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Enterocin M and Sage Supplementation in Post-weaning Rabbits: Effects on Growth Performance, Caecal Microbiota, Fermentation and Enzymatic Activity

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Abstract

The effects of enterocin (Ent) M and sage extract applied separately and in combination were investigated. EntM (E 50 μL/animal/day in water) and sage extract (S 10 μL/animal/day in water) were applied individually and in combination (E+S) to rabbits during 21 days of treatment. The rabbits' growth was not significantly influenced by the additives. Lower feed conversion (FC) was noted in the experimental groups compared with controls, with the lowest data detected in E. The antimicrobial activity of EntM was noted (in E+S: lactic acid bacteria—$P < 0.01$; in E, E+S: enterococci, enterobacteria—$P > 0.05$; in E: clostridia—$P > 0.05$). The most significant changes in fermentation between weaned and older rabbits were noted in amylolytic activity at day 21 (E $P < 0.05$; E + S $P < 0.05$); prolonged reduction effect of sage extract on amylolytic activity was observed. The activity of cellulase, pectinase and xylanase was higher in older than in younger animals. Decrease in lactic acid and volatile fatty acids was noted during EntM administration, with significant effect on propionic acid concentration (E $P < 0.05$; E+S $P < 0.001$). The sage extract reduced propionic acid (S $P < 0.001$) and butyric acid levels (S $P < 0.05$) and increased the concentrations of butyric, iso-valeric, valeric, caproic acids and lactic acid ($P < 0.001$). It seems to be that EntM and sage supplementation may improve the economy of rabbit farms (increased FC) and the health status of rabbits (reduction of spoilage microbiota, enhanced enzymatic activities in caecum).

Keywords Enterocin · Sage · Microflora · Enzymes · Organic acids

Introduction

In rabbits, the caecum and the proximal colon are the primary fermenters; approximately 40% of digested organic matter of the feed is digested in the caeco-colic segment [1]. The digestion of nutrients is localised in the small intestine by the digestive enzymes of the host. However, some components, e.g. plant cell walls and fibres (mainly lignins, cellulose, hemicellulose, pectins) are hydrolyzed by bacterial enzymes into soluble smaller compounds (e.g. monosaccharides, amino acids), which are fermented into the end products—such as volatile fatty acids (VFA: acetic, propionic and butyric acid), ammonia, intermediary metabolites (lactic, succinic, formic acid) and gas (CO_2, CH_4, H_2; [2]). Changes and stabilisation of the bacterial population from birth through weaning to slaughter age cause changes in the digestive physiology, fermentative activities, quantities and proportions of end products. Before weaning, bifidobacteria, lactobacilli and colibacilli are the predominant flora which is typical for milk-fed animals. On weaning, the substrate for caecal fermentation is continually changed into solid feed, containing large amounts of polysacharide, and this leads to development of a microbial population with dominance of enterobacteriaceae and anaerobic bacteria. These bacteria have cellulolytic, fibrolytic, pectinolytic, xylanolytic and starch degrading activities [3, 4], which are usually increased and stabilised from 15 to 44–49 days of age, also influencing the levels of end products, mainly VFA. Because of the complexity of caecal digestion, stable microbial fermentation is essential for rabbit

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health, and only small dietary and environmental changes can lead to microbial dysbiosis, digestive disturbances, susceptibility to bacterial/viral agents and increased morbidity and/or mortality. Control of the microbiota could therefore improve digestive efficiency, and consequently also the immune status and digestive health. One of the ways of preventing those disturbances is the supplementation of the rabbits' diet with natural substances: probiotics, prebiotics, organic acids, enterocins and herbal extracts [5–10]. Despite many studies demonstrating the beneficial results of the application of these substances in rabbits, there is a need to extend the existing knowledge as well as to find new possibilities concerning the improvement of the caecal environment and rabbit health. On the other hand, results of studies based on bacteriocin/enterocin administration in rabbits are still in the initial phase, especially compared with probiotics addition [5, 7, 8, 11, 12]. To the best of our knowledge, this is the first study helping to link the effects of enterocin addition with characteristics of the caecal microbiota composition and enzymatic activity in rabbits. The objective of this work was to test the effects of enterocin M and sage extract applied individually and in combination on growth performance, caecal microbiota, fermentation and enzymatic activity in vivo in rabbits.

Material and Methods

The experiment was performed in co-operation with our colleagues in Nitra (National Agricultural and Food Centre - NAFC). All care and experimental procedures involving animals followed the guidelines stated in the Guide for the Care and Use of Laboratory Animals approved by the Slovakian State Veterinary and Food Administration and the Ethical Committees of both institutions.

Animals and Housing

Sixty-four rabbits (meat line M91, aged 5 weeks after weaning, both sexes) were divided into three experimental groups (E—EntM, S—sage extract, E+S—EntM in combination with sage extract) and one control group (C), 16 animals in each group. The rabbits were kept in standard cages (0.61 m × 0.34 cm × 0.33 m, D-KV-72 supplied by the Kovobel company, Domažlice, Czech Republic), two animals per cage. A cycle of 16 h light, 8 h dark was used throughout the experiment. Temperature 16 ± 4 °C and humidity 70 ± 5% were maintained throughout the experiment by heating and ventilation systems and were recorded continuously with a digital thermograph positioned at the same level as the cages.

Experiment Design

The animals were fed a commercial granulated diet for growing rabbits (Table 1) ad libitum during the experiment. The rabbits in group E were administered EntM (prepared according to Mareková et al. [13]; a dose of 50 μL/animal/day, with activity 12,800 AU/mL, from day 0–1 to day 21) in their drinking water. Activity of EntM was tested by means of the agar spot test according to De Vuyst et al. [14] against the principal indicator strain *Enterococcus avium* EA5 (isolated from piglet, in our laboratory). Rabbits in group S received (from day 0–1 to day 21) sage plant extract (*Salvia officinalis* extract containing 24% thujone, 18% borneol and 15% cineole; the Calendula company, Nová Ľubovňa, Slovakia) in their drinking water at a dose of 10 μL/animal/day. The animals in E+S were administered (from day 0–1 to day 21) the combination of EntM (50 μL/animal/day, 12,800 AU/mL) strain and sage plant extract (10 μL/animal/day). The doses of additives and manner of their application resulted from our previous in vitro studies testing the inhibitory activity of EntM and sage extract against target bacteria and another experiment with rabbit-derived bacteriocin-producing strain *E. faecium* CCM7420 [5, 15]. On the basis of our previous experiments, we had information about the volume of water drunk by rabbits, so firstly, the rabbits consumed 100 mL of drinking water with additives, and after consuming the total volume, they had access to water ad libitum. Control rabbits (group C) were fed the commercial diet only. The experiment lasted for 35 days.

Performance Traits, Slaughtering and Sampling

Body weight and feed consumption were measured every week during the experiment; average daily weight gain and feed conversion were calculated mathematically. Mortality and morbidity were also recorded in groups daily. At days 21 and 35, rabbits were randomly selected for slaughter ($n = 4$); caeca were sampled to test microbiota, organic acid analyses and enzymatic activity. The rabbits were stunned with electronarcosis (90 V for 5 s), immediately hung by the hind legs on the processing line and quickly bled by cutting the jugular veins and the carotid arteries.

Bacterial Enumeration

To test the microbiota, samples of caecal content (1 g) were treated using the standard microbiological dilution method

Table 1 Ingredients and determined chemical composition of the commercial diet

Feed ingredients (%)	Diet	Chemical analysis, minerals and vitamins (g[a], mg[b]/kg feed)	Diet
Dehydrated lucerne meal	36.0	Dry matter[a]	882.1
Extracted sunflower meal	5.5	Crude protein[a]	164.2
Oats	13.0	Crude fibre[a]	155.5
Wheat bran	9.0	Fat[a]	33.3
Dry malting sprouts	15.0	Ash[a]	73.0
Extracted rapeseed meal	5.5	Nitrogen free extract[a]	457.1
Barley	8.0	Organic compounds[a]	810.0
DDGS	5.0	Acid detergent fibre (ADF)[a]	191.1
Sodium chloride	0.3	Neutral detergent fibre(NDF)[a]	339.5
Premix minerals[1]	1.7	Lignine[a]	42.3
Limestone	1.0	Hemicellulose[a]	148.5
		Cellulose[a]	148.8
		Starch[a]	127.2
		Calcium[a]	6.0
		Phosphorus[a]	5.9
		Magnesium[a]	2.3
		Sodium[a]	1.7
		Potassium[a]	7.5
		Iron[b]	403.9
		Zinc[b]	166.4
		Manganese[b]	156.7
		Cuprum[b]	22.9
		Metabolic energy (MJ/kg)	11.0

DDGS dried distillers grains with solubles

[1] Premix contains per kg: calcium 6.73 g; phosphorous 4.13 g; magnesium 1.90 g; sodium 1.36 g; potassium 11.21 g; iron 0.36 g; zinc 0.13 g; copper 0.03 g; selenium 0.2 mg. Vitamin mixture provided per kg of diet: vitamin A 1500000 IU; vitamin D3 125,000 IU; vitamin E 5000 mg; vitamin B1 100 mg; vitamin B2 500 mg; vitamin B6 200 mg; vitamin B12 0.01 mg; vitamin K3 0.5 mg; biotin 10 mg; folic acid 25 mg; nicotinic acid 4000 mg, choline chloride 100,000 mg

according to the International Organization for Standardization (ISO). The appropriate dilutions in Ringer solution (pH 7.0; Oxoid Ltd., Basingstoke, Hampshire, England) were spread on the following media: M-Enterococcus agar (NF-V04503, Difco Laboratories, Detroit, USA) for enterococci, De Man-Rogosa-Sharpe agar (ISO 15214, Merck, Germany) for lactic acid bacteria (LAB), Mannitol Salt agar for coagulase-negative staphylococci (CoNS, ISO 6888, Difco), Baird-Parker agar enriched with egg yolk tellurite supplement (ISO 21527-1, Difco) for coagulase-positive staphylococci (CoPS), *Clostridium difficile* agar with the supplement SR0096E 7% (*v*/*v*) defibrinated horse blood (SR0050, ISO 15883, Oxoid) for *Clostridium* species (anaerobic cultivation), MacConkey agar (ISO 7402, Oxoid) for coliforms and CLED agar (Conda, Spain) for enterobacteria. Pseudomonads were isolated on Pseudomonas agar (Biomark, India). Cultivation was performed at 30 °C and/or 37 °C for 24–48 h depending on the bacterial genera. The bacterial counts were expressed in log 10 of colony-forming units per gram (log10 CFU/g ± SD).

Organic Acid Analyses and Measurement of Enzymatic Activity

Lactic acid (g/100 g) and volatile fatty acid (VFA) values (acetic, propionic, butyric, iso-butyric, valeric, iso-valeric and caproic acids) were determined (mmol/L) using gas chromatography (Perkin Elmer gas chromatograph, USA) from samples of caecal content (15 g) on days 21 and 35. A glass column (average diameter 3 mm, length 180 cm) was filled with N_2 (30 mL), H_2 (20 mL) and air (240 mL) and a sample (1 μL) for diffusion. As the standard column was used, isocapron acid (SP 1200 H_3PO_4) on Chromosorbe WAW was separated at 130 °C and at 125 °C on Chromatone N-AW-DMCS. The value of pH was measured with a Jenwey 3310 pH metre (Germany).

The degradation of plant carbohydrates (cellulose, starch, inulin, pectin, xylan) was determined using the method previously described by Miltko et al. [16].

Statistical Analysis

Statistical analysis of the results was performed with one-way analysis of variance (ANOVA) and the Tukey's post hoc test with the level of significance set at $P < 0.05$. The results are quoted as means ± SD. The results were compared between groups within the same days of samples collections to check the changes during the experiment within individual experimental groups (small letters a, b, c, d).

Results

Application of EntM (E) and sage extract (S) separately did not influence the growth of the rabbits; the animals were in good health throughout the experiment. During combined application (E+S), minimal mortality was recorded compared with the control group (C; Table 2). On the other hand, the highest body weight gain was also noted in this group (up by 0.5% compared with C). Lower feed conversion was noted in the experimental groups compared with C (day 21), with the lowest data detected in E.

In the caecum only in the E+S group, counts of LAB were reduced significantly ($P < 0.01$, Table 3) compared with C. Lower LAB counts in the S group (difference 0.79 log cycle) and enterococci in E, S and E+S groups (difference 0.90, 1.18 and 1.43 log cycles, respectively) were also enumerated compared with C. Enterobacteria were decreased in E and E+S groups with differences of 0.27 and 0.37 log cycles, respectively. Clostridia were also decreased in E (difference 0.58 log cycle).

Increased enzymatic activity was noted in the caecum, except the cellulolytic activity in each group and the pectinolytic activity in S and E+S rabbits (day 21, Table 4). Cellulolytic activity was comparable with the activity of pectinase and xylanase, being higher in older than in younger animals. The most significant changes were noted in amylolytic activity at day 21 (E, E+S $P < 0.05$). After additives cessation (between days 21 and 35), higher activity of tested enzymes, including cellulolytic activity, was noted in the experimental groups, except for starch and inulin degradation in E+S rabbits.

In general, lower levels of LA and VFA in the chymus were measured in E and E+S groups compared with C (day 35, Table 4); significant reduction in propionic acid (E $P < 0.05$; S, E+S $P < 0.001$; Table 4) and butyric acid levels (S $P < 0.05$) was noted. In contrast, in rabbits receiving sage extract, the concentration of butyric, iso-valeric, valeric, caproic acids and lactic acid ($P < 0.001$) were increased.

Discussion and Conclusion

Good health status of rabbits was noted during the whole experiment. While Lauková et al. [17] presented improved weight gain during lantibiotic nisin application in rabbits (about 9.4%), Ent2019 administration did not influence the rabbits´ growth [5], which is in accordance with our findings. Regarding the feed conversion ratio, beneficial impact of Ent2019 and sage extract application in rabbits was noted, and similarly by us; moreover, increased weight gain and consumption during sage administration was presented [15]. The improved feed conversion ratio could be explained in terms of more intensive metabolism in the caecum.

In general, lower counts of tested bacteria in the caecum than in faeces (data not shown) and their inhibition/reduction were detected, similar to our previous results [7, 11, 15, 17]. The antimicrobial effects of bacteriocins and sage extract are well known, including mostly Gram-positive but also Gram-negative bacteria. While Szabóová et al. [7] presented better caecal bacteria reduction using sage and Ent4231 in combination, compared with the results of their individual use, EntM and sage together inhibited only enterococci, LAB, enterobacteria and pseudomonads. Caecal microbiota is able to synthesise enzymes, which help in nutrient digestion through hydrolysis of the diet components of plant cell walls or fibres (mainly lignins, cellulose, hemicellulose and pectins), which cannot be decomposed by the host's digestive enzymes. Development of the ability to ferment carbohydrates depends on the complexity of the carbohydrates concerned and the types of bacteria dominating the microbiota. Changes in bacterial population depend on age (before and after weaning) and diet composition (change from milk to solid feed; [4]). The bacterial community increases with age and reaches stable levels around the weaning period [3]; the higher enzymatic activity in the rabbits´ caecum at the end of the experiment (day 35 of experiment; day 70 of age) confirmed this fact. Sirotek et al. [18] described the production of amylases by *Clostridium butyricum* and also by *Bacterocides* and *Bifidobacterium* cells. Because the counts of clostridia were not influenced by EntM and sage addition, we assume higher amylase activity of anaerobic bacteria, including *Bacteroides* and *Bifidobacterium* species, the bacterial counts of which, however, were not enumerated by us. Strictly anaerobic bacteria in the caecum are also characterised by fibrolytic and pectinolytic activity [19]. Because no changes in the counts of the tested aerobic bacteria were observed by us, we hypothesise higher fermentative activity by the anaerobic caecal microbiota. The cellulolytic potential of clostridia in vivo was noted at day 35 (70 days of age; higher clostridial counts, higher cellulolytic activity); Gupta et al. [20] demonstrated the potential of *Clostridium* sp. to convert cellulose into reducing sugars under in vitro conditions. Despite several modern diagnostical methods and intensive testing of rabbit caecum and GIT, most rabbit caecal bacteria still correspond to new uncultivated bacterial species not found in the databases [2].

Table 2 The effect of enterocin M (E), sage extract (S) and their combinative (E+S) application on the growth performance of rabbits

	E	S	E+S	C	*P* value
Number of rabbits	(*n* = 16)	(*n* = 16)	(*n* = 16)	(*n* = 16)	
Initial live weight (35 days), g	940.6 ± 180.8	993.1 ± 300.0	1002.3 ± 162.3	1042.5 ± 315.7	0.7167
Intermediate live weight (56 days), g	1687.8 ± 171.7	1737.5 ± 347.4	1664.4 ± 170.0	1856.9 ± 361.4	0.2185
Final weight (70 days), g	2280.0 ± 174.6	2175.6 ± 259.5	2206.7 ± 164.6	2319.2 ± 164.6	0.2645
Average daily gain (g/day)	40.45	40.47	42.70	42.50	
Feed conversation ratio between 35 and 56 days of age (g/g)	2.58	2.67	2.97	3.03	
Feed conversation ratio between 56 and 77 days of age (g/g)	4.39	3.70	4.28	4.29	
Feed conversation ratio per kg gain	3.30	3.35	3.48	3.57	
Mortality (*n*)	0	0	1	0	

35–56 days of age—application of enterocin M, sage and their combination

56–70 days of age—after the enterocin M and sage cessation

Specific activities of all tested enzymes were lower in young than in adult rabbits. The tested enzyme activities showed a tendency to increase till the end of the treatment, except for amylolytic activity in the group with sage administration (lower activity at day 35). Marcin et al. [21] observed enhancement of amylolytic activity during 42 days of treatment using sage essential oil in chickens at days 16 and 29, but a decrease at day 42, similarly to us. The ability of α-amylase activity inhibition by sage extract could be explained by the presence of the flavone compound chrysoeriol in *Salvia virgata* [22]. In contrast to our results, Lavrenčič [23] presented significantly higher starch fermentability at slaughter age (78 days of age) than at weaning age (36 days of age). This researcher also noted lower intensity of pectin, xylan and cellulose fermentation with increasing age, which does not correspond with our findings. Marounek et al. [24] presented lower amylase activity in young rabbits than in adults, in accordance with our results, but higher activity of inulinase at younger age, contrary to us. Our results are in accordance with those demonstrating higher xylanolytic and pectinolytic activities than cellulolytic activity in the caecum, also dependent on the age of rabbits (increased activity between 21 and 35 days of slaughtering; [4]). Marounek et al. [24] found

Table 3 The effect of enterocin M (E), sage extract (S) and their combinative (E+S) application on the bacterial counts ($\log_{10}$ CFU/g ± SD) in caecum of rabbits

Caecum	E	S	E+S	C	*P* value
	Day 21				
Enterococcus sp.	1.72 ± 1.12	1.44 ± 1.08	1.19 ± 0.45	2.62 ± 0.95	0.2071
LAB	3.59 ± 0.26^{a}	2.62 ± 1.21^{ab}	1.18 ± 0.48^{b}	3.41 ± 0.61^{a}	0.0021
CoNS	3.69 ± 0.18^{a}	3.68 ± 0.18^{a}	3.79 ± 0.34^{a}	2.45 ± 0.83^{b}	0.0044
CoPS	2.16 ± 0.19	2.72 ± 0.52	2.24 ± 0.53	2.02 ± 0.48	0.1995
Enterobacteria	3.80 ± 0.42^{ab}	4.45 ± 0.32^{a}	3.70 ± 0.37^{b}	4.07 ± 0.02^{ab}	0.0277
Coliform bacteria	1.35 ± 0.77^{ab}	2.73 ± 0.80^{a}	1.58 ± 0.83^{ab}	0.90 ± 0.00^{b}	0.0171
Pseudomonas-like sp.	4.46 ± 0.64	4.21 ± 0.50	3.70 ± 0.84	4.11 ± 0.07	0.3623
Clostridium-like sp.	4.46 ± 0.64	5.09 ± 0.03	5.10 ± 0.00	5.04 ± 0.07	0.0432
	Day 35				
Enterococcus sp.	1.90 ± 0.63	1.76 ± 0.97	1.32 ± 0.72	1.84 ± 0.85	0.7358
LAB	3.38 ± 0.05	3.80 ± 1.09	3.59 ± 0.29	4.00 ± 1.01	0.6906
CoNS	3.72 ± 0.42	3.71 ± 0.18	3.71 ± 0.44	3.54 ± 0.41	0.8867
CoPS	2.44 ± 0.11	2.59 ± 0.18	2.40 ± 0.10	2.57 ± 0.37	0.5477
Enterobacteria	3.72 ± 0.07	3.67 ± 0.02	4.20 ± 0.82	4.40 ± 1.02	0.3508
Coliform bacteria	0.90 ± 0.00	1.03 ± 0.25	0.93 ± 0.05	1.82 ± 1.10	0.1192
Pseudomonas-like sp.	3.63 ± 0.20	3.56 ± 0.16	3.43 ± 0.16	3.58 ± 0.11	0.3776
Clostridium-like sp.	5.43 ± 0.67	5.99 ± 0.23	6.10 ± 0.00	6.02 ± 0.34	0.1148

CoNS coagulase-negative staphylococci, *CoPS* coagulase-positive staphylococci, *LAB* lactic acid bacteria, *ND* not detected

^{a,b,c,d} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

Table 4 The effect of enterocin M (E), sage extract (S) and their combinative (E+S) application on organic acids concentrations and on the digestion rate of carbohydrates in caecum of rabbits

	E	S	E+S	C	*P* value
Organic acids	Day 35				
Acetic acid (mmol/100 mL)	7.560 ± 1.140	8.031 ± 0.576	7.716 ± 0.961	9.143 ± 0.673	0.0914
Propionic acid (mmol/100 mL)	0.509 ± 0.083^{a}	0.477 ± 0.096^{ab}	0.455 ± 0.064^{ab}	0.775 ± 0.148^{c}	0.0028
Iso-butyric acid (mmol/100 mL)	0.008 ± 0.002	0.011 ± 0.004	0.006 ± 0.001	0.012 ± 0.007	0.2133
Butyric acid (mmol/100 mL)	1.846 ± 0.268^{a}	2.740 ± 0.148^{b}	2.105 ± 0.250^{ac}	2.536 ± 0.362^{c}	0.0020
Iso-valeric acid (mmol/100 mL)	0.028 ± 0.006	0.032 ± 0.007	0.026 ± 0.005	0.031 ± 0.004	0.4418
Valeric acid (mmol/100mLl)	0.066 ± 0.012^{a}	0.112 ± 0.026^{b}	0.087 ± 0.015^{ab}	0.095 ± 0.024^{ab}	0.0461
Caproic acid (mmol/100 mL)	0.049 ± 0.025	0.059 ± 0.026	0.073 ± 0.024	0.053 ± 0.033	0.6301
NH_3 (mmolLl)	11.037 ± 1.741	8.944 ± 1.170	10.386 ± 0.912	11.168 ± 2.789	0.3266
Lactic acid (g/100 g)	0.009 ± 0.002^{a}	0.021 ± 0.003^{b}	0.009 ± 0.004^{a}	0.009 ± 0.002^{a}	0.0001
pH	0.056 ± 0.008	0.065 ± 0.017	0.065 ± 0.017	0.065 ± 0.185	0.9987
Carbohydrates	Day 21				
CMC (μmol glucose/g DM of ceacum digesta/min)	8.47 ± 2.46	9.53 ± 0.74	8.84 ± 1.70	10.44 ± 2.16	0.3934
Xylan (μmol xylose/g DM of ceacum digesta/min)	11.72 ± 3.66	11.86 ± 0.52	11.28 ± 1.26	11.22 ± 2.76	0.5968
Pectin (μmol glucric acid/g DM of ceacum digesta/min)	9.33 ± 0.99	9.14 ± 1.84	8.52 ± 0.98	9.27 ± 1.13	0.8655
Starch (μmol glucose/g DM of ceacum digesta/min)	16.98 ± 2.36^{a}	14.45 ± 3.85^{ab}	17.61 ± 3.60^{a}	10.19 ± 1.61^{b}	0.0080
Inulin (μmol fructose/g DM of ceacum digesta/min)	5.13 ± 0.65	4.85 ± 0.46	4.68 ± 0.87	4.42 ± 0.47	0.1521
	Day 35				
CMC (μmol glucose/g DM of ceacum digesta/min)	10.78 ± 2.39	9.40 ± 1.63	9.87 ± 1.83	8.44 ± 1.86	0.1781
Xylan (μmol xylose/g DM of ceacum digesta/min)	20.96 ± 1.90^{a}	15.51 ± 3.32^{b}	15.98 ± 2.19^{ab}	14.73 ± 3.38^{b}	0.0052
Pectin (μmol glucaric acid/g DM of ceacum digesta/min)	10.88 ± 2.08	11.04 ± 3.77	10.97 ± 2.44	9.55 ± 2.08	0.8435
Starch (μmol glucose/g DM of ceacum digesta/min)	21.76 ± 3.90^{a}	13.96 ± 3.90^{b}	11.26 ± 2.16^{b}	11.82 ± 1.84^{b}	0.0002
Inulin (μmol fructose/g DM of ceacum digesta/min)	5.87 ± 1.20	5.80 ± 1.27	5.26 ± 0.64	5.75 ± 0.94	0.7862

$^{a, b, c, d}$ Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

relatively higher pectinolytic and xylanolytic and low cellulolytic activity in the rabbit caecum, similarly to us. The lower cellulolytic activity observed during additives application could be explained in terms of the fast rate of passage of cellulose through the caeco-colic segment, which does not allow its complete hydrolysis [1].

Fermentation processes lead to volatile fatty acid production (VFA), which can cover 30 to 50% of maintainance of adult rabbits´ energy requirements [1]. Results concerning lactic acid (LA) and VFA concentrations in the caecum are not uniform; some researchers describe no influence on caecal pH and total VFA concentrations [17, 25], while others note higher total VFA production during probiotic treatments [26]. Although we did not observe any significant changes in VFA caecal content between experimental groups, the proportion of propionic acid was lower in all of them (around 5%) compared with the relative concentration of VFA in the caecum (75% acetate, 15% butyrate and 10% propionate; [2]). It is known that acetate production is connected with structural carbohydrate fermentation by cellulolytic bacteria, while propionate results from non-structural carbohydrates due to amylolytic bacteria [27]. In contrast to these facts, we noted higher amylase and cellulolytic activity at day 35 of the experiment compared while control data, while concentrations of propionate and acetate were lower than in the C group. Szabóová et al. [7, 15] also found increased levels of LA, butyric and acetic acids through sage administration and higher values of LA during combined application of Ent4231 and sage in rabbits, in accordance with us. On the other hand, no influence of nisin addition was observed on caecal VFA content [17]. In general, increase in caecal VFA concentration in rabbits presupposes a reduction in caecal pH, which might have an adverse effect on the intestinal pathogens and a beneficial effect on nutrient digestibility. Despite the higher LA, butyric, valeric, iso-valeric and caproic acid concentrations, their increase did not lead to reduction in caecal pH and the bacterial population, as described by Phuoc and Jamikorn [25]. Ultimately based on the rabbits' good health conditions, lower feed conversion ratio during additives application and improved jejunal morphology (data not shown), we hypothesise a positive correlation between weight gain and caecal fermentation due to improved gut functionality (jejunal morphology) and nutrient uptake.

The application of EntM and sage did not affect rabbit growth, but it did improve the feed conversion ratio. Differences between young and adult rabbits were noted in their caecal enzymatic activity, with higher fermentation processes in adults stimulated by EntM and sage extract administration. The counts of caecal bacteria were not significantly influenced by the additives, except for the significant reduction in LAB found in the E+S group ($P < 0.01$).

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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