

Article

Effect of *Enterococcus faecium* AL41 (CCM8558) and Its Enterocin M on the Physicochemical Properties and Mineral Content of Rabbit Meat

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Abstract: Improving rabbit meat quality using natural substances has become an area of research activity in rabbit nutrition due to stabilization of husbandry health and economy. The present study evaluates the effect of bacteriocin-producing, beneficial strain *Enterococcus faecium* AL41/CCM8558 and its enterocin M (EntM) on the quality and mineral content of rabbit meat. Seventy-two Hycole rabbits (aged 35 days) were divided into EG1 (CCM8558 strain; 1.0×10^9 CFU/mL; 500 µL/animal/d), EG2 (EntM; 50 µL/animal/d), and control group (CG). The additives were administrated in drinking water for 21 days. Significant increase in meat phosphorus (EG1: $p < 0.05$; EG2: $p < 0.0001$) and iron (EG1, EG2: $p < 0.001$) contents was noted; sodium and zinc levels were only slightly higher in experimental groups compared with control data. The calcium (EG1, EG2: $p < 0.001$), potassium, and copper (EG1: $p < 0.01$) concentrations were reduced. The treatment did not have a negative influence on physicochemical traits of rabbit meat. Based on these results, we conclude that diet supplementation with beneficial strain *E. faecium* CCM8558 and its EntM could enhance the quality and mineral content of rabbit meat, with the focus on its iron and phosphorus contents.

Keywords: enterocin; beneficial strain; meat quality; minerals; rabbit meat



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1. Introduction

In the last decade, the number of well-educated consumers focusing on a healthy lifestyle, diet and food sources is increasing. Rabbit meat is tender white meat, suitable for preparing delicious, nutritious, and mainly, healthy food, and greatly valued for its high protein level (20–21%) with essential amino acids of high digestibility, low fat content with a favorable proportion among saturated, monounsaturated, and polyunsaturated fatty acids, and it is almost free of cholesterol. Rabbit meat also provides a moderate amount of energy and low sodium content, but it is rich in potassium, magnesium, phosphorus, selenium, and B vitamins (as the richest source of vitamin B₁₂ [1–3]). Due to these dietary properties, its frequent consumption is highly recommended, e.g., for pregnant women, children, elderly people, and patients with cardiovascular disorders. Moreover, the fortification of the rabbit diet with functional compounds has an increasing tendency and, in this way, rabbit meat can represent a functional food [2]. The majority of studies have been focused on the qualitative parameters and sensory properties of rabbit meat [1,4–6]. Other works present the fatty acid profile, oxidative stability and lipid metabolism of rabbit meat, and their influence using natural additives [7–9]. Although the mineral content of rabbit meat has been investigated by several researchers [2,10–13], in vivo studies about the influence of natural feed additives on rabbit meat minerals are scarce [14,15].

In recent years, many alternatives—probiotics, prebiotics, enzymes, bacteriocins, organic acids, herbs, and their extracts have been tested in rabbits as feed additives to

enhance their productivity and health [2]. It is well known that probiotics—beneficial bacteria can improve gut microbial balance, positively influence metabolism and nutrient digestibility as well as mucosal immunity, and maintain the health, growth, and productivity of animals. The majority of studies suggested a positive effect of dietary natural feed additives' inclusion on meat quality and that these compounds could be useful to improve nutritional properties—minerals, fatty, and amino acids of rabbit meat. While most of the works present the environmental, feeding, genetics, biological factors, and technological (pear-slaughter, transportation, processing) effects on rabbit carcass and meat quality [1], only a few of them have presented the effect of probiotics and/or bacteriocins on the rabbit meat mineral composition [14,15]; information about probiotic influence on meat of other monogastric animals, such as chickens and pigs is also rare [16,17]. Therefore, this study aimed to investigate the impact of feed administration by bacteriocin-producing and beneficial strain *Enterococcus faecium* AL41 (deponed to Czech Culture Collection of Microorganisms in Brno, Czech Republic, CCM8558) and its enterocin M (EntM) on the mineral content and quality of rabbit meat. Moreover, EntM is a new, not commercial bacteriocin, which can help to extend existing knowledge about bacteriocins' application in animal husbandry with a focus on the meat's nutritional quality—this is the novelty of this study.

2. Materials and Methods

2.1. Experiment Schedule, Diet, Slaughtering, and Sampling

The experiment was performed in co-operation with our colleagues at the National Agricultural and Food Centre (NAFC, Nitra, Slovakia). All applicable international, national and/or institutional guidelines for the care and use of animals were followed appropriately, and all experimental procedures were approved by the Slovak State Veterinary and Food Administration and Ethics Committees of both (permission code: SK CH 17016 and SK U 18016).

Seventy-two Hycole rabbits (weaned at age of 35 days, both sexes, equal male to female ratio per treatment) were used in this experiment. Rabbits were divided into 2 experimental groups (EG1, EG2) and 1 control group (CG), with 24 animals in each group. The average live weight of rabbits at the start of the experiment were $830.0 \text{ g} \pm 165.2$ in EG1, $833.0 \text{ g} \pm 116.9$ in EG2, and in CG it was $729.0 \text{ g} \pm 152.5$. The rabbits were kept in standard cages (type D-KV-72, $61 \times 34 \times 33 \text{ cm}$, supplied by Kovobel company, Domažlice, Czech Republic) with two animals per cage. A cycle of 16 h of light and 8 h of dark was used through the experiment. Temperature ($20 \pm 4 \text{ }^{\circ}\text{C}$) and humidity ($70 \pm 5\%$) were maintained throughout the experiment by heating and ventilation systems, and data were recorded continuously with a digital thermograph positioned at the same level as the cages. Animals were fed a commercial pelleted diet for growing rabbits (ANPRO-FEED, VKZII Bučany, Slovakia; Table 1) during the whole experiment with access to water ad libitum. Rabbits in group EG1 received bacteriocin-producing *E. faecium* CCM8558 strain possessing probiotic properties ($1.0 \times 10^9 \text{ CFU/mL}$) in their drinking water at a dose of $500 \text{ }\mu\text{L/animal/day}$ for 21 days. It was marked by rifampicin to differ it from the total enterococci and prepared as described previously by Stropfová et al. [18]. The animals in group EG2 were administered EntM (prepared according to Mareková et al. [19]), a dose of $50 \text{ }\mu\text{L/animal/day}$, with activity $12,800 \text{ AU/mL}$ for 21 days. Activity of EntM was tested by the agar spot test according to De Vuyst et al. [20] against the principal indicator strain *E. avium* EA5 (isolate from feces of piglet, our laboratory). The doses of additives and their manner of application were decided based on our previous in vitro studies testing the inhibitory activity of EntM against target bacteria and an experiment with rabbit-derived bacteriocin-producing strain *E. faecium* CCM7420 [21]. Based on our previous experiments, that these additives can be dissolved in distilled water and/or phosphate buffer [19], the additives were applied firstly to 100 mL of drinking water in all cages, and after consuming this volume, the rabbits had access to water ad libitum. Control rabbits (group CG) had the same conditions, but without additives being applied to their drinking water, and they

were fed a commercial diet. Drinking water was provided through nipple drinkers. The experiment lasted for 42 days.

Table 1. Ingredients, chemical composition, and nutritive value of diets.

Ingredients	(%)		(g.kg ⁻¹)
Extracted clover (grass) meal	27.00	Mineral and vitamin premix ¹	3.00
Extracted sugar beet	10.00	Crude protein	189.38
Oats	13.00	Crude fiber	191.88
Wheat bran	6.00	Fat	39.00
Soybean meal	7.50	Ash	80.00
Sunflower meal	14.00	Organic compounds	921.00
Monocalcium phosphate	0.60	Starch	178.00
Dicalcium carbonate	0.90	Lysin	7.50
Salt	0.30	Methionine + cysteine	6.50
Carob	2.50	Choline chloride	0.80
DL-Methionine + wheat meal	0.10 + 0.10	ME	11.92 MJ

¹ Premix provided per kg diet: vitamin A, 10,000 IU; vitamin D₃, 2000 IU; vitamin E acetate, 30 mg; vitamin B₂, 5 mg; vitamin B₆, 2 mg; vitamin B₁₂, 8 mg; Ca, 9.25 g; P, 6.2 g; Na, 1.6 g; Mg, 1.0 g; K, 10.8 g; Fe, 327.5 mg; Mn, 80 mg; Zn, 0.7 mg.

At days 21 and 42, eight animals from each group were randomly selected for slaughter; they were stunned with electronarcosis (50 Hz, 0.3 A/rabbit/4 s) in an experimental slaughterhouse, immediately hung by the hind legs on the processing line, and quickly bled by cutting the jugular veins and the carotid arteries. After the bleeding, the *Longissimus thoracis* and *lumborum* (LTL) muscles were separated by removing the skin, fat and connective tissue, chilled, and stored 24 h at 4 °C until physicochemical analysis started.

2.2. Physico-Chemical, Mineral, and Statistical Analysis

The ultimate pH was determined 48 h postmortem (p.m.) with a Radelkis OP-109 (Jenway, Essex, UK) with a combined electrode penetrating 3 mm into the LTL. Color measurements were taken on MLD surface of the carcass at 24 h after bleeding. Color characteristics were expressed using the CIE L*a*b system (lightness-L*, 0: black and 100: white), (redness and greenness-a*; yellowness and blueness-b*) using a Lab. Miniscan (HunterLab, Reston, VA, USA). Lightness measurements at room temperature were also taken. Total water, protein and fat contents were estimated using an INFRATEC 1265 spectroscope (FOSS, Tecator AB, Höganäs, Sweden) and expressed in g/100 g. The Near Infrared Transmission (NIT) principle is based on the fact that the measured sample absorbs the Near Infrared light at different wavelengths according to different characteristics such as fat or protein content [22]. From these values, the energy content was calculated [EC (kJ/100 g) = 16.75 × Protein content (g/100 g) + 37.68 × Fat content (g/100 g)]; [23]. Water holding capacity (WHC) was determined by compress method at constant pressure [24]. The analyzed samples (0.3 g in weight) were placed on filter papers (Schleicher and Shuell No. 2040B, Dassel, Germany) with tweezers previously weighed. Together with the papers, samples were sandwiched between Plexiglass plates and then subjected to a pressure of 5 kg for 5 min. The results were calculated from the difference in weight between the slips with aspirating spot and the pure filter paper. The ash content was determined by burning in Muffle furnace at 530 ± 20 °C according to STN 570185.

For macro and micro element analysis, samples were ashed at 550 °C, the ash was dissolved in 10 mL of HCl (1:3), and minerals were determined by AAS iCE 3000 (Thermo Fisher Scientific, Waltham, MA, USA). Phosphorus content was determined by molybdovanadate reagent on Camspec M501 (Spectronic Campes Ltd., Leeds, UK).

Treatment effects on tested parameters were analyzed using one-way analysis of variance (ANOVA) with Tukey post hoc test. All statistical analyses were performed using GraphPad Prism statistical software (GraphPad Prism version 6.0, GraphPad Software, San Diego, CA, USA). Differences between the mean values of the different dietary treatments were considered statistically significant at $p < 0.05$. Data are expressed as means and standard deviations of the mean (SD).

3. Results

Mineral Profile of Rabbit Meat

The effect of dietary supplementation with *E. faecium* CCM8558 and its EntM on mineral profile of rabbit meat is presented in Table 2. Feeding of CCM8558 strain and EntM significantly increased the phosphorus (EG1: $p < 0.05$; EG2: $p < 0.0001$) and iron concentrations (EG1, EG2: $p < 0.001$), while sodium and zinc levels were only slightly higher in experimental groups, compared with control. On the other hand, calcium (EG1, EG2: $p < 0.001$) and copper (EG1: $p < 0.01$) values significantly decreased during additives' application. The lowest calcium and the highest phosphorus level were measured in EG1.

Table 2. Mineral levels (mg/100 g fresh matter/muscle, means \pm SD) in *Longissimus thoracis* and *lumborum* of rabbits.

	EG1	EG2	CG	p-Value
Day 21 of the Experiment (at 56 Days of Age)				
Calcium (mg/100 g)	6.73 \pm 0.01 ^a	12.07 \pm 0.54 ^b	17.83 \pm 1.50 ^c	<0.0001
Phosphorus (mg/100 g)	259.50 \pm 22.06 ^a	215.13 \pm 36.94 ^b	193.33 \pm 5.20 ^b	0.0012
Magnesium (mg/100 g)	23.30 \pm 0.02	23.53 \pm 0.03	24.10 \pm 1.40	0.2430
Sodium (mg/100 g)	31.10 \pm 0.06	30.70 \pm 0.06	29.53 \pm 4.10	0.5063
Potassium (mg/100 g)	382.87 \pm 8.54	372.80 \pm 10.47	409.10 \pm 39.89	0.0542
Iron (mg/100 g)	0.487 \pm 0.013 ^a	0.495 \pm 0.067 ^a	0.390 \pm 0.040 ^b	0.0019
Manganese (mg/100 g)	0.061 \pm 0.022 ^a	0.065 \pm 0.003 ^b	0.062 \pm 0.058 ^b	0.9800
Zinc (mg/100 g)	1.203 \pm 0.415	1.216 \pm 0.149	1.158 \pm 0.259	0.9385
Copper (mg/100 g)	0.188 \pm 0.039 ^a	0.215 \pm 0.021	0.251 \pm 0.030 ^b	0.0104

^{a,b,c} Means with a different superscript in the same row are significantly different ($p < 0.05$). EG1: experimental group 1 (*E. faecium* CCM8558 strain); EG2: experimental group 2 (enterocin M); CG: control group.

Within microminerals, the iron content significantly increased in EG1 and EG2 ($p < 0.01$), while reduced copper level was noted in both experimental groups.

Higher pH values ($p < 0.01$, Table 3) were noted through EntM application (EG2), compared with CG. The color measurements differed in all tested parameters: increase of lightness (EG1: $p < 0.01$, EG2: $p < 0.05$) and yellowness was noted, whereas the redness was decreased. No significant differences were found in protein, lipid, energy, ash, and water contents during additives' administration.

Table 3. Physicochemical properties of *Longissimus thoracis* and *lumborum* of rabbits (means \pm SD).

	EG1	EG2	CG	p-Value
Day 21 of the Experiment (at 56 Days of Age)				
Average live weight (g)	1575.5 g \pm 189.5 ^a	1729.7 g \pm 252.5 ^b	1451.8 g \pm 202.5 ^a	<0.0001
pH 48 h postmortem	5.37 \pm 0.02 ^a	5.48 \pm 0.09 ^b	5.36 \pm 0.02 ^a	0.0034
L* (lightness)	55.53 \pm 3.66 ^a	52.79 \pm 1.80 ^a	47.87 \pm 3.56 ^b	0.0024
a* (redness)	0.55 \pm 0.24	0.57 \pm 0.08	0.62 \pm 0.11	0.7398
b* (yellowness)	8.30 \pm 0.69	8.08 \pm 0.59	7.37 \pm 0.78	0.0820
Water content (g/100 g)	75.90 \pm 0.17 ^a	76.27 \pm 0.15 ^a	75.87 \pm 0.23 ^b	0.0033
Protein content (g/100 g)	21.37 \pm 0.21	21.03 \pm 0.23	21.40 \pm 0.66	0.2707
Fat content (g/100 g)	1.73 \pm 0.23	1.70 \pm 0.10	1.73 \pm 0.49	0.9824
Energy value (kJ/100 g)	426.53 \pm 4.21 ^a	416.35 \pm 1.98 ^b	423.76 \pm 8.65 ^{a,b}	0.0196
Water holding capacity (g/100 g)	25.99 \pm 2.18	30.35 \pm 1.46	28.68 \pm 5.28	0.1151
Ash (g/100 g)	1.033 \pm 0.003	1.033 \pm 0.003	1.033 \pm 0.003	1.0000
Day 42 of the Experiment (at 77 Days of Age)				
Average live weight (g)	2321.0 g \pm 317.8 ^a	2626.4 g \pm 192.9 ^b	2174.0 g \pm 243.0 ^a	0.0005
pH 48 h post mortem	5.54 \pm 0.03 ^a	5.63 \pm 0.09 ^b	5.65 \pm 0.04 ^b	0.0134
L* (lightness)	51.78 \pm 4.14	50.04 \pm 1.71	51.79 \pm 1.19	0.4468
a* (redness)	0.68 \pm 0.29 ^{a,b}	1.22 \pm 1.21 ^a	0.08 \pm 0.05 ^b	0.0470
b* (yellowness)	7.34 \pm 0.74	7.56 \pm 0.61	7.89 \pm 0.68	0.3918
Water content (g/100 g)	75.33 \pm 0.38	75.70 \pm 0.46	75.50 \pm 0.44	0.3511
Protein content (g/100 g)	21.93 \pm 0.15	21.83 \pm 0.38	22.00 \pm 0.26	0.5825
Fat content (g/100 g)	1.73 \pm 0.23	1.47 \pm 0.25	1.50 \pm 0.20	0.1301
Energy value (kJ/100 g)	432.69 \pm 11.14	420.97 \pm 11.49	425.02 \pm 11.27	0.2224
Water holding capacity (g/100 g)	31.09 \pm 5.16 ^{a,b}	25.75 \pm 2.82 ^a	33.55 \pm 5.18 ^b	0.0267
Ash (g/100 g)	1.000 \pm 0.000	1.000 \pm 0.000	1.000 \pm 0.000	1.0000

^{a,b} Means with a different superscript in the same row are significantly different ($p < 0.05$). EG1: experimental group 1 (*E. faecium* CCM8558 strain); EG2: experimental group 2 (enterocin M); CG: control group.

4. Discussion

There is a great variety in macrominerals and trace elements content of rabbit meat among different studies. Whereas the potassium content framed within those reported in the literature [1,2,10], phosphorus, sodium and manganese level were below, and calcium was over the range presented by formerly mentioned authors. Moreover, Hermida et al. [10] presented higher iron and manganese, and lower zinc and copper concentrations in rabbit meat, compared with our findings.

The lowest level of calcium in EG1 was similar to the values presented by Dalle Zotte [1]. On the other hand, Nistor et al. [13] reported higher calcium content of rabbit meat (21.4 mg/100 g). Opposite to calcium, the phosphorus level in EG1 (259.50 mg/100 g) was the highest. The indigestible carbohydrates compounds like oligofructose, galactooligosaccharides, and inulin have been found to cause improved mineral retention/absorption by the host organism because of their ability to bind and sequester the minerals, and these carbohydrate-mineral complexes pass unabsorbed through the small intestine onto the colon when the minerals are released from the complex and absorbed. The application of probiotics acts on the nondigestible carbohydrates, causing the short chain fatty acids (SCFAs) rise which can affect an increased absorption of minerals like magnesium and calcium. Moreover, the probiotic enhanced SCFAs can stimulate Vitamin D receptor expression on the eukaryotic cells, which regulate the absorption of calcium from diet, and its metabolism in mammals [25]. The probiotics can also increase the calcium transporters like TRPV6 and calcitriculin Sp100 in the intestine [26]. Phosphorus is needed for the growth of muscle tissue for being involved in the energy metabolism. Its content was higher compared to our previous experiment with autochthonous *E. faecium* CCM7420 rabbit-derived strain [14] as well as to data reported by the other authors [1,2,10] but still lower than it was reported by Nistor et al. [13]. Wang et al. [27] also demonstrated improved phosphorus absorption and utilization in broilers after *E. faecium* CGMCC 2516 microcapsules' application. Rabbit meat contains more potassium than other types of meat and is recommended for hypertension diet. Although the potassium concentrations decreased in both experimental groups compared to CG, the data were comparable to values reported by Dalle Zotte and Szendrő [2].

Increased iron content was noted similarly to our previous results achieved during beneficial *E. faecium* CCM7420 strain application [14]. Other authors presented higher iron values in rabbit meat: 1.1–1.3 mg/100 g [1] and 0.66–0.99 mg/100 g [11]. It is well known that iron is better absorbed in solution in an acidic environment. In the intestine, the pancreas pours a very alkaline fluid into the upper small intestine and makes the whole contents alkaline; this creates a problem for mineral and trace elements, e.g., iron, zinc absorption. In this case, probiotics could improve the intestinal microbial balance due to lactic acid bacteria, create a beneficial acidic environment in the gastrointestinal tract, and increase the minerals' absorption and their subsequent transferring/inclusion into the meat. The acidic environment can enhance the ionization of minerals that in turn results in passive diffusion [28]. Some studies have been considered showing better iron, copper, and zinc absorption due to improvement of the microbial balance by dietary inclusion of microorganisms—yeasts—in several animals [29–31]. We also hypothesized enhanced enzymatic activity due to improved intestinal microbiota during CCM8558 strain and its EntM application. Another possible way of better mineral absorption could be the enlargement of the absorption surface by proliferation of enterocytes. This fact is also confirmed by improved morphometry parameters, recorded during our previous experiments when beneficial *E. faecium* CCM7420 strain and the EntM were applied to rabbits [32,33]. Copper is an essential trace mineral which performs important biochemical functions, which is usually deficient in the typical human diet. Regarding the copper content, the results vary widely [10,11,34]; our findings agree with those reported by Hermida et al. (0.03–0.21 mg/100 g; [10]). Despite the reduced (but still in the range characteristic for rabbit meat) copper level in both experimental groups compared with

control data, the dietetic value of rabbit meat was not negatively influenced during the beneficial CCM8558 strain and its EntM application.

The majority of reports present higher pH_{24} (24 h postmortem) values than 5.70. Lower pH_{48} (48 h postmortem) values (in the range 5.37–5.65) obtained in this experiment could be explained by the depletion of glycogen reserve in muscles during refrigeration and by longer storage time. Similar pH_{48} values were detected also during the beneficial *E. faecium* CCM7420 strain (5.34–5.65) and phyto-additives' (5.61–5.71) application in rabbits [14,35]. Lower values of lipids were determined, but the protein content of rabbit meat was not influenced using the CCM8558 strain and its EntM. Redness is influenced with the degree of iron oxidation in the heme pigment in myoglobin. At high pH levels, oxymyoglobin is rapidly turned into dark red color reduced myoglobin, showing a positive relationship between these parameters [36]; our findings also confirm this relationship. Higher values of yellowness could be related to free radicals, produced by lipid oxidation during storage and/or manipulation, which can oxidize heme pigments, causing discoloration of meat and meat products [37]. Lower lipid value than the average [1] was determined by us, similarly to Lauková et al. [38] after gallidermin application in rabbits, but contradictory to results achieved during probiotic BioPlus 2B[®] preparation or phyto-additives' supplementation in rabbits [35,39]. Positive correlation between lipids and energy was found in rabbits of EG1 group, mainly at day 42 (after probiotic strain cessation). The protein content of rabbit meat was not influenced using CCM8558 strain and its EntM, similarly, to results noted through gallidermin administration in rabbits [38]. Improved energy values were previously described also during phyto-additives' application in rabbits [35].

5. Conclusions

This study contributes to updating data in the literature concerning the effect of bacteriocin-producing and beneficial strain *E. faecium* CCM8558, and its enterocin M on the quality and mineral content of rabbit meat. Higher phosphorus and iron levels were recorded during both CCM8558 strain and EntM administration in rabbits. During EntM application, improved pH_{48} , lightness, and water content was noted. In general, physicochemical parameters and nutritional value of rabbit meat were not negatively influenced during bioactive compounds' administration. This study has impact for basic research primarily due to spreading knowledge regarding the rabbit meat's mineral composition and secondarily due to the opportunity to compare the beneficial effect of different natural additives—probiotics and enterocins in rabbits under in vivo conditions. Nevertheless, further investigations are needed to assess the efficacy of CCM8558 and EntM in rabbit husbandries.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the State Veterinary and Food Administration of the Slovak Republic on 1 December 2016 (approval numbers SK CH 17016 and SK U 18016).

Data Availability Statement: Data are available upon reasonable request to the corresponding author.

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