase in the MALT activity with age indicate that the digestion of carbohydrates at intestinal level may not be the limiting for broiler digestion as previous suggested by Gilbert et al. (2007). The genetic selection for growth of the breed studied could have favored cell reprogramming to lower protein degradation and increased MALT synthesis throughout development for better digestion of carbohydrates.

We also investigated the temporal gene expression profile of genes related with absorption of hexoses (*SLC2A2*, *SLC5A1* and *ATP1A1*), and obtained higher

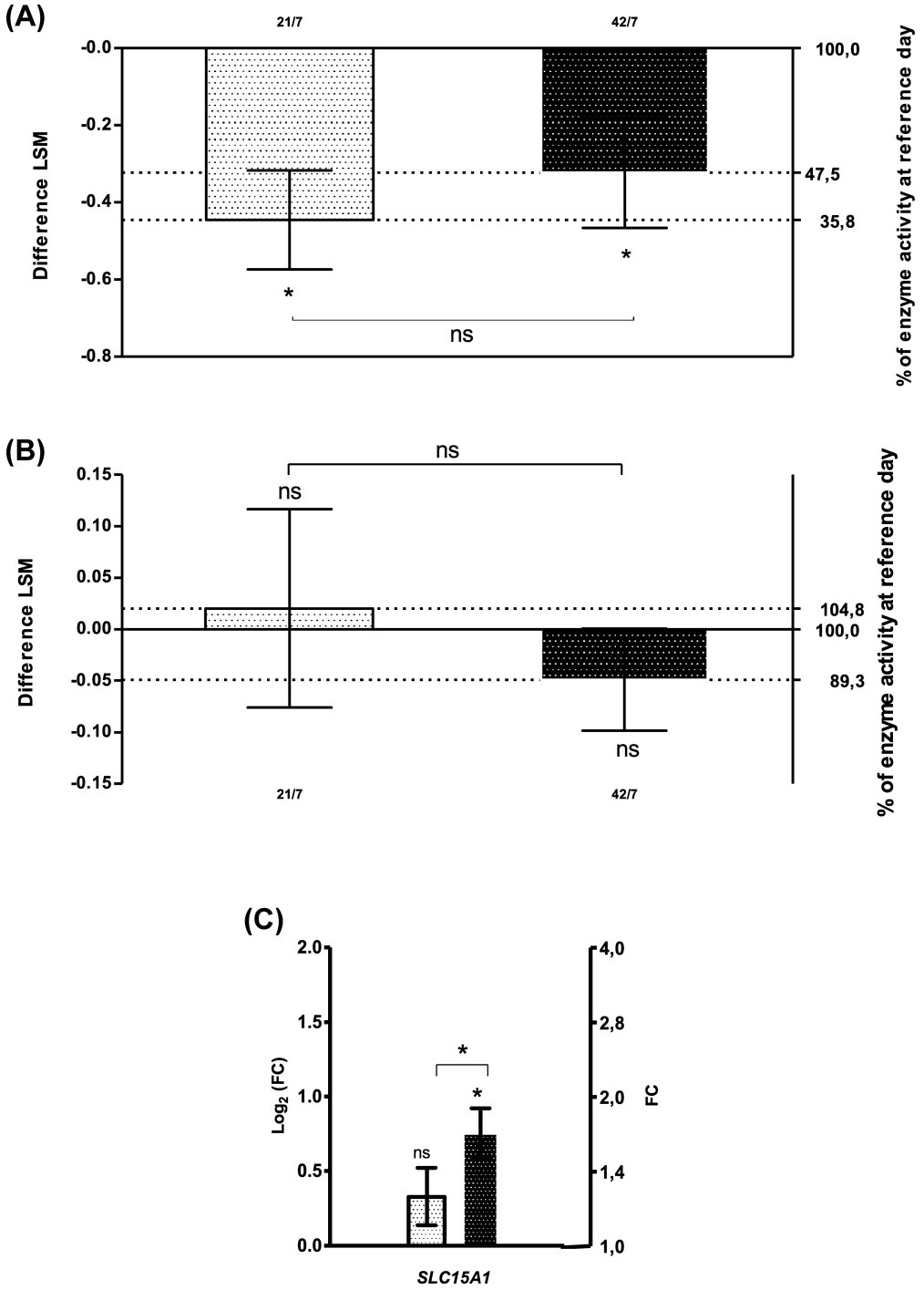

Figure 4. Enzyme activity estimated comparing days for IAP **(Panel A)** and APN **(Panel B)**. The difference LSM for: ▭ = 21 and ▬ = 42. The contrast 42/21 is indicated by the bracket. Percent in relation to reference day is included on the right axis. **Panel C:** Gene expression fold changes estimated comparing days for *SLC15A1*. The $\log_2$-fold changes: ▭ = 21 and ▬ = 42. The contrast 42/21 is indicated by the bracket. Fold-change (FC) scale is included on the right axis. There was a main effect of day ($P < 0.05$) as indicated by *. An asterisk above the bracket indicates significant difference ($P < 0.05$) between 21 and 42 d by *t* test. ns = not significant.

expression with age for genes *SLC2A2* and *SLC5A1* (Figure 3A). From 7 to 21 d, *SLC2A2* and *SLC5A1* mRNA abundance increased, but not significantly. In contrast, from 21 to 42 d *SLC2A2* and *SLC5A1* mRNA abundance significantly increased 1.62-fold and 1.73-fold, respectively (Figure 3A). Our data also supports results from Gilbert et al. (2007), who observed that *SLC2A2* and *SLC5A1* mRNA increased in the jejunum with time from hatch to 14 d in Aviangen commercial broilers.

Regarding expression of genes related to glucose absorption, the results of this study indicate that the increase of mRNA of genes *SLC2A2* and *SLC5A1*, between 21 and 42 d (Figure 3A), indicates a response to

increased levels of carbohydrate in the diet, similar to *SI* and *MGAM* mRNA levels and MALT activity in the same period (Figure 3A, B).

Age Influence in Digestion and Transport of Proteins in the Small Intestine Mucosa

The expression of the gene *ANPEP* assessed herein, associated with protein digestion, remained stable over age as well as the protein activity of APN (Figure 4B), indicating that protein digestion may be limiting during the broiler life. However, although Gilbert et al. (2007) observed that quantities of *ANPEP* mRNA increased from d 1 to d 14 in Arbor Acress chicks, the stability of APN verified in our study could be an advantage compared to the lower enzyme activity reported in other studies that show reduced APN with age in Steggles × Ross broilers (Iji, et al., 2001a).

The IAP activity significantly decreased from d 7 to 21 and remained unchanged from d 22 to 42 (Figure 4A). Therefore, the pattern of IAP until 21 d in this experiment is consistent with the findings of Iji et al. (2001b) in broiler chicks. These authors found decreased specific activity of IAP along small intestine from hatching to 21 d of age and higher IAP activity towards the villus tip than at crypt or mid-villus. The drop in IAP activity between 7 and 21 d may be a consequence of the reduction in the rate of migration of enterocytes in the same period (Iji et al., 2001a), that led to the accumulation of senile and less functional enterocytes in the villus tip (Iji et al., 2001b). The similar values of IAP activity from 21 to 42 d observed in this study reflects that intestinal maturation as early as 21 d of age, since it is known that IAP play a major role maintaining gut homeostasis and barrier function (Lalles, 2010).

We also investigated the temporal gene expression profile of genes related with absorption of di and tripeptides (*SLC15A1* and *ATP1A1*), and obtained higher expression with age for *SLC15A1* gene (Figure 4C). From 7 to 21 d, *SLC15A1* mRNA abundance increased, but not significantly. In contrast, from 21 to 42 d, *SLC15A1* mRNA abundance significantly increased 1.69-fold, independent of treatment (Figure 4C).

These findings corroborate those obtained by Chen et al. (2005), who showed that abundance of jejunum *SLC15A1* mRNA tends to increase from hatch to 35 d in Cobb broilers. Our data also supports results from Gilbert et al. (2007), who observed that *SLC15A1* mRNA increased in the jejunum with time from hatch to 14 d in Aviangen commercial broilers.

The increased mRNA levels of *SLC15A1* do not appear to be related to regulation at substrate level, since the expression of *ANPEP* and APN activity remained stable for the same period (Figure 4B, C). Usually there is a general increase in the absolute levels of uptake of nutrients with age linked with absorptive area (Rayo et al., 1992), but the efficiency of uptake, when related to intestinal and body weights declines (Iji et al., 2001c). The reduction in transport rate in older animals was associated with an increase in transport affinity and a decrease in transporter capacity (Navab and Winter, 1988). In general dietary uptake capacity matches dietary load in animals, however may impose a "bottleneck" on availability of nutrients in animals selected for growth, with high nutrient intake (Obst and Diamond, 1992). Therefore, increasing the number of carriers of nutrients would be a way to offset the decline of its functionality with the development.

Diet Influence in Growth Performance

We did not find any influence of treatments on performance at different ages (Table 3) in the present study, which resembles published data using BS based DFM (Willis and Reid, 2008; Lee et al., 2010) or EO (Bozkurt et al., 2012; Abudabos and Alyemni, 2013). Bozkurt et al. (2012) also found no difference in performance between birds from the control diet and those supplemented with monensin or herbal extract (24.5% carvacrol, 20.1% 1,8-cineole, camphor 12.1% and 6.0% thymol), however improvements in performance in response to both feed additives were detected after a challenge with a mixture of Eimeria. Indeed, it has been raised that the contribution of DFM and EOs on performance is linked with the presence of pathogens (Giannenas et al., 2013; Murugesan et al., 2014a).

In the present study, the birds were housed in an aviary that was unutilized for 6 months and properly disinfected before receiving the chicks. Furthermore the chicks were housed in pens with unused wood shavings as litter. These measures have been adopted in order to isolate the influence of the additives tested in intestinal physiology and access their contribution to performance, without the influence that high pathogenic load in the environment can exert in the gut. AGPs are expected to have higher efficacy with increased infection pressure, such in certain ages, husbandry conditions, and regions (Niewold, 2007). Thus, the similar performance between AGP and CON indicates that we were successful in rearing birds under reduced pathogenic load. This situation also supports the assumption that BS DFM and EO efficacy as growth promoter is related with the control of pathogenic microorganisms.

Final Remarks

To our knowledge, this is the first study to investigate the effect of probiotic and EO on intestinal expression of genes encoding proteins related with digestion and absorption of carbohydrates and proteins in broiler chickens. The increase in the activity of the brush border enzymes in birds receiving AVI or EO should be more investigated, especially in adverse situations. Remarkably, birds might have less energy available for growth when reared in poor sanitary conditions

or when fed diets with lower levels of nutrients. Therefore, any improvement in the utilization of carbohydrates and proteins might be a considerable advantage in such situations. Furthermore, we found that BS DFM enhanced *SLC15A1* mRNA which suggests enhanced trans-cellular transport of amino peptides molecules. Improvements in the utilization of dietary protein can translate into huge savings for the commercial livestock industry and can reduce the impact of nitrogen excretion into environment (Aletor et al., 2000; Bregendahl et al., 2002). In this research the birds were raised in adequate conditions of hygiene and received a balanced diet with highly digestible ingredients which may have limited the potential for such changes promote better performance and nutrient utilization. Thus, further studies should assess the capacity of the BS DFM and EO in improving the utilization of carbohydrates and protein in adverse conditions.

Conclusions: 1) The use of BS DFM did not enhance the activities of carbohydrate digestion and protein brush border digestion in the small intestine mucosa. In the other hand, although BS DFM did not interfere over carbohydrate transport, effect was evident over protein transport due to the up regulation of the *SLC15A1* gene. 2) The use of EO enhanced the activities of carbohydrate digestion (MALT) and protein brush border digestion (APN), reflecting improvement in digestive physiology of birds. 3) Changes performed by BS DFM and EO did not favor performance. 4) Age had positive effect over genes encoding enzymes responsible for carbohydrate digestion (*MGAM* and *SI*) and MALT activity, which presented higher expression at d 42. In contrast, the expression of *ANPEP* and activity of APN, associated with protein digestion, remained stable over age, indicating that protein digestion may be limiting during the broiler life. Also, IAP activity significantly decreased from d 7 to 21. Higher expression profile of genes related with absorption of hexoses (*SLC2A2* and *SLC5A1*) and di- and tripeptides (*SLC15A1*) were obtained at d 42. Therefore, intestine undergoes specific changes suggesting higher ability to digest maltose and higher expression of transporters of hexose and peptides in order to accomplish the increasing daily feed consumption and reach the expected growth of the modern broiler. The results of the analysis can help to formulate diets that best accommodate the profile of digestive enzymes and nutrient transporters expressed in the small intestine over time.

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