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Dietary supplementation of microencapsulated botanicals and organic acids enhances the expression and function of intestine epithelial digestive enzymes and nutrient transporters in broiler chickens

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ABSTRACT Organic acids and botanicals have shown protective effects on gut barrier and against inflammation in broilers. However, their effects on intestinal digestive enzymes and nutrients transporters expression and functions have not been fully studied. The objective of this study was to understand how a microencapsulated blend of botanicals and organic acids affected intestinal enzyme activities and nutrient transporters expression and functions in broilers. A total of 288 birds were assigned to a commercial control diet or diet supplemented with 500 g/MT (metric ton) of the microencapsulated additive. Growth performance was recorded weekly. At d 21 and d 42, jejunum and ileum were isolated for enzyme (maltase, sucrase, and aminopeptidase) and transporter (SGLT1, GLUT2, GLUT1, EAAT3, B^0AT1, and PepT1) analyses. Jejunum specific nutrients (glucose, alanine, and glutamate) transport activities were evaluated by Ussing chamber. Protein expression of nutrient transporters in small intestine were measured in mucosa and brush-border membrane (**BBM**) samples by western blot. Intestinal gene expression of the transporters was determined by RT-PCR. Statistical analysis was performed using Student's t-test comparing the supplemented diet to the control. The feed efficiency was significantly improved through the study period in the supplemented group ($P \leq 0.05$). Significant changes of intestinal histology were shown in both jejunum ($P \leq 0.10$) and ileum ($P \leq 0.05$) after 21 d of treatment. At d21, jejunal maltase activity was upregulated ($P \leq 0.10$). The Ussing chamber transport of glucose and alanine was increased, which was in line with increased gene expression (GLUT2, GLUT1, EAAT3, and B^0AT1) ($P \leq 0.10$ and $P \leq 0.05$, respectively) and BBMV protein levels (B^0AT1, $P < 0.10$). At d21, ileal sucrase and maltase activities were upregulated ($P \leq 0.05$). Increased expressions of GLUT1, EAAT3, and B^0AT1 were observed in both mRNA and protein levels ($P \leq 0.05$). Similar pattern of changes was also shown at d42 of age. Our results suggest that feeding microencapsulated additives improves intestinal nutrient digestion and transporter expression and function in broilers, thereby enhancing feed efficiency.

Key words: botanical, organic acid, intestinal nutrient transport, digestive enzyme, broiler

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INTRODUCTION

Optimal gut health is considered central to animal health, growth performance, and feed efficiency. A healthy gut is characterized by a balance of 3 key components: a strong mucosal and immune barrier, a well-balanced microbiota, and efficient nutrient absorption (Diaz Carrasco et al., 2019). With limitations on the use of growth-promoting antibiotics in poultry, recent research has emphasized the importance of the immune and mucosal barrier. However, strategies focusing on nutrient digestion and transport have been relatively understudied. Modern birds require high-nutrient feed to meet their heightened growth and immune function demands. An efficient gut is essential for digesting and absorbing these dietary nutrients, particularly of carbohydrates and proteins, thereby preventing potential malnutrition, nutrient-induced inflammation, and gut dysbiosis (Kogut and Arsenault, 2016; Aruwa et al., 2021). Over the years, researchers have looked into the relationship between nutrition and immune competence and how it affects the overall well-being of animals

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(Adedokun and Olojede, 2019). Changes in nutrient utilization and growth performance are a result of changes in intestinal morphology and microflora, which reflect intestinal functionality and regulate nutrient digestion and absorption. Modification of all these factors can be achieved through specific diet interventions, such as the addition of specific feed additives. In particular, the addition of a microencapsulated blend of organic acids and botanicals (**OA+B**) has already been underlined as an effective tool not only to ameliorate intestinal architecture, barrier functions, and microbial population in chickens (Feye et al., 2020; Bialkowski et al., 2023), but also to improve the bird's response to necrotic enteritis and salmonella infection (Stingelin et al., 2023; Dittoe et al., 2023). However, there is a lack of knowledge about the possible role of natural feed additives in ameliorating intestinal health in terms of nutrient digestibility and absorption.

The efficiency of animals obtaining nutrient from feed highly relies on the intestinal digestion and transport of nutrients. The dietary digestion process takes place both within the small intestine's lumen and in the brush-border membrane (**BBM**) of enterocytes. To digest and absorb carbohydrate, maltase and sucrase breaks down maltose and sucrose into glucose, respectively. After the digestion process, glucose is ready to be absorbed through intestinal epithelium via specific transporters, as sodium glucose cotransporter-1 (**SGLT1**) and glucose transporters (**GLUT**) (Shibata et al., 2019). The digestion of proteins and amino acids (**AAs**) relies on interorgan collaboration among stomach, pancreas, and intestine. After major digestion of enzymes, such as pepsin and trypsin, the small peptides are further hydrolyzed by peptidases that are bound primarily to the BBM, and to a lesser extent, in the intestinal lumen to form free AAs, dipeptides, and tripeptides (He et al., 2021). Ileum and jejunum are the active sites for AAs absorption, where tripeptides and dipeptides are transported across the BBM of the intestine via H^+-dependent peptide transporter 1 (**PepT1**), while free AAs are mainly transported using both Na^+-dependent and Na^+-independent transporters in response to acidic, neutral, and/or basic AAs. In chickens BBM, B^0AT1 is the major Na^+-coupled neutral AAs transport system, excitatory AAs transporter 3 (**EAAT3**) is the mainly transporter of anionic AAs (aspartate and glutamate), and $B^{0/+}AT$ is the Na^+-independent transporter of cationic and neutral AAs (Gilbert et al., 2007; Shibata et al., 2020). The efficiency of intestinal nutrient uptake is influenced by several factors, including BBM maturation and the relative abundance of transporters expressed. Higher expression of SGLT1, GLUT, PepT1, and EAAT would maximize glucose and nitrogen absorption, with a special mention to glutamate which is the fuel source of enterocytes, facilitating the development of a healthy intestinal epithelium and a rapid growth rate. Also, as the Na^+/K^+-ATPase enzyme is responsible for maintaining membrane potential, it plays a central role for Na^+-nutrient co-transport processes in the BBM, particularly SGLT1 and B^0AT1 (Gal-Garber et al., 2003; Mott et al., 2008; Reicher and Uni, 2021; Pirahanchi et al., 2024). It is interesting to note that glucose and AAs may be competing for intestinal uptake, probably for co-absorption with sodium via their respective Na^+-dependent transport systems (Vinardell, 1990).

As mentioned, the potential of natural feed additives to influence intestinal health via nutrient absorption is not well-understood. Some feed additives seem able to modulate the nutrient transport mechanisms, implementing the animal health and well-being. OAs are known to be capable to reduce the pH of intestinal digesta and increase digestive enzymes activity (Khan and Iqbal, 2016). For example, citric acids seem able to increase activities of sucrase, maltase, and alkaline phosphatase in the small intestinal mucosa of piglets, which implies efficient uptake of sugar and AAs (Deng et al., 2021). Aguiar and colleagues (2023) found greater secretion of digestive enzymes and upgraded metabolism of glucose and lipids using peppermint oil in fishes. Hashemipour et al. (2013) demonstrated that, under certain circumstances, the supplementation of thymol and carvacrol would trigger the secretion of digestive enzymes, enhancing the nutrient digestion at the intestinal level in chickens. Considering the increasing importance of feed additives due to the elimination of growth-promoting antibiotics from broiler chickens' production, the aim of the present study was to evaluate the effect of a microencapsulated blend of citric acid, sorbic acid, thymol, and vanillin on growth performance and to enhance our comprehension of its potential role on intestinal nutrient transport function.

MATERIALS AND METHODS

Animals Husbandry

All animal procedures followed guidelines established by the University of Delaware Institutional Animal Care and Use Committees and were approved by this committee (AUP#101R-2019). The experiments were conducted in accordance with the recommended code of practice for the care and handling of poultry and followed the ethical principles according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). A total of 288 Ross 308 eggs were obtained from a local commercial hatchery (Moyer's Chicks, Inc., Quakertown, PA) and incubated at 37.5°C and 60% humidity for 21 d. After hatch, the straight-run (mixed male and female) chickens were given *ad libitum* access to water and an unmedicated 2 phases of broiler diet (the starter, d 1−21; and the grower, d 22−42) (Supplementary Table 1) that met or exceeded the established nutrient requirements (National Research Council, 1994). All the birds were raised under the same condition, recommended by commercial broiler industry. Each house was maintained around 32°C for the first week and then reduced by 1.5° C weekly until a final temperature of 21°C was reached. The birds were provided with 24 h of light for the entire length of the trial.

Experimental Design and Treatments

On the hatching day, birds (straight-run) were removed from the incubator, weighed, and randomly assigned to 2 dietary treatments, with 18 birds per pen for 8 replicate floor pens per treatment ($n = 8$), body weight (**BW**) balanced. Birds assigned to the OA+B supplement-diet were given free access to the same basal diet supplemented with 500 g/MT (metric ton) of a microencapsulated blend of citric (25%) and sorbic (16.7%) acids, thymol (1.7%), and vanillin (1.0%) (AviPlusP; Vetagro S.p.A., Reggio Emilia, Italy. AviPlusP, EU feed additive N. 4d3, European Patent EP 1391155 B1, US Patent US 7.258.880 B2, Canadian Patent CAN 2.433.484 C, more patents pending). The birds did not receive any medications nor vaccine during the study. The BW and feed intake (**FI**) were recorded weekly, then were calculated average daily feed intake (**ADFI**), average daily gain (**ADG**), and feed conversion ratio (**FCR**).

On 21 and 42 d of age, birds ($n = 16$) were randomly selected and euthanized by cervical dislocation. Jejunum and ileum were collected by using landmarks of duodenal loop and the Meckel's diverticulum. A 2 cm length segment from distal end of jejunum and ileum was fixed in 10% neutral buffered formalin for histological analysis; the next 4 cm segment was used for Ussing chamber analysis; the next 5 to 7 cm segment was frozen in liquid nitrogen and stored at -80° C until further analysis. Also, serum samples were collected and stored at -80°C until further glucose quantification analysis.

Blood Glucose Assay

The frozen serum was thawed on ice and aliquoted for dilution in phosphate buffered saline (**PBS**). The glucose assay was conducted following commercial kit instructions (Glucose Colorimetric Detection Kit, ThermoFisher Scientific). The data was expressed in mmol/L.

Histological Analysis

Segment from distal end of jejunum and ileum were fixed and stained as previously reported from Bialkowski et al. (2023). Briefly, stained slides were scanned onto an imaging analysis software (Leica). The villus height (**VH**) was measured from the tip to the villus-crypt junction. Villus width (**VW**) was measured at one-third and two-thirds of the length of the villus and reported as the average between these 2 values. The crypt depth (**CD**) was defined as the base of the villus to the mucosa. A total of 10 to 15 measurements per animal ($n = 6$) were taken for each trait from intact well-oriented crypt-villus units. VH and VW were measured at 4-times objective magnification and the CD at 10-times objective magnification.

Ussing Chamber Nutrient Transport

Electrophysiological nutrient transport of jejunum ($n = 16$) at 21 of age were evaluated. The Ussing chamber experiment was set-up following the previous design Bialkowski et al. (2023). Briefly, immediately postmortem a 5 cm piece of jejunum was cut open along the mesentery and put into cold Ringer's solution, which was pregassed with carbogen gas (95% O_2-5% CO_2). The opened intestinal sheet was pinned down and the circular muscle layer was carefully stripped under a dissecting microscope. A piece of 0.5 cm^2 was mounted on the Ussing chamber. The tissue was bathed 5 mL of 40°C Ringer's solution, with continuously gassed with carbogen to allow for oxygenation and circulation of the buffer by gas lift. The temperature was maintained at 40°C by a circulating thermostatic water jacket. A 10 mmol/L glucose was added to the serosal side as tissue energy source, and 10 mmol/L mannitol was added to the mucosal side for balancing osmotic pressure. After 15-20 min of equilibration under open-circuit conditions, the tissue was short-circuited by clamping the voltage to zero. The short-circuit current (I_{SC}, $\mu A/cm^2$), and transepithelial resistance (R_T, Ω x cm^2) were continuously measured by the chamber software. The electrogenic nutrients transport were measured by the ΔI_{SC} changes in response to 10 mmol/L of each selective nutrient (glucose, alanine, and glutamate), which were administered to the luminal side separately with 10 min interval between nutrients.

Gene Expression Analysis

A commercially available kit (RNeasy Mini kit, Qiagen, Germantown, MD) was used for RNA isolation from jejunum and ileum samples. All purification steps were performed according to the manufacturer's instructions. A NanoDrop (ThermoFisher Scientific, Waltham, MA) was used to determine RNA quantity and a 1.5% agarose gel denatured with formaldehyde was run to check RNA quality. Synthesis of single-stranded complementary DNA (**cDNA**) from total RNA was performed using a Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific). Reactions were run using a C1000 Touch thermal cycler (Bio-Rad). The DNase treatment was incubated at 37°C for 3 min.

Real-Time quantitative PCR of cDNA was performed using a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific) using 96-well PCR plates. Primers are listed in Table 1. All reactions were made at a 10 μL final volume and consisted of 5 μL PowerUp SYBR^{TM} Green Master mix (Applied Biosystems, Waltham, MA), forward and reverse primers each at a final concentration of 300 nmol/L or 600 nmol/L (0.3 μL or 0.6 μL of a 10 μmol/L stock), 2.5 μL of cDNA (15 ng/μL per reaction), and nuclease-free water (Invitrogen, ThermoFisher Scientific). Each reaction was performed in duplicate. The amplification program used was a hold stage for 1 cycle of 50°C for 2 min and 95°C for 2 min, a PCR stage for 40 cycles of 95°C for 1 s and 60°C for 30 s, and a

Table 1. Primers used for gene expression experiments in this study.

Gene	Accession number	Sequences (5′→3′)	Product length (bp)
HMBS	XM_417846	F: GGTTGAGATGCTCCGTGAGTTT R: GGCTCTTCTCCCCAATCTTAGAA	153
RPLP1	NM_205322.1	F: TCTCCACGACGACGAAGTCA R: CCGCCGCCTTGATGAG	63
TBP	NM_205103	F: CTTCGTGCCCGAAATGCT R: GCGCAGTAGTACGTGGTTCTCTT	82
SGLT1	NM_001293240.2	F: GCCATGGCCAGGGCTTA R: CAATAACCTGATCTGTGCACCAGTA	71
GLUT1	NM_205209.1	F: AGTTCGGCTACAACACCGGC R: CCAACCGAGAAGATGGCGAC	151
GLUT2	NM_207178.1	F: ATGACGGTTGGACTTGTGCT R: CAATGAAGTTGCAGGCCCAG	202
EAAT3	XM_424930.8	F: TGCTGCTTTGGATTCCAGTGT R: AGCATGACTGTAGTGCAGAAGTAATATAT	78
B^0AT1	XM_419056.6	F: AGGTGGGAGGAGCGGAATTT R: TGCGGGTGCTCTCATGTATT	153
PepT1	NM_204365.2	F: AGACTGGGCAAGCGAGAAGT R: TGCCCAGAACATCGGGAGAG	100

Abbreviations: B^0AT1, Sodium-dependent neutral amino acid transporter; EAAT3, excitatory AAs transporter 3; F = forward; GLUT 2, glucose transporter 2; GLUT1, glucose transporter 1; HMBS, hydroxymethylbilane synthase; PepT1, peptide transporter 1; R, reverse; RPLP1, ribosomal protein lateral stalk subunit P1; SGLT1, sodium/glucose cotransporter 1; TBP, TATA-box binding protein.

melt curve stage with 1 cycle of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The melt curve was analyzed at the end of the run to determine single product amplification. The comparative Ct method ($2^{-\Delta\Delta Ct}$ method) was used to quantify PCR results. The Ct values of each gene were compared to the geometric mean of 3 housekeeping genes (HMBS, RPLP1, and TBP) (Schmittgen and Livak, 2008).

Isolation of Brush-border Membrane Vesicles

Brush-border membrane vesicles (**BBMV**) were isolated according to standard protocol. Briefly, proximal jejunum or ileum samples were homogenized with buffer (100 mmol/L mannitol, 2 mmol/L HEPES, 2 mmol/L Tris and 0.25 mmol/L PMSF), centrifuged as baseline homogenized samples, and stored at -80°C until analysis. BBMV was purified by adding 10 mmol/L $MgCl_2$ to the supernatant, then BBMV was pelleted by ultracentrifuge (30,000 x *g*) for 30 min with buffer (100 mmol/L mannitol, 2 mmol/L HEPES, 2 mmol/L Tris, and 0.1 mmol/L $MgSO_4$). The BBMV pellet was dissolved in another buffer (300 mmol/L mannitol, 20 mmol/L HEPES, and 0.1 mmol/L $MgSO_4$) and stored at -80°C until further use.

Western Blotting Analyses of Nutrient Transporter

Western blot was performed with a standard procedure from (Li et al., 2022). Total protein was isolated from the proximal jejunum and ileum with ice-cold RIPA buffer with a protease inhibitor and PMSF. The tissues were homogenized and total protein samples or BBMV samples were prepared in 4X laemmli buffer with 10% 2-mercaptoethanol and denatured with heat. A 12% stain free gel and 7.5% stain free gel was exposed to 302 nm UV light for 5 min. After transfer, the PVDF membrane was exposed with 302 nm UV light to obtain the total protein level on the membrane. This membrane was blocked with 5% BSA and dissolved in TBS buffer with 0.1% Tween-20. The membrane was incubated with anti-SGLT1 (PA5-88282, Invitrogen), anti-EAAT3 (12686-1-AP, Proteintech), anti-B^0AT1 (PA560276, Invitrogen), and anti-rabbit IgG (31460, ThermoFisher Scientific) antibodies at 4°C overnight. An ECL kit was used for imaging. ImageJ was used to quantitate the protein expression levels.

Digestive Enzyme Activity

Enzymatic activity of sucrase and maltase was measured according to previous protocols with some adaptations (Hansen, 1978; Peral et al., 1995; Rueda et al., 2007). Briefly, 56 mmol/L sucrose or maltose substrates were dissolved in 0.1 M sodium maleate. The samples were allowed to react with the substrate at 40°C for 1 h. The reaction was stopped by heating the samples with boiling water. Glucose levels were measured with a Glucose Colorimetric Detection Kit (EIAGLUC, Invitrogen). The enzyme activity unit (U) was defined as μmol/L product/μg protein/h.

Aminopeptidase activity was measured according to Mercado-Flores et al. (2006) with some modifications. Briefly, 2 mmol/L L-lysine p-nitroanilide was dissolved in 0.1 mol/L Tris buffer and allowed to react at 37°C for 1 h. The reaction was stopped by the addition of 5% $ZnSO_4$ and the pH was neutralized with 7.5% $Ba(OH)_2$. The sample was centrifuged (10,000 x *g*) for 10 min and supernatant was collected. The OD values was measured at 405 nm. The enzyme activity unit (U) was defined as μmol/L product/μg protein/h.

Na^+/K^+-ATPase activity was adapted from previous studies (Hansen, 1978; Peral et al., 1995). Briefly, 12 mmol/L p-nitrophenylphosphate with or without 700 μmol/L Ouabain was dissolved in buffer (50 mmol/L Tris, 10 mmol/L $MgSO_4$, 5 mmol/L EDTA, and

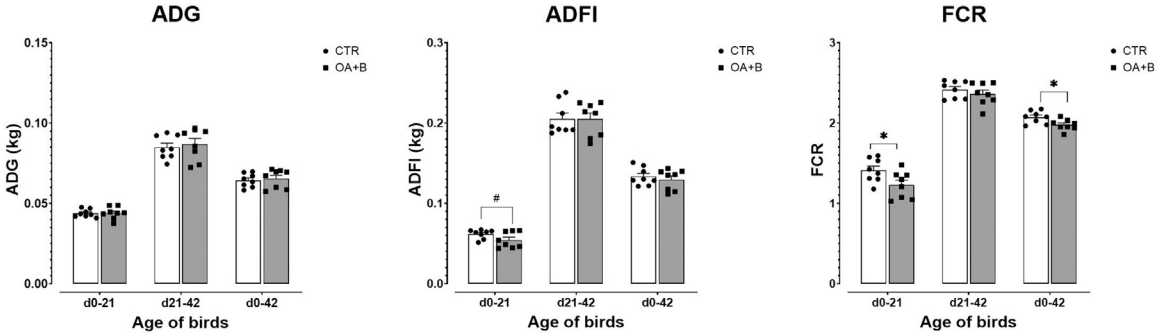

Figure 1. Growth performance results of birds fed with control or supplemented diet. ADG = Average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio. Difference between 2 groups at each growth period was labeled. *Indicates significant difference ($P \leq 0.05$), #indicates tendency ($P \leq 0.10$). Dietary treatments were as follows: CTR, basal diet; OA+B, basal diet + 500 ppm microencapsulated organic acids and botanicals.

90 mmol/L KCl) and allowed to react at 37°C for 30 min. The reaction was stopped with 30% trichloroacetic acid. The pH was neutralized with 1 N NaOH and the OD value was measured at 410 nm. The enzyme activity unit (U) was defined as μmol/L product/μg protein/h.

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism v.9.5.0 (GraphPad Software, Inc., San Diego, CA). Data are displayed on graphs as means ± SEM. For the growth performances analysis, the pen was used as an experimental unit ($n = 8$); for all other analyses, each individual animal was considered as an experimental unit (n = 6 ~16, depended on the measurements). The Shapiro−Wilk test was performed for normality check. Significance was determined using a Student's t-test, comparing the means of treated group (OA+B) against the untreated control (**CTR**). Additionally, False Discovery Rate test was performed with the 2-stage step-up (Benjamini, Krieger, and Yekutieli) methods. Differences were considered significant when $P \leq 0.05$ (*), and trends were identified when $0.05 < P \leq 0.1$ (#).

RESULTS

Growth Performance

Growth performance results are reported in Figure 1. No significant variations in the ADG were observed in the chickens fed OA+B diet. A tendency of reduction of ADFI was shown at the first 21 d of age in OA+B group ($P = 0.08$). However, during the first 3 wk and the overall growth period, the FCR was significantly improved in the OA+B-treated birds compared with the control ($P \leq 0.05$).

Intestinal Morphology

At d21, the jejunum VH and VH:VW ratio were tented to be higher ($P < 0.10$) in the dietary treatment group. In the ileum at d21, the VH, VH:VW ratio, VH:CD ratio, and villus surface area were significantly increased ($P \leq 0.05$) in the supplemented diet group. No significant differences were found in the intestinal mucosal morphology at d 42 (Table 2).

Digestive Enzyme Activity in Jejunum

Enzymatic activity of sugar digestive enzymes was positively modulated by the supplementation of OA+B (Figure 2). In particular, mucosa of jejunum showed significant increase of maltase activity at both 21 and 42 d of age birds. Similarly, maltase activity tended to be increased in BBMV ($P = 0.09$) in d 42 birds treated with OA+B. Concerning sucrase, the addition of OA+B allowed an increase of enzyme levels in both BBMV ($P = 0.07$) and mucosa ($P < 0.05$) after 42 d of treatment. No significant

Table 2. Intestinal mucosal morphology.

Sample	Treatment	VH (μm)	VW (μm)	CD (μm)	VH:VW	VH:CD	Villus area, (mm^2)
Jej d21	CTR	984 ± 38	138 ± 7	241 ± 7	7.7 ± 0.5	4.2 ± 0.2	0.43 ± 0.03
	OA+B	**1091 ± 47**†	126 ± 6	256 ± 13	**9.3 ± 0.6**†	4.4 ± 0.2	0.43 ± 0.03
Ile, d21	CTR	421 ± 14	122 ± 15	209 ± 14	3.9 ± 0.6	2.0 ± 0.3	0.15 ± 0.03
	OA+B	**709 ± 32***	99 ± 6	174 ± 25	**7.2 ± 0.6***	**4.3 ± 0.5***	**0.22 ± 0.01***
Jej d42	CTR	1336 ± 66	126 ± 6	324 ± 14	10.7 ± 0.7	4.1 ± 0.1	0.53 ± 0.04
	OA+B	1328 ± 45	125 ± 9	312 ± 11	11.0 ± 1.0	4.3 ± 0.1	0.52 ± 0.04
Ile, d42	CTR	805 ± 75	134 ± 7	218 ± 11	6.2 ± 0.8	3.7 ± 0.2	0.33 ± 0.02
	OA+B	806 ± 42	118 ± 12	230 ± 14	7.1 ± 0.8	3.5 ± 0.2	0.30 ± 0.03

Abbreviations: CD, crypt depth; CTR, control; Ile, ileum; Jej, jejunum; OA+B, basal diet + 500 ppm microencapsulated organic acids and botanicals; VH, villus height; VH:CD, villus height: crypt depth ratio. VH:VW, villus height: villus width ratio; VW, villus width.

*Indicates significant difference ($P \leq 0.05$).

†Indicates tendency ($P \leq 0.10$).

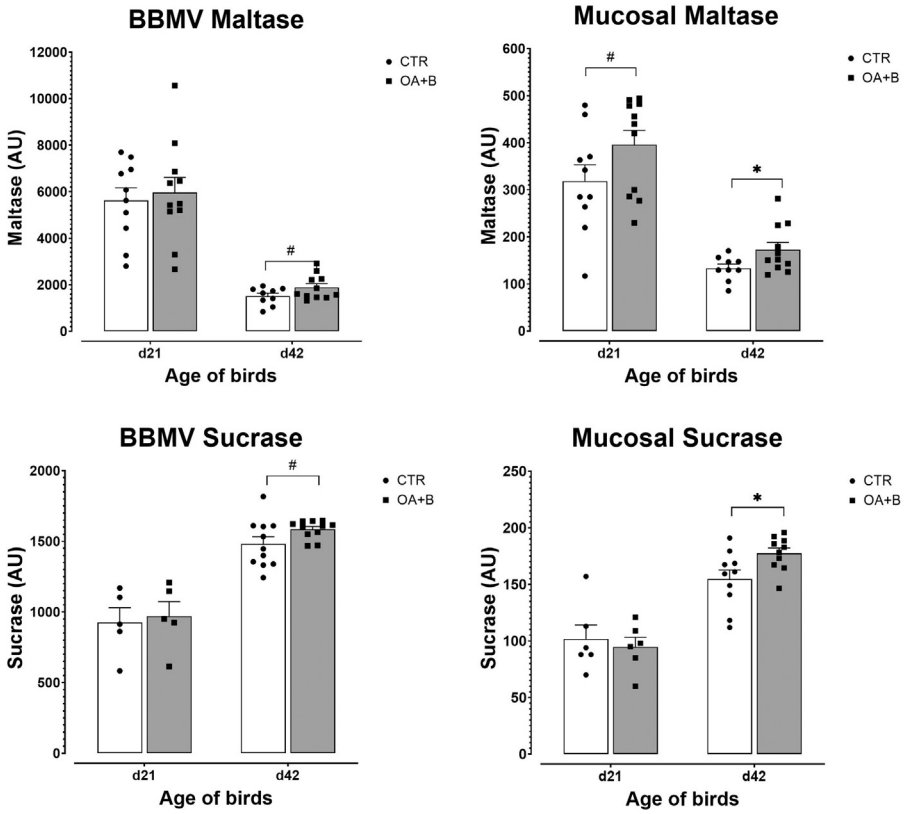

Figure 2. Digestive enzyme activity in jejunum of birds fed with control or supplemented diet. Maltase activity in BBMV and mucosa and sucrase activity in BBMV and mucosa are reported. BBMV = brush-border membrane vesicles. Difference between 2 groups at each growth period was labeled. *Indicates significant difference ($P \leq 0.05$), #indicates tendency ($P \leq 0.10$). Dietary treatments were as follows: CTR, basal diet; OA +B, basal diet + 500 ppm microencapsulated organic acids and botanicals.

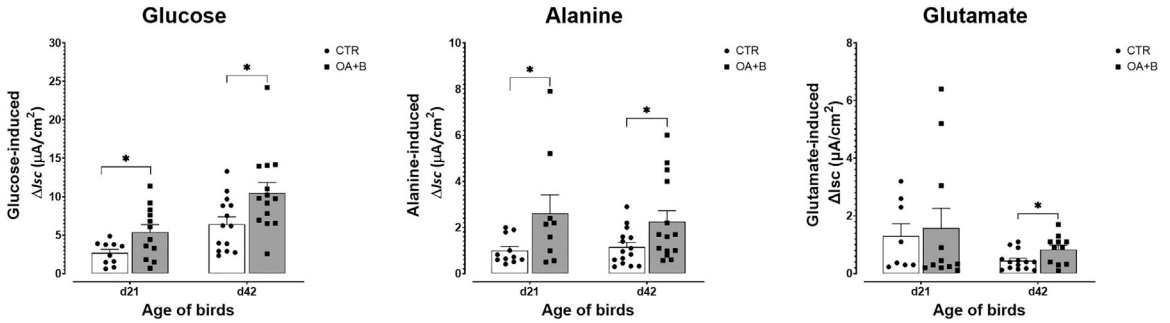

Figure 3. Nutrient transporter function in jejunum of birds fed with control or supplemented diet. Difference between 2 groups at each growth period was labeled. *Indicates significant difference ($P \leq 0.05$), #Indicates tendency ($P \leq 0.10$). Dietary treatments were as follows: CTR, basal diet; OA+B, basal diet + 500 ppm microencapsulated organic acids and botanicals.

differences in aminopeptidase activity were observed (data not shown).

Nutrient Transporter Function in Jejunum

Nutrient transporter function resulted augmented due to the supplementation of OA+B (Figure 3). In particular, glucose-induced ΔI_{SC} showed a significant increase at both 21 and 42 d of age birds when treated with supplemented diet. The AAs transport activities were upregulated in the supplementation group, with significant increase of alanine at both d21 and d42, while glutamate transport was increased at d 42.

Nutrient Transporter Expression in Jejunum

In spite of higher electrogenic glucose transport, none of the SGLT1 transporter gene expression nor BBMV protein location were significantly changed between treatments (Figures 4 and 5). However, the mRNA expression of sugar transporter GLUT1 and GLUT2 were both upregulated in the supplement group

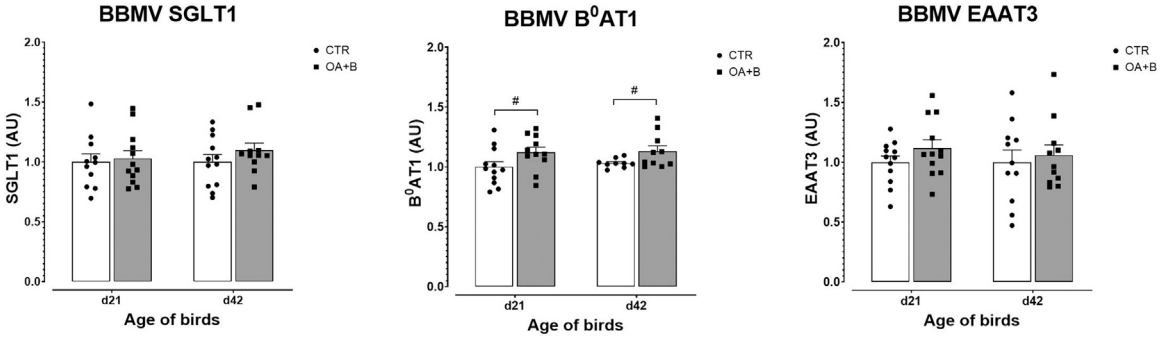

Figure 4. Nutrient transporter protein levels in jejunum of birds fed with control or supplemented diet. BBMV = brush-border membrane vesicles; SGLT1 = sodium/glucose cotransporter 1; B^0AT1 = Sodium-dependent neutral amino acid transporter; EAAT3 = excitatory AAs transporter 3. Difference between 2 groups at each growth period was labeled. *Indicates significant difference ($P \leq 0.05$), #indicates tendency ($P \leq 0.10$). Dietary treatments were as follows: CTR, basal diet; OA+B, basal diet + 500 ppm microencapsulated organic acids and botanicals.

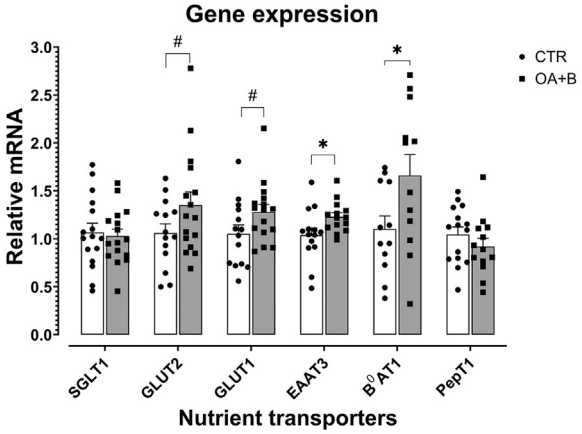

Figure 5. Nutrient transporter mRNA levels in jejunum of birds fed with control or supplemented diet. SGLT1 = sodium/glucose cotransporter 1; GLUT1 = glucose transporter 1; GLUT 2 = glucose transporter 2; EAAT3 = excitatory AAs transporter 3; B^0AT1 = Sodium-dependent neutral amino acid transporter; PepT1 = peptide transporter 1. Difference between 2 groups at each growth period was labeled. *Indicates significant difference ($P \leq 0.05$), #indicates tendency ($P \leq 0.10$). Dietary treatments were as follows: CTR, basal diet; OA+B, basal diet + 500 ppm microencapsulated organic acids and botanicals.

(Figure 5). The gene expression and BBMV protein of B^0AT1 were upregulated, while only mRNA expression of EAAT3 was increased in supplement group (Figures 4 and 5).

Digestive Enzyme Activity in Ileum

Similar with jejunum, the mucosal sucrase and maltase activities in ileum was positively modulated by the supplementation of OA+B at d42 of age (Figure 6). At d21, BBMV enriched maltase activity was higher, while mucosal sucrase was higher in the supplement group.

Nutrient Transporter Expression and Function in Ileum

Unlike in the jejunum, the ileal BBMV SGLT1 protein levels were increased in the supplement group at both d21 and d42 (Figure 7). The mRNA level of sugar transporter GLUT1 was upregulated (Figure 9). In spite that the AA transporter EAAT3 and B^0AT1 were both upregulated at d21 (Figure 8), while only BBMV and EAAT3 at d21 was higher in the supplemented group (Figure 7).

Ileal Na^+/K^+-ATPase Activity and Serum Glucose

In order to understand the potential Na^+ gradient, which is the driving force of aforementioned Na^+-dependent glucose and AA transport, the ileal Na^+/K^+-ATPase activity measured. This enzyme activity was increased in group fed with OA+B (Table 3), suggesting an increased potential of the nutrient transport. To confirm the overall systemic importance of intestinal transport changes, serum glucose level was quantified. Serum glucose levels were found higher in animal treated with OA+B (Figure 9).

DISCUSSION

Due to the high growth rate required in the poultry industry, broiler chickens are often bred with high-calorie diets to meet the objective of meat production. An efficient gut is essential to digest and absorb dietary nutrients, thus meet the high nutritional demands for growth and immune functions in modern birds. Feeding antibiotic alternatives improves broiler weight gain and gut health through various mechanisms, including reducing inflammation and modifying gut microbiota, yet their effects on intestinal epithelial nutrient transport was not fully understood. In this study, broiler chickens were fed with a microencapsulated blend of citric acid, sorbic acid, thymol, and vanillin (OA+B) for 42 d; then growth performance and nutrient digestion and absorption parameters were evaluated. The feed efficiency was significantly improved throughout the experimental period even though the growth rate of the birds did not change with OA+B supplementation. This beneficial effect of OA+B supplementation on growth performance and feed efficiency has been reported consistently in previous findings (Grilli et al., 2010; Mohammadi et

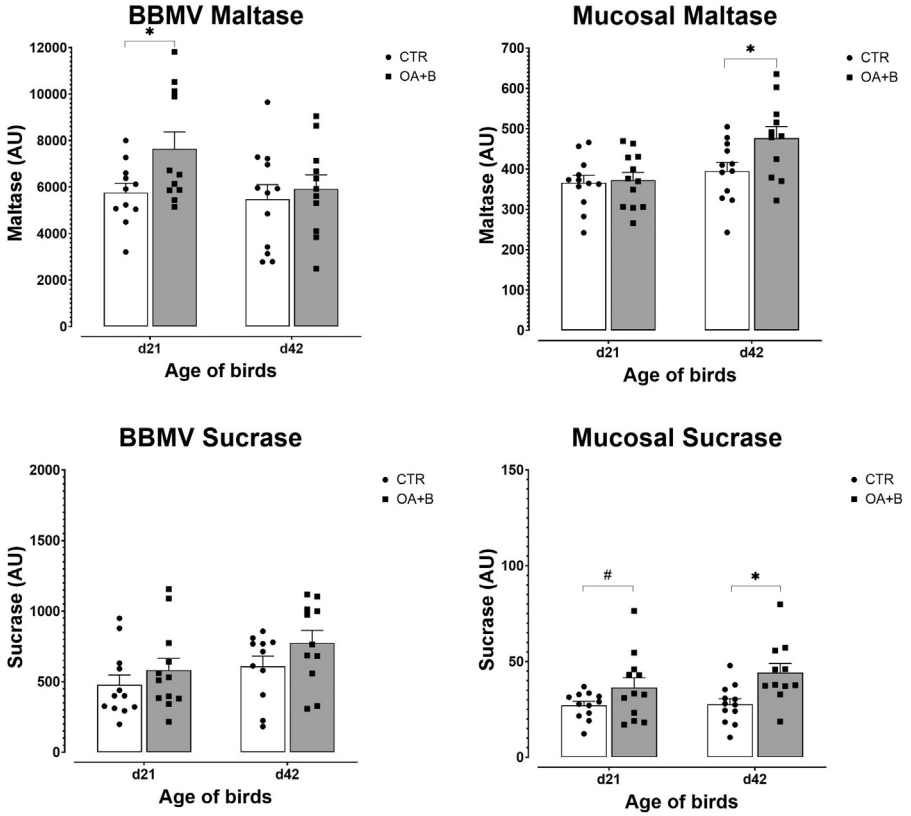

Figure 6. Digestive enzyme activity in ileum of birds fed with control or supplemented diet. Maltase activity in BBMV and mucosa and sucrase activity in BBMV and mucosa are reported. BBMV = brush-border membrane vesicles. Difference between 2 groups at each growth period was labeled. *Indicates significant difference ($P \leq 0.05$), #indicates tendency ($P \leq 0.10$). Dietary treatments were as follows: CTR, basal diet; OA+B, basal diet + 500 ppm microencapsulated organic acids and botanicals.

al., 2015; Hutchens et al., 2020; Bialkowski et al., 2023). We further evaluated intestinal health status in terms of epithelial morphology, nutrient digestive enzymes activity, and transport efficiency.

Villus morphology determines the balance between nutrient absorbing villus cells and secretory crypt cells and the effective area of nutrient absorption. In the present study, OA+B supplementation increased VH in the small intestines, and improved VH:CD ratio and total villus surface area in ileum at d 21 in broilers. Similar results of villus morphology from addition of essential oil and organic acids have been reported previously (Khodambashi Emami et al., 2012; Stamilla et al., 2020; Sun et al., 2022). In general, the reduced inflammation and a modified gut microbiota, suggested mechanisms for this improvement, likely protect the intestinal lining from damage, promoting overall epithelial cell health and a balanced population of epithelial cells. As an indication of epithelial cell function, further, we investigated the epithelial cell-membrane bound digestive enzyme activity.

The intestinal BBM is the functional site of nutrient digestive enzymes and transporters. Unlike total mucosal enzyme activity, which reflects the combined influence of epithelial cell function and the number and size of villi, BBM enzyme activity offers a more specific

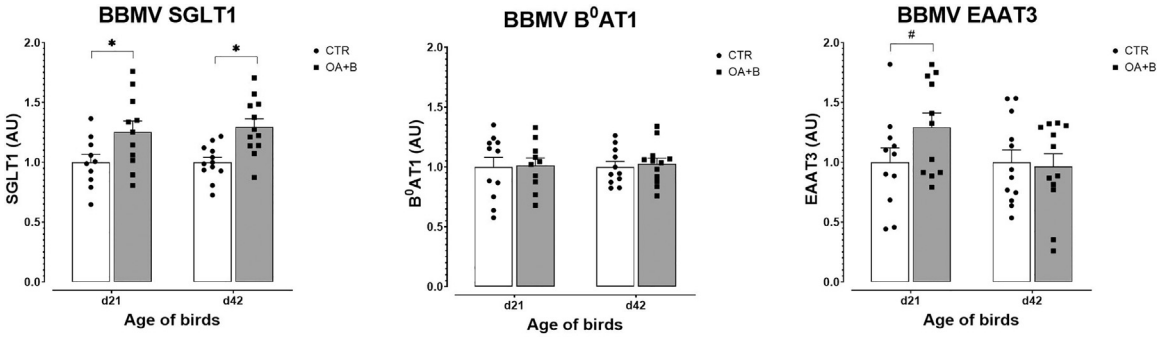

Figure 7. Nutrient transporter protein levels in ileum of birds fed with control or supplemented diet. BBMV = brush-border membrane vesicles; SGLT1 = sodium/glucose cotransporter 1; B^0AT1 = Sodium-dependent neutral amino acid transporter; EAAT3 = excitatory AAs transporter 3. Difference between 2 groups at each growth period was labeled. *Indicates significant difference ($P \leq 0.05$), #indicates tendency ($P \leq 0.10$). Dietary treatments were as follows: CTR, basal diet; OA+B, basal diet + 500 ppm microencapsulated organic acids and botanicals.

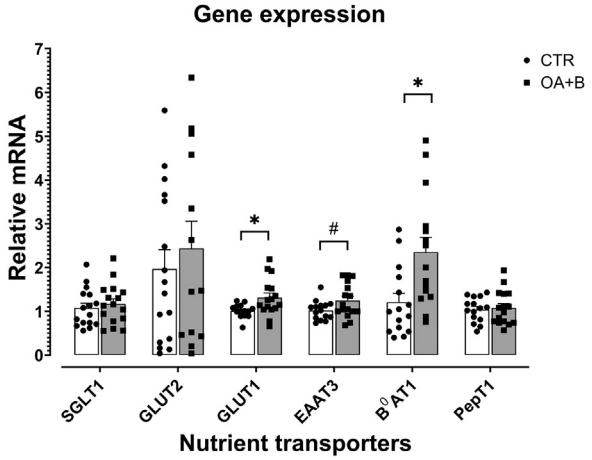

Figure 8. Nutrient transporter mRNA levels in ileum of birds fed with control or supplemented diet. SGLT1 = sodium/glucose cotransporter 1; GLUT1 = glucose transporter 1; GLUT 2 = glucose transporter 2; EAAT3 = excitatory AAs transporter 3; B^0AT1 = Sodium-dependent neutral amino acid transporter; PepT1 = peptide transporter 1. Difference between 2 groups at each growth period was labeled. *Indicates significant difference ($P \leq 0.05$), #indicates tendency ($P \leq 0.10$). Dietary treatments were as follows: CTR, basal diet; OA+B, basal diet + 500 ppm microencapsulated organic acids and botanicals.

measure of individual epithelial cell function. In this study, OA+B supplement upregulated the ileal BBM maltase activity at d21, while the jejunal BBM maltase and sucrase activities were not affected until d 42. While other studies using essential oil and organic acid additives observed increased digestive enzyme activity, their focus was primarily on the upper gastrointestinal tract and pancreas (Jang et al., 2007; Khodambashi Emami et al., 2012; Hashemipour et al., 2013; Deng et al., 2021; Su et al., 2021). Very few research in poultry studied the intestinal cell membrane bound enzymes. Since OA+B was delivered in a protective matrix in this study, we anticipate observing its initial effects in the lower intestine. After 42 d of supplement, the upregulation of mucosal enzymes activities in jejunum and ileum in the present study indicated an improvement in the overall gut digestion of nutrients. Interestingly, not all membrane bound enzymes were affected in the same manner, since no changes were observed with aminopeptidase activity. More studies are needed in the future to reveal this mechanism.

Nutrient absorption in the gastrointestinal tract is a key point in achieving the right energy and nutrients intake, with a particular interest in carbohydrates and AAs uptake (Lenard and Berthoud, 2008). Therefore, we further evaluated the expression and function of specific transporters for these nutrients. There are 3 glucose transporters of our interest. SGLT1 and GLUT2 are expressed specifically on the epithelial cells, where the former actively absorb low dose of luminal glucose and generate electric current, whereas the latter passively diffuse high dose of postprandial glucose in gut lumen and release epithelial glucose to the bloodstream (Shibata et al., 2019). GLUT1, in the other hand, is ubiquitously expressed in most of cells in the intestine and brings blood glucose into cells and its expression level reflects the available glucose surrounding the cells. Our findings suggest that OA+B could upregulate the expression of GLUT1 and GLUT2, but not SGLT1 in the jejunum at d21 of age. Therefore, the treated intestines were equipped with high capacity of obtaining dietary glucose and supporting tissue needs. Additionally, OA+B was able to enhance the mRNA expression of major Na^+-coupled AAs transporters, EAAT3 and B^0AT1. In particular, B^0AT1 transports majority of electroneutral AAs, while EAAT3 is mainly responsible for glutamate uptake. Our data suggested that the addition of OA+B in the diet might support the uptake of the primary fuel source of enterocyte. The upregulated expression of these nutrient transporters may be one of the reasons for the improved FCR in treated broilers. Remarkably, Mott and colleagues (2008) reported greater expression of EAAT3, SGLT1, and PepT1 in female chickens, in order to increase AAs and sugars assimilation to be more metabolically efficient and prepare for the energy expenditure of reproduction. This suggests that the supplementation of OA+B could make chickens more metabolically efficient, improve the positive energy balance, and ameliorate the growth performances due to the increase of abundance of nutrient transporters as a possible mode of action. The differences in the magnitude of results between the 2 intestinal tracts analyzed is related with the different expression of nutrient transporters in the intestinal segments. In the chicken intestine, the jejunum is where sugar assimilation is most likely to occur, as mRNA levels increase in

Table 3. Amino peptidase and Na^+/K^+-ATPase activity.

Sample	Treatment	BBMV Aminopeptidase	Mucosal Aminopeptidase	Mucosal Na^+/K^+-ATPase
Jejunum,	CTR	165 ± 19	37 ± 4	40 ± 8
d 21	OA+B	206 ± 27	38 ± 5	35 ± 9
Ileum,	CTR	369 ± 41	29 ± 5	11 ± 1
d 21	OA+B	367 ± 42	35 ± 6	**14 ± 2**[†]
Jejunum,	CTR	178 ± 11	33 ± 3	50 ± 7
D 42	OA+B	182 ± 11	33 ± 2	57 ± 10
Ileum,	CTR	285 ± 21	24 ± 4	9 ± 1
D 42	OA+B	342 ± 55	29 ± 4	**20 ± 6**[†]

Abbreviations: BBMV, brush-border membrane vesicles; CTR, control; OA+B = basal diet + 500 ppm microencapsulated organic acids and botanicals.

[†]Indicates tendency ($P \leq 0.10$).

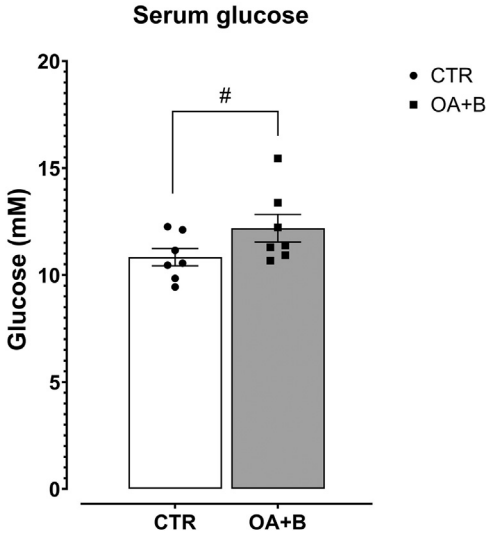

Figure 9. Serum glucose level in birds fed with control or supplemented diet. *Indicates significant difference ($P \leq 0.05$), #indicates tendency ($P \leq 0.10$). Dietary treatments were as follows: CTR, basal diet; OA+B, basal diet + 500 ppm microencapsulated organic acids and botanicals.

the jejunum with age, showing a correlation of glucose transporters abundance (GLUT2, GLUT5, SGLT1) and intestinal segments (Garriga et al., 2002; Gilbert et al., 2007). Likewise, EAAT3 is highly express in the ileum compared to other segments (Iwanaga et al., 2005; Mott et al., 2008), suggesting that ileum is mainly responsible for the absorption of AAs, while jejunum is responsible for the absorption of carbohydrates.

Given the known discrepancies between gene transcription, protein translation, and post-translational modifications, evaluating transporter function provides a more biologically relevant measure of their activity than simply measuring their expression levels. The well-established Ussing chamber technique enables real-time measurement of epithelial Na^+-coupled glucose and AA absorption activity by monitoring I_{SC} changes upon specific nutrient application (Li et al., 2017, 2022; Karaki, 2023). In the present study, OA+B supplementation for 21 d significantly increased electrogenic absorption of glucose, alanine, and glutamate in the jejunum. In addition, the transporter function was in line with the transporter protein level in BBM sample. It is worth noting that the alanine and glutamate are not the only substrates for this transporter system, they serve as representatives for most dietary electroneutral and negatively charged AAs. Typically, increased surface area of villi results in enhanced digestion and absorption performance, which is accompanied by increased production of BBM enzymes and higher availability of nutrient transporters. In contrast, villus nutrient transport function could be independent with its structure or morphology (Boudry et al., 2007; Li et al., 2017, 2022). In this study, OA+B allowed an increase of nutrient transporters abundance and functions. It is probable that the microencapsulated blend treatment resulted in improved enterocyte maturation and nutrient transporter efficiency. Another possibility is that the treatment could enhance the affinity of transporters for a nutrient, then cells are more competent and efficient to prepare in case nutrients become scarce. If affinity increases, the new transporter can generate a greater import flux than the original transporter at sufficiently low concentrations of substrate because the increase in affinity dominates the decrease in rate (Montaño-Gutierrez et al., 2022).

Most of the transport gene expression and BBM protein level in the ileum exhibited in the same pattern compared that in the jejunum. Due to the limitation in lab capacity, we were not able to measure the ileal transporter function on the Ussing chamber. Alternatively, ileal Na^+/K^+-ATPase activity was measured. This enzyme maintains the Na^+ gradient across the cell membrane, which serves as the driving force for Na^+-coupled nutrient transport. The increased Na^+/K^+-ATPase activity in OA+B group indicated a strong absorption capacity. Another limitation of this study is that it focused solely on the electrogenic component of nutrient transport. The intestine likely employs other mechanisms, such as passive or electroneutral transport, that were not evaluated.

It is possible that certain feed additives affect the nutrient transport mechanisms, while the exact mechanism of action remains unknown, there are some speculations that can be made. Cell metabolism may be impacted by the role of citric acid, which is an intermediate in the Krebs cycle (Krebs and Johnson, 1980), whereas the signaling of insulin-like growth factor (IGF) pathway can be influenced by sorbic acid, a medium-chain unsaturated fatty acid that is metabolized through β-oxidation (Luo et al., 2011). Moreover, OAs plays a crucial role in digestion, absorption, and metabolism due to a change in microbial diversity in broilers, which could potentially enhance the chickens' energetic balance (Yin et al., 2023; Dittoe et al., 2023; Hu et al., 2024). Also, the use of thymol and vanillin in animal nutrition is widespread due to their botanical compounds, which have anti-inflammatory and antioxidant activities both *in vitro* and *in vivo* (Windisch et al., 2008; Toschi et al., 2020; Rossi et al., 2020). All these factors could lead to an improvement in the maturation of enterocytes, as a stronger BBM, high efficiency of BBM-binding disaccharide enzymes, and a high number and efficiency of transporters can be achieved, and improvements in transportation activities can eventually be observed through the use of the Ussing chamber. The overall upregulation in sugar digestive enzyme and transporter function might contribute to the increase in blood glucose level, thus support the higher feed efficiency and growth performance.

CONCLUSION

In conclusion, the results of the present study indicate that dietary supplementation with organic acids and botanicals can improve overall feed efficiency and enhance intestinal health. The beneficial effects of these supplements may be partially attributed through

improved intestinal morphology, enhanced activities of epithelial cell membrane-bound enzymes, and increased expression and function of nutrient transporters. This study provides a novel perspective on evaluating supplements that benefit intestinal health. The inclusion of antibiotic alternatives can improve nutrient digestion and the absorptive capacity of the intestine, which, in turn, facilitates animal growth and production efficiency.

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DISCLOSURES

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ester Grilli reports financial support was provided by Vetagro S.p.A. Ester Grilli reports a relationship with Vetagro Inc. that includes: board membership. Ester Grilli reports a relationship with University of Bologna that includes: employment. Andrea Toschi reports a relationship with Vetagro S.p.A. that includes: employment.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.104237.

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