

Article

Assessment of Postbiotic, Mundtacin-like Substance EM 41/3 Application in Broiler Rabbits

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Featured Application: Postbiotic, Mundtacin-like substance EM 41/3 looks as a promising additive for health status sustainability in animal husbandry.

Abstract: Nowadays, the use of probiotics, postbiotics, parabiotics and other beneficial natural substances dominate health status sustainability in animals. Mundtacin-like substance EM 41/3 (MLS) is a thermo-stable substance of proteinaceous character—a bacteriocin (postbiotic) with a broad antimicrobial (inhibitory) spectrum produced by the horse-derived strain *Enterococcus mundtii* EM 41/3. The aim of this study was to find if this new one bacteriocin can have an effect and/or a similar effect in rabbits as was achieved previously by using enterocins detected and characterized in our laboratory. In addition, these tests have been conducted to secure the health of rabbits as food-derived animals. Moreover, the aim of this study was also to test the effect of MLS EM 41/3 in broiler rabbits as an animal model before its use in horses. The parameters tested were analyzed by validated methods. Administration of MLS EM 41/3 lead to a significant increase in the non-specific immunity parameter (phagocytic activity, $p < 0.05$), while microbiota in feces, caecum and appendix were not negatively influenced. However, the total enterococcal count was significantly increased in the EG compared to the CG ($p < 0.001$). Application of MLS EM 41/3 did not have a negative impact on blood biochemistry. No oxidative stress was noted. However, higher growth parameters were noted and also hydrolytic activity was increased after MLS EM 41/3 application. Finally, to check physico-chemical parameters, no negative influence on rabbit meat quality was noted. Postbiotic MLS EM 41/3 administration seems to be a contributing factor in the feeding strategy for animal health status sustainability.

Keywords: bacteriocin; broiler rabbits; postbiotic effect; health status



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1. Introduction

Nowadays, various innovative routes have been searched to sustain the healthy status of animals, including food-derived animals. Among food-derived animals also broiler rabbits can be included because of their easily digestible meat [1]. It could be recommended for people, e.g., in the period after illness convalescence. Rabbits can convert an approximately 20% of the protein they eat into edible meat, which is a higher amount than, e.g., in pig meat (16–18%) [2]. Taking into account innovative strategies, the use of probiotics, postbiotics, parabiotics and other beneficial natural substances has dominated [3]. Recently, postbiotics have been aimed more frequently for this purpose. Postbiotic components are

diverse and outperform live probiotics in terms of technology, safety, cost due to their sufficient absorption, metabolism and organismal distribution [4]. This term has been officially established by the ISAPP (The International Scientific Association of Probiotics and Prebiotics consensus statement on the definition and scope of postbiotics) [5]. A wide range of future perspectives for postbiotics has been reported, including health care.

Mundtacin-like substance (MLS) EM 41/3 is a thermo-stable substance of proteinaceous character. It is produced by horse-derived strain *Enterococcus mundtii* EM 41/3. This MLS producer strain was isolated from Slovak horse breed Norik from Muráň. It possess a gene for Enterocin P and a gene for Mundtacin KS [6]. Under in vitro conditions, MLS EM 41/3 has shown a broad antimicrobial (inhibitory) spectrum also inhibiting the growth of listeriae and staphylococci with activity up to 800 AU/mL [6]. Based on the origin of the MLS EM 41/3 producer strain, MLS EM 41/3 has been aimed predominantly for use in horse breeding [6]. However, before its use in horses, its functionality has been assessed in broiler rabbits. Rabbits are a food-derived animal category and they also represent suitable model animals. Reasons for this are, e.g., the small body size, short generation interval, rapid growth rate, high productive capacity, and their meat as has already been mentioned [1,7]. Resulting from our previous experiments using broiler rabbits and, e.g., postbiotics characterized at our laboratory (Enterocin M, Durancin ED 26E/7) and/or their producer strains, we have hypothesized that MLS EM 41/3 would have beneficial effects in animals without side effects [7–10]. Enterocin M and Durancin ED 26E/7 application lead to a decrease in coliforms and pseudomonads ($p < 0.05$) ($p < 0.001$) in feces of rabbits. Both postbiotics (bacteriocins) mentioned stimulated enzymatic activity in caecum [7–10]. In the case of Ent M, higher daily weight gains were also noted in rabbits, and stimulation of phagocytic activity (PA) ($p < 0.01$, $p < 0.001$) not only in the case of Ent M but also in the case of its producer strain EFAL41 = CCM8558 [9]. Therefore, the aim of this study was to test the effect/functionality of MLS EM 41/3 in broiler rabbits with a focus on the following parameters: microbiota, phagocytic activity (PA) as a non-specific immunity parameter, glutathione-peroxidase activity (GPx) to assess oxidative stress, growth parameters, biochemistry in blood serum, jejunal morphology, organic acids in cecal chyme, fecal enzymatic activity, and meat quality. Based on previous achievements with our enterocins and hypothesis formerly indicated, the study aim was to find if this new enterocin can have similar effect in rabbits to previously detected and tested enterocins. In addition, these tests have been conducted to ensure rabbit health as food-derived animals and their meat as functional food. And as mentioned, the aim was also to test MLS EM 41/3 in a broiler rabbit animal model before its use in horses.

2. Materials and Methods

2.1. Preparation of Mundtacin-like Substance EM 41/3 for Application

Mundtacin-like substance EM 41/3 (MLS) is a bacteriocin produced by the strain *Enterococcus mundtii* EM 41/3 [6]. This bacteriocin-producing strain was isolated from feces of Slovak horse breed Norik from Muráň. To prepare bacteriocin substance for application in broiler rabbits, the producer strain was inoculated in MRS broth (Merck, Darmstadt, Germany, pH 6.9, 0.1% inoculum). Based on previous analyses, the strain was cultivated up to its late log phase in an incubator at 37 °C. Broth culture was centrifuged ($10,000\times g$) for half an hour. Supernatant (pH 5.5) was treated with EDTA/Chelaton III (centrifuge MR 1812, Society Jouan, Saint Herblain, France Sigma-Aldrich, Darmstadt, Germany). It was exposed to 80 °C for 10 min to inactivate other organic components than bacteriocin. Treated supernatant was precipitated with ammonium sulphate at 4 °C for 18 h (40% saturation). Precipitate was re-suspended in a minimal volume of phosphate buffer (10 mM, pH 6.5). The inhibitory activity of partially purified bacteriocin, Mundtacin-like was tested by the agar spot method [11] against two indicator strains—*Enterococcus avium* EA5 (fecal piglet strain, from our laboratory) and *Listeria monocytogenes* LMP 7223 (State Veterinary Institute in Olomouc, Czech Republic). The inhibitory activity of this precipitate reached 25,600 AU/mL (and/or 102,400 AU/mL).

2.2. Experimental Design and Sampling

A total of 48 rabbits (meat lines M91 and P91) after weaning (in age 35 days), of both sexes (equal male-to-female ratio per treatment), were divided into an experimental group (EG) and a control group (CG), with 24 animals in each. At the start of the experiment, the average body weight of rabbits was 1041 g (EG)–1078 g (CG). The rabbits were placed in the menagerie at the National Agricultural and Food Centre (NAFC) in Nitra-Lužianky (Slovakia), with which we have co-operated almost for 20 years. The guidelines stated in the Guide for the Care and Use of Laboratory Animals approved by the Slovak Veterinary and Food Administration and Ethical Commissions of both institutions (permission code: SK CH 17016 and SK U 18016) were accepted for care and experimental procedures. The animals were fed a commercial diet for growing rabbits (SIGI Trade, Dvory nad Žitavou, Slovakia), with the following nutritional values: dry matter 896.79 g/kg, crude fiber 140.29 g/kg, fat 23.84 g/kg, N-substances 172.83 g/kg, ash 93.68 g/kg, organic matter 803.11 g/kg, and starch 167.66 g/kg. The diet was supplemented with minerals in the following amounts: magnesium—3.27 g/kg, sodium—1.25 g/kg, kalium—13.22 g/kg, iron—626.97 mg/kg, and zinc—173.02 mg/kg. The energy value of the diet reached 11.22 MJ/kg. Broiler rabbits were kept in standard cages (type D-KV-72; 0.61 m × 0.34 m × 0.33 m; Kovobel company, Domažlice, Czech Republic), with two animals per cage. A cycle of 16 h light and 8 h dark was applied throughout the experiment. The temperature and humidity in the menagerie were recorded continuously by a digital thermograph positioned at the same level as the cages. The heating and ventilation systems allowed the menagerie an air temperature maintained at 16 ± 4 °C and a relative humidity of approximately $70 \pm 5\%$ throughout the experiment as previously reported by Pogány Simonová et al. [12]. The rabbits had ad libitum access to drinking water.

In addition, animals in the EG were administered Mundtacin-like substance (MLS) EM 41/3 in drinking water (activity concentration 25,600 AU/mL), 50 µL for each animal per day over 21 days (3 weeks). The rabbits in the CG were only administered a commercial diet. The duration of the experiment was 42 days. Fecal sampling was performed at day 0/1 (mixed samples of all animals, $n = 10$, before MLS application), at day 21 (mixed sample of all animals, $n = 5$, 3 weeks of MLS application), and at day 42 (mixed samples of animals, $n = 5$, 3 weeks of MLS cessation). In addition, at days 21 and 42, caecum ($n = 4$) and appendix ($n = 4$) were also sampled after the rabbits were slaughtered as previously described by Pogány Simonová et al. [12], with one rabbit/one replicate. They were selected based on daily weight measurements to ensure similar weights of animals. Moreover, fecal samples were also taken to test hydrolytic (enzymatic) activity.

Musculus longissimus thoracis and lumborum (MLTL) was separated by removing skin, connective tissue, chilled and stored at 4 °C for 24 h until analysis. The rabbits were also regularly weighed. Rabbit blood (*vena auricularis*) was sampled into Eppendorf tubes (with and without heparin according to parameter analyzed) at days 0/1, 21 and 42.

2.3. Microbiota Analyses

Microbiota were checked in feces, caecum and appendix. To count microbiota in feces, sampling was provided at day 0/1 (before MLS EM 41/3 application), then at day 21 (after 3 weeks of MLS application) and finally at day 42 (3 weeks of MLS cessation). In the case of caecum and appendix, sampling was performed at days 21 and 42. For microbiota enumeration, the standard dilution microbiological method (ISO, International Organization for Standardization) was used with the selective media (ISO). Samples of feces, caecum and appendix (one g) were mixed with Ringer solution (ratio 1:9, Merck, Darmstadt, Germany, pH 7.0) and treated using Stomacher-Masticator (IUL Instruments, Barcelona, Spain). The appropriate dilutions were spread on M-Enterococcus agar (Becton and Dickinson, Difco, Detroit, MI, USA) to count enterococci. Mannitol salt agar (MSA, Difco) was used to enumerate staphylococci. To count coliform bacteria, Mac Conkey agar (Oxoid, Basingstoke, UK) was used. Amylolytic streptococci were counted on M17

agar (Difco) enriched with starch. The agar plates were incubated at 37 °C for 24–48 h. Bacterial counts were expressed in colony forming unit per gram (CFU/g) $\log 10 \pm \text{SD}$.

After re-isolation of colonies from different agar media, colonies were treated with MLS EM 41/3 using the agar spot test [11]. Inhibitory activity was expressed in arbitrary units per milliliter (AU/mL).

2.4. Phagocytic Activity Analysis, Glutathione-Peroxidase (GPx) Evaluation, and Biochemistry in Blood Serum

For phagocytic activity (PA), blood was sampled from *vena auricularis* into Eppendorf tubes with micro-spheric hydrophilic (MSH) particles and heparin [13]. Sampling was performed at day 0/1 ($n = 8$), at day 21 ($n = 8$), and at day 42 ($n = 8$), meaning there were 8 mixed blood samples from animals. A volume 50 μL of MSH particle suspension (ARTIM, Prague, Czech Republic) was mixed with 100 μL of blood in an Eppendorf tube and incubated at 37 °C for 1 h. Blood smears were prepared and stained with May–Gruenwald and Giemsa–Romanowski stains. To validate PA, the direct microscopic counting procedure was performed with the calculation of the number of white cells containing at least three engulfed particles per 100 white cells (monocyte/granulocytes). PA was expressed in percentage (%). The index of phagocytic activity was also involved in analysis (IPA).

GPx (glutathione-peroxidase) activity ($n = 8$, meaning at the start of the experiment, at days 21 and 42, in each of the 8 mixed blood samples of all animals from each group) was determined by the colorimetric method (Spectrophotometer UV-2550 Shimadzu San Dimas, CA, USA) using the commercial kit Randox RS504 (Randox Laboratory Ltd., Crumlinh, UK) after blood sampling in the tube with heparin.

For biochemistry, blood samples were taken into Eppendorf tubes. They were centrifuged ($3000 \times g$, 30 min) and delivered to the SK-Lab company Lučenec (Slovakia) for analysis. In this analysis, validated methods were used. The following parameters were analyzed: total proteins (TP in g/L), albumin (g/L), creatinine ($\mu\text{mol/L}$), alanine aminotransferase (ALT in $\mu\text{kat/L}$), aspartate transferase (AST in $\mu\text{kat/L}$), alkaline phosphatase (ALP in $\mu\text{kat/L}$), glucose (mmol/L), cholesterol (mmol/L), triglycerides (mmol/L), sodium (Na), kalium (K), chlorides (CL) calcium (Ca), phosphorus (P), and magnesium (Mg) in mmol/L. Reference values for rabbits are summarized in Table 4.

2.5. Hydrolytic Activity, Jejunal Morphometry Growth Performance, Organic Acids in Chyme, and Quality of Meat

Hydrolytic activities in feces (amylolytic, cellulolytic, xylanolytic, pectinolytic and inulolytic expressed in $\mu\text{mol/g/DM/min}$ meaning in micromol per gram of dry matter per minute) were processed as previously described by Lauková et al. [14]. The enzymes were extracted using the procedure of Huhtanen and Khali [15] and they were measured according the procedure of Miltko et al. [16]. The degradation of the following substrates was measured: cellulose, starch, inulin, pectin, and xylan.

Body weight (BW) was measured every week during the experiment; average daily weight gain (ADWG) and feed conversion ratio (FCR) were calculated mathematically.

MLTL (100 g) were stored for 24 h *post mortem* at 4 °C. The following parameters were analyzed: total water content (g/100g), total proteins (g/100 g), total fat, water holding capacity, pH 24, and energy value (kJ/100 g) using validated methods (analyzer Infratec 1265, Copenhagen, Denmark) in co-operation with colleagues in Nitra-Lužianky as well as a method according to Ouhayoun [17] by gas chromatography (for organic acids). The pH values were measured 24 h *post mortem* with a Radelkis OP-109 pH meter (Jenway, UK). Morphometry testing was performed as previously described by Žitňan et al. [18]. Briefly, a part of tissue (1 cm^2) from proximal jejunum was fixed in 4% neutral formaldehyde solution. After rinsing in water, they were dehydrated in a graded series of ethanol and absolute ethanol, cleared in benzene and embedded in paraffin. Sections of 5 μm thickness with 10 slices of each sample were stained using hematoxylin/eosin and analyzed under a light microscope. The height, circumference and cut surface area of 30 villi and depth of 30 crypts were determined by the computer-operated Image C picture analysis system

(Limtronic, GmbH, Limburg, Germany). The interactive measurements included the IMES analysis program, a color video camera (Sony 3 CCD, Sony Electronics Ltd., Bratislava, Slovakia) and a light microscope (Axiolab, Carl Zeiss AG, Jena, Germany).

2.6. Statistical Analysis

Statistical analysis of the treatment included one-way analysis of variance (ANOVA) with Tukey's post hoc test. Data are expressed as the means and standard deviation ($\pm$ SD) of the mean. Different superscripts indicate significant differences, $p < 0.05$. Statistical analyses were performed using GraphPad Prism version 6.0 (San Diego, CA, USA).

3. Results

3.1. Microbiota Evaluation

The total count of enterococci in rabbit feces reached 5.81 ± 0.22 CFU/g on average (log 10) at day 21 (3 weeks of MLS EM 41/3 application (Table 1)). At that time, their counts were significantly higher in EG rabbits compared to day 0/1 ($^{ab} p < 0.001$). But the enterococcal amount was also significantly higher in EG rabbits compared to CG rabbits ($^{bc} p < 0.001$). At day 42, higher counts of the total enterococci were found in EG rabbits compared in CG rabbits (difference 1.19 cycle) and also compared to day 0/1 (difference 1.35 cycle). The total counts of LAB, staphylococci, amylolytic streptococci and coliforms in feces of broiler rabbits were not reduced by MLS EM 41/3 (Table 1).

Table 1. Bacterial counts in feces of rabbits after MLS EM 41/3 application (21 day) and its cessation (day 42) expressed in colony forming units per g (CFU/g, log 10).

	Day 0/1 ($n = 10$)	Day 21 ($n = 5$)—EG	Day 21 ($n = 5$)—CG	Day 42 ($n = 5$)—EG	Day 42 ($n = 5$)—CG
Enterococci	3.39 ± 1.45^a	5.81 ± 0.22^b	4.40 ± 0.79^c	4.74 ± 0.47	3.39 ± 0.48
Lactic acid bacteria	3.79 ± 1.23	5.67 ± 0.22	4.94 ± 0.75	4.44 ± 0.82	3.25 ± 0.40
Staphylococci	3.98 ± 0.50	4.28 ± 0.34	3.91 ± 0.33	4.25 ± 0.28	4.43 ± 0.57
Amyl. streptococci	5.71 ± 0.21	5.76 ± 0.48	5.24 ± 0.61	5.99 ± 0.37	5.89 ± 0.24
Coliform bacteria	2.28 ± 1.72	5.37 ± 0.73	4.83 ± 1.16	4.18 ± 1.27	3.85 ± 0.64

Average value of bacteria in CFU/g (log 10) $\pm$ SD; enterococci; Day 21—EG: Dy 0/1, $^{ab} p < 0.001$; Day 21—EG: Day 21—CG, $^{bc} p < 0.001$; Amyl. streptococci-amylolytic streptococci. Day 0/1 means sampling before application of Mundicin-like substance MLS EM 41/3; Day 21—EG means the experimental group at day 21 (3 weeks of MLS EM 41/3 application); Day 21—CG means the control group at day 21; Day 42—EG means the experimental group at day 42 (3 weeks of MLS EM 41/3 cessation); Day 42—CG means the control group at day 42.

The count of total enterococci in caecum was lower than in feces. At days 21 and 42, enterococci reached up to 1.43 CFU/g (log 10) in caecum (Table 2). At day 21, LAB count decreased in caecum of the EG compared of the CG (difference 0.67 cycle). Staphylococci were found almost in the same amount in caecum and feces of the EG at day 21 (Table 2) and they were slightly reduced in caecum at day 21 (difference 0.11 cycle). At day 42, even a significant decrease in staphylococci was found in rabbits of the EG ($^{ab} p < 0.05$) compared to rabbits in the CG. Amylolytic streptococci in caecum were almost 1.0 log cycle lower than in feces (Table 2). At days 21 and 42, the count of amylolytic streptococci was slightly higher in rabbits of the EG than in the CG (difference 0.19, respectively 0.22 cycle).

In appendix, enterococci reached up to 2.54 CFU/g (log 10) at day 21 (Table 2). At day 42, enterococci reached up to 1.0 CFU/g (0.90) in appendix. Their counts were higher in rabbits of the EG than in rabbits of the CG. At day 21, amount of LAB was higher in rabbits of the EG than in rabbits of the CG. However, at day 42, LAB count decreased up to almost 1.0 CFU/g. Coliform bacteria and amylolytic streptococci reached almost similar amounts in appendix as counted in caecum at days 21 and 42. Coliform bacteria and amylolytic streptococci were not reduced in appendix.

A total of 50% of re-isolated bacterial colonies from feces, caecum and appendix were susceptible to MLS EM 41/3.

Table 2. Bacterial counts in caecum and appendix of rabbits after MLS EM 41/3 application and its cessation.

	Day 21 (n = 4)—EG	Day 21 (n = 4)—CG	Day 42 (n = 4)—EG	Day 42 (n = 4)—CG
Caecum				
Enterococci	0.93 ± 0.05	0.93 ± 0.05	1.43 ± 1.05	0.09 ± 0.0
Lactic acid bacteria	1.82 ± 1.41	2.55 ± 1.93	1.05 ± 0.17	0.95 ± 0.06
Staphylococci	3.96 ± 0.29	4.07 ± 0.51	2.48 ± 1.72 ^b	4.09 ± 0.30 ^a
Amylolytic streptococci	4.15 ± 0.21	3.96 ± 0.39	4.43 ± 0.19	4.21 ± 0.16
Coliform bacteria	2.42 ± 1.25	1.32 ± 0.33	3.54 ± 1.04	2.42 ± 1.25
Appendix				
Enterococci	2.54 ± 1.07	0.93 ± 0.05	0.90 ± 0.00	0.90 ± 0.0
Lactic acid bacteria	3.30 ± 1.63	2.55 ± 1.93	0.90 ± 0.00	0.95 ± 0.06
Staphylococci	3.78 ± 0.24	4.07 ± 0.51	3.59 ± 0.23	4.09 ± 0.30
Amylolytic streptococci	4.47 ± 0.42	3.96 ± 0.39	4.66 ± 0.38	4.21 ± 0.16
Coliform bacteria	3.97 ± 0.17	1.32 ± 0.33	4.95 ± 0.11	2.42 ± 1.25

Average value of bacteria in CFU/g (log 10) ± SD; Day 0/1 means sampling before MLS EM 41/3 application, Day 21—EG, experimental group at day 21 (3 weeks of MLS EM 41/3 application), Day 21—CG, control group at day 21, Day 42—EG, experimental group at day 42 (3 weeks of MLS EM 41/3 cessation), Day 42—CG, control group at day 42. Caecum-staphylococci, Day 42—EG: Day 42—CG ^{ab} $p < 0.05$.

3.2. Phagocytic Activity, GPx and Biochemistry

PA values from blood reached $57.13 \pm 3.14\%$ at day 0/1 (Table 3). At day 21, PA increased in rabbits of the EG compared to PA measured at day 0/1 (^{ba} $p < 0.05$). But PA also increased in rabbits of the EG compared to rabbits in the CG (^{bb} $p < 0.05$). And PA value was significantly higher in rabbits of the EG at day 21 compared to PA value in rabbits of the EG at day 42 (^{bc} $p < 0.05$). The increase in PA was also noted in rabbits of the CG at day 21 compared to day 0/1 (^{ab} $p < 0.05$) and it was also increased in rabbits of the CG at day 42 compared to day 0/1 (^{ac} $p < 0.05$). At day 21, PA values reached almost the same level in rabbits of the CG compared to rabbits in the CG at day 42 (Table 3). The values of IPA were not influenced by MLS EM 41/3.

Table 3. The values of phagocytic activity (PA), the index of phagocytic activity (IPA), and GPx (U/gHb).

n = 8	Day 0/1	Day 21	Day 42
EG/PA	57.13 ± 3.14 ^a	64.38 ± 1.30 ^b	60.13 ± 0.99 ^c
CG/PA	57.13 ± 3.14 ^a	60.13 ± 0.99 ^b	60.38 ± 0.92 ^c
EG/IPA	3.55 ± 0.38	3.88 ± 0.05	3.40 ± 0.20
CG/IPA	3.55 ± 0.38	3.86 ± 0.05	3.88 ± 0.05
EG/GPx	151.98 ± 27.32	185.98 ± 37.60	153.05 ± 29.14
CG/GPx	151.98 ± 27.32	190.86 ± 28.98	171.49 ± 27.66

EG—experimental group of rabbits, CG—control group of rabbits, GPx (glutathione-peroxidase), ± SD. Day 0/1 means at the start of the experiment before application; Day 21 (3 weeks application of substance MLS EM 41/3), EG/PA at day 21: Day 0/1 (^{ba} $p < 0.05$); Day 21 EG/PA:CG/PA (^{bb} $p < 0.05$); Day 21 EG/PA: Day 42 EG/PA (^{bc} $p < 0.05$); Day 21 CG/PA: Day 0/1 (^{ab} $p < 0.05$); Day 42 (3 weeks after cessation) CG/PA: Day 0/1 (^{ac} $p < 0.05$). GPx = NS; however, an increase in the CG was noted compared to in the EG at days 21 and 42.

At day 21, the values of GPx increased in both groups of rabbits (EG, CG) (Table 3) compared to day 0/1. However, GPx was higher in rabbits of the CG than in rabbits of the EG (no oxidative stress stimulation). At day 42, GPx values decreased; in the EG, GP values almost reached initial levels (Table 3), and still lower in the EG than in the CG.

The total protein values measured were lower than the limit. However, they were increased in both groups at day 21 compared to day 0/1 and they were stable in the EG also at day 42 (Table 4). The same situation was noted in albumin status. Its measured values were within the limit. In the case of creatinine, a significant increase was noted (Table 4) within the limit (Day 0/1: Day 21/EG, ^{ab} $p < 0.001$; Day 0/1: Day 21/CG, ^{ad} $p < 0.001$; Day 0/1: Day 42 EG, ^{ac} $p < 0.01$; Day 42EG: Day 42/CG, ^{dc} $p < 0.01$). Bilirubin values in rabbit blood reached less than 0.3 $\mu\text{kat/L}$, under the limit. Glucose values were within the limit. They significantly decreased at days 21 and 42 compared to day 0/1; still within the top limit level (Day 0/1: Day 21/EG, ^{ca} $p < 0.001$, Day 01/: Day 42, ^{cb} $p < 0.001$; Day 0/1: Day 42/EG ^{ab} $p < 0.001$; Day 0/1: Day 42/CG ^{ad} $p < 0.001$) (Table 4). Cholesterol was measured within the limit and significantly decreased ($p < 0.01$). Triglycerides were slightly higher at day 0/1 but within the limit. At days 21 and 42 they were slightly decreased to limiting range value (Table 4). ALP value was the highest at day 0/1. Then, it decreased to the value of the limit. AST values were low and values of ALT were within the limit (Table 4). Na and K values were balanced, and Ca, Mg and p values were assessed as within the limit. Chlorides were also in the optimal range, while slightly increased for all days.

Table 4. Biochemistry at days 0/1, 21 and 42.

<i>n</i> = 8	Day 0/1	EG/21	CG/21	EG/42	CG/42
Total protein (g/L)	42.65 ± 3.06	47.41 ± 7.39	49.33 ± 6.36	47.03 ± 3.29	48.18 ± 5.30
Albumin (g/L)	31.69 ± 2.22	33.61 ± 5.60	34.35 ± 4.39	33.44 ± 2.42	32.83 ± 2.46
Creatinine ($\mu\text{mol/L}$)	27.97 ± 3.21 ^a	43.53 ± 5.16 ^b	38.96 ± 8.10 ^d	48.93 ± 5.41	52.81 ± 7.48 ^c
Glucose (mmol/L)	8.7 ± 0.78 ^a	7.55 ± 0.85	7.35 ± 0.82 ^c	6.86 ± 0.58 ^b	6.8 ± 0.48 ^d
Cholesterol (mmol/L)	1.89 ± 0.30 ^a	1.40 ± 0.32	1.62 ± 0.64 ^b	1.05 ± 0.29 ^c	1.06 ± 0.30 ^d
Triglycerides (mmol/L)	1.76 ± 0.68	1.09 ± 0.28	1.19 ± 0.54	0.75 ± 0.22	1.01 ± 0.22
ALT ($\mu\text{kat/L}$)	0.13 ± 0.03	0.14 ± 0.03	0.17 ± 0.04	0.19 ± 0.08	0.15 ± 0.05
AST ($\mu\text{kat/L}$)	0.12 ± 0.07	0.15 ± 0.03	0.20 ± 0.05	0.11 ± 0.00	0.17 ± 0.07
ALP ($\mu\text{kat/L}$)	2.44 ± 0.40	1.94 ± 0.53	1.81 ± 0.63	2.00 ± 0.40	1.81 ± 0.44
Na (sodium, mmol/L)	132.9 ± 2.96	136.1 ± 10.74	136.9 ± 9.72	134.1 ± 7.36	130.5 ± 7.09
K (kalium, mmol/L)	4.60 ± 0.44	4.52 ± 0.39	4.03 ± 0.49	3.96 ± 0.18	4.60 ± 0.44
Ca (calcium, mmol/L)	3.30 ± 0.15	3.20 ± 0.47	3.42 ± 0.36	3.16 ± 0.20	3.06 ± 0.22
P (phosphorus, mmol/L)	2.27 ± 0.28	2.23 ± 0.31	2.35 ± 0.28	1.29 ± 0.24	2.04 ± 0.38
Mg (magnesium, mmol/L)	0.80 ± 0.04	0.83 ± 0.09	0.86 ± 0.10	0.82 ± 0.06	0.79 ± 0.09
Chlorides (Cl, mmol/L)	92.00 ± 3.51	95.94 ± 8.43	93.24 ± 7.28	95.19 ± 6.94	90.63 ± 5.20

Day 0/1, sampling before application of MLS EM 41/3, EG/21, experimental group at day 21 (3 weeks of MLS EM 41/3 application), CG/21, control group at day 21, EG/42, experimental group at day 42 (3 weeks of MLS EM 41/3 cessation), CG/42, control group at day 42; Reference ranges: total proteins, 53–85 g/L, albumin (24–46 g/L), creatinine (44–141 $\mu\text{mol/L}$), glucose (5.5–8.6 mmol/L), cholesterol (0.28–2.1 mmol/L), triglycerides (1.44 $\mu\text{kat/L}$), ALT (alanine aminotransferase, 0.33–1.19 $\mu\text{kat/L}$), AST (aspartate aminotransferase, 0.23–0.93 $\mu\text{kat/L}$), ALP (alkalic phosphatase, 0.3–2.13 $\mu\text{kat/L}$), bilirubin (4.3–8.5 $\mu\text{kat/L}$), Na (natrium/sodium), K (kalium, mmol/L), Ca (calcium, 2.2–4.2 mmol/L), P (phosphorus, 1.2–2.4 mmol/L), CL (chlorides, 92–120 mmol/L), and Mg (magnesium, 0.8–1.20 mmol/L). Creatinine, Day 0/1: Day 21/EG, ^{ab} $p < 0.001$; Day 0/1: Day 21/CG, ^{ad} $p < 0.001$; Day 0/1: Day 42 EG, ^{ac} $p < 0.01$; Day 42EG: Day 42/CG, ^{dc} $p < 0.01$. bilirubin values in rabbits reached less than 0.3 $\mu\text{kat/L}$. Glucose, Day 0/1: Day 21/EG, ^{ca} $p < 0.001$; Day 0/1/: Day 42, ^{cb} $p < 0.001$; Day 0/1: Day 42/EG ^{ab} $p < 0.001$; Day 0/1: Day 42/CG ^{ad} $p < 0.001$. Cholesterol, Day 21/CG: Day 42/EG, ^{cb} $p < 0.01$; Day 0/1: Day 42/EG, ^{ac} $p < 0.01$; Day 0/1: Day 42/CG ^{ad} $p < 0.01$.

3.3. Hydrolytic Activity, Jejunal Morphometry, Growth Performance, Organic Acids in Chyme, Quality of Meat

Fecal hydrolytic activity measurements are involved in Table 5. The highest value was measured for amylolytic activity at days 0/1, 21 and 42. Their values were increased from days 0/1, 21 and 42 in both groups. However, those values were higher in the EG

than in the CG and almost stable at days 21 and 42 (Table 5). Xylanolytic activity was also high and increased in both groups at day 21, reaching almost the same values with a slight decrease at day 42. The value of pectinolytic activity was only slightly increased at day 21 compared to day 0/1 and pectinolytic activity was slightly higher in the EG than in the CG at days 21 and 42 (Table 5). Cellulolytic activity and inulolytic activity reached the lowest value at the start of the experiment and at day 21 as well; at day 21, those activities slightly increased but they were still balanced. Inulolytic activity was not influenced.

Table 5. Fecal hydrolytic activity in μmol released products/g dry matter of feces/min.

	Day 0/1 (<i>n</i> = 10)	Day 21/EG (<i>n</i> = 5)	Day 21/CG (<i>n</i> = 5)	Day 42/EG (<i>n</i> = 5)	Day 42/CG (<i>n</i> = 5)
Amylolytic activity	3.76 $\pm$ 0.53	5.92 $\pm$ 0.96	4.60 $\pm$ 0.67	5.34 $\pm$ 1.00	4.22 $\pm$ 0.63
Cellulolytic activity	1.68 $\pm$ 0.15	2.63 $\pm$ 0.56	2.18 $\pm$ 0.38	2.04 $\pm$ 0.23	2.10 $\pm$ 0.46
Xylanolytic activity	3.50 $\pm$ 0.39	4.88 $\pm$ 0.84	4.23 $\pm$ 0.36	3.09 $\pm$ 0.52	3.52 $\pm$ 1.05
Inulolytic activity	1.19 $\pm$ 0.09	1.64 $\pm$ 0.05	1.44 $\pm$ 0.29	1.46 $\pm$ 0.11	0.91 $\pm$ 0.10
Pectinolytic activity	2.66 $\pm$ 0.29	3.08 $\pm$ 0.63	2.86 $\pm$ 0.39	2.84 $\pm$ 0.18	2.29 $\pm$ 0.58

Day 0/1 (mixed fecal samples of all animals before MLS EM 41/3 application), Day 21/EG-experimental group at day 21 (3 weeks of MLS EM 41/3 application, 5 mixed samples from all animals in group), Day 21/CG, control group at day 21, Day 42/EG, experimental group at day 42 (3 weeks of MLS EM 41/3 cessation), and Day 42/CG, control group at day 42.

Regarding jejunal morphometry, the stimulating effect of MLS EM 41/3 was noted at day 21 and also after cessation at day 42 when the villi height to crypt depth ratio was higher (EG/21, 4.0 $\pm$ 0.16; CG/21, 3.93 $\pm$ 0.98). At day 42, the ratio did not change in the EG (4.0 $\pm$ 0.16) and it even decreased in the CG (3.86 $\pm$ 0.96).

Live weight of rabbits reached 2671 $\pm$ 335 g in the CG at the end of the experiment. In rabbits of the EG, live weight reached 2753 $\pm$ 200 g. ADWG reached 45.05 g in rabbits of the EG at day 42 and 39.84 g in rabbits of the CG. Feed conversion was balanced (3.557 $\pm$ 0.378 kg mass per kg ADWG in the CG and 3.662 $\pm$ 0.336 mass per kg in the EG). The values evaluated were not significantly changed. The values of acetic acid were slightly higher in the EG than in the CG at day 21 (Table 6). At day 42, the values of acetic acid decreased in both groups of rabbits. The values of other individual organic acids and/or ammonia in cecal chyme were not influenced, respectively; they were almost the same values in the EG as in the CG (Table 6) at day 21. The pH value was slightly higher in the EG than in the CG and it was not changed in the EG at day 42. At day 42, lower values of acetic, butyric, caproic and lactic acids were measured (Table 6).

Table 6. The values of organic acids, ammonia-NH₃ and pH in cecal chyme (mmol/100 mL) at days 21 and 42.

<i>n</i> = 4	Day 21/EG	Day 21/CG	Day 42/EG	Day 42/CG
Acetic acid	10.65 $\pm$ 1.57	12.09 $\pm$ 1.82	7.65 $\pm$ 2.27	8.03 $\pm$ 1.79
Propionic acid	0.47 $\pm$ 0.07	0.41 $\pm$ 0.08	0.47 $\pm$ 0.24	0.62 $\pm$ 0.13
Isobutyric acid	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.0	0.02 $\pm$ 0.01
Butyric acid	3.20 $\pm$ 0.96	2.97 $\pm$ 0.67	0.09 $\pm$ 0.05	1.65 $\pm$ 0.48
Isovaleric acid	0.08 $\pm$ 0.05	0.09 $\pm$ 0.02	0.05 $\pm$ 0.01	2.07 $\pm$ 0.76
Caproic acid	0.15 $\pm$ 0.06	0.18 $\pm$ 0.03	0.06 $\pm$ 0.01	0.06 $\pm$ 0.01
Valeric acid (mmol/L)	0.12 $\pm$ 0.04	0.13 $\pm$ 0.02	0.12 $\pm$ 0.05	0.01 $\pm$ 0.00
Lactic acid (g/100 kg)	0.09 $\pm$ 0.00	0.02 $\pm$ 0.00	0.02 $\pm$ 0.00	0.02 $\pm$ 0.00
Ammonia (NH ₃ , mmol/L)	12.49 $\pm$ 2.29	12.41 $\pm$ 2.10	8.14 $\pm$ 1.66	11.76 $\pm$ 3.68
pH	6.13 $\pm$ 0.09	5.86 $\pm$ 0.19	6.01 $\pm$ 0.12	6.24 $\pm$ 0.34

Day 21/EG means the experimental group at day 21 (3 weeks of MLS EM 41/3), Day 21/CG means the control group at day 21, Day 42/EG means the experimental group at day 42 (3 weeks of MLS EM 41/3 cessation), and Day 42/CG means the control group at day 42.

Higher-energy values were noted in the meat of EG rabbits (Table 7). Measured values were not significant (NS). WHC was increased. The other physico-chemical parameters measured in meat were not negatively influenced.

Table 7. Physico-chemical values in *Musculus longissimus thoracis* and *lumborum*.

<i>n</i> = 4	Day 21/EG	Day 21/CG	Day 42/EG	Day 42/CG
Water content (g/100 g)	74.93 ± 0.35	74.76 ± 0.11	74.29 ± 0.87	74.76 ± 0.31
Protein content (g/100 g)	22.89 ± 0.33	22.78 ± 0.19	23.85 ± 0.43	23.22 ± 0.35
Fat content (g/100 g)	1.47 ± 0.00	1.42 ± 0.36	1.19 ± 0.15	1.25 ± 0.17
pH 24 p.m.	5.96 ± 0.03	5.99 ± 0.04	5.96 ± 0.05	6.02 ± 0.02
Water holding capacity g/100 g)	26.33 ± 3.32	29.02 ± 4.81	27.08 ± 6.42	27.94 ± 1.03
Energy value (kJ/100 g)	443.07 ± 10.75	435.16 ± 9.17	444.48 ± 7.96	436.10 ± 10.19

Day 21/EG means the experimental group at day 21 (3 weeks of MLS EM 41/3 application), Day 21/CG means the control group at day 21, Day 42/EG means the experimental group at day 42 (3 weeks of MLS EM 41/3 cessation), and Day 42/CG means the control group at day 42, NS (not significant).

4. Discussion

Assessing the microbiota, the bacterial count in feces of broiler rabbits was not reduced by Mundticin-like substance (MLS) EM 41/3 application. Only the enterococcal count was influenced—increased—at day 21. The bacterial count in caecum was lower than in feces for all but staphylococci and amylolytic streptococci. In appendix, there were almost the same bacterial amounts as in feces and/or they were found in lower amounts. However, re-isolated bacterial colonies from feces, caecum and appendix were mostly susceptible to MLS EM 41/3. The appendix is related to rabbit immunity via the development of gut-associated lymphoid tissue (GALT). It can be enhanced/improved by microbiota which play an important role in rabbit appendix development and diversity in the primary antibody repertoire [7,10].

Enterococci are a group of LAB which constitute a large proportion of autochthonous microbiota in the gastrointestinal tract of human and animals [19]. Many of them can produce antimicrobial active proteinaceous substances, bacteriocins, which have recently been found to be involved in a group of postbiotics [3,20–22]. The Mundticins produced by strains isolated from plants described to date showed limited inhibitory activity mostly against Gram-positive bacteria, including listeriae [22]. In vitro studies with MLS EM 41/3 will be continued in addition to the MLS purification process to determine the possibility of using more concentrated substances with supposed higher (broader) inhibitory effects.

The hepatal profile in rabbits represented by the enzymes ALT, AST and ALP showed low values, meaning MLS EM 41/3 did not have a negative influence on those parameters. ALT is a liver-specific enzyme in cytoplasm, the level of which is increased in the case of hepatal cell membrane damage associated with liver attack. AST is a liver non-specific enzyme in mitochondria and cell cytoplasm. This enzyme is also leaked after damage [23]. Natural additives can influence blood parameters depending on the length and dosage of their administration. The hypoproteinemia could be explained by reduced exogenous protein intake. Similarly, total proteins were not significantly increased after Ent 4231 [24]. The hypocholesterolemic effect of enterocins was previously noted, e.g., after Durancin ED 26E/7 administration as well as Ent M [25]. The mineral profile of rabbits from blood was not influenced in this study and its values were within the limit. Some disbalances could be caused by unbalanced mineral content in feeds and/or by supposing better intestinal absorption and metabolism of tested minerals in the gastrointestinal tract [25]. In our previous study, the energy profile, including glucose values, was supported by lantibiotic bacteriocin-nisin and Ent 2019 application [25,26].

In our previous experiments using enterocins, Ent 2019 and Ent M, an increase in PA ($p < 0.01$) was noted, and even prolonged stimulation of PA was measured [27]. Moreover,

PA also increased in the case of Ent 4231 [24], independently of the origin of the enterocin producer strain [27].

Similar to this study, no oxidative stress stimulation was noted also after Ent M application when the lowest GPx values were noted in EG rabbits; the same situation was also found after Ent 2019 and Ent 4231 application [24,27].

Rabbit health was maintained in good condition during the whole experiment. Diet supplementation with bioactive compounds usually produces improved ADWG and feed conversion ratio in rabbits [28], which was also noted after MLS EM 41/3 application.

In general, higher hydrolytic activity is detected in young than adult rabbits [29]. Hydrolytic activity in caecum of rabbits after Ent M and ED 26E/7 application was high at day 21 and then it decreased. In our case, hydrolytic activity was measured in feces and these values were lower than in caecum as also reported by Pogány Simonová et al. [10]. But these values were higher at day 21 than at day 42. Cecal microbiota and change in their representatives can influence the fermentation process in caecum and this is the same in feces.

Pogány Simonová et al. [10] presented almost the same pH values after Ent M and Durancin ED 26E/7 application in rabbits. Ammonia values in the mentioned study were comparable with values reached at day 21 in this study; however, at day 42, they were higher in the studied groups than in our study (up to 22.0 g/100 g and up 0.1 g/100 g in our study). The other organic acid values were almost the same as presented Pogány Simonová et al. [10]. Lactic acid is important for beneficial digestion and immunity. It is fully absorbed in the rabbit body and this could be due to the low values of LA measured in rabbits in this study. It is also seen in association with a low amount of LAB detected in caecum. However, coliforms reached up to 10^3 CFU/g and/or 10^4 CFU/g.

The increased tendency in jejunal morphology after MLS EM 41/3 application is in accordance with previously reported results [27,30,31].

Regarding meat quality, an important parameter is water content, which influences water holding capacity, meat color and digestibility/fragility of meat. These parameters are key points for meat quality, mainly during storage. Water content, fat content and water holding capacity are associated parameters. In this study, correlation between the pH values and water holding capacity values was found. The energy value is associated with protein content and fat content. In our case, energy values were higher in meat from EG rabbits. Energy values are important for consumers because that meat is high dietetic with beneficial nutritional and biological value. Energy values in fresh rabbit meat is comparable with red meats [32]. As we do not have an exact explanation for influencing the meat quality using postbiotics, our hypothesis can be based only on an idea of total postbiotic influence (MLS EM 41/3) on, e.g., animal immunity.

The indigenous intestinal microbiota plays an important role in the regulation of intestinal development and in supporting the host against potential pathogenic microbiota colonization [33]. But external factors (e.g., diet changes and weaning period, which is a stressed period) also influence this stability. So, using postbiotics as an innovative feed strategy could help to improve digestive immunity and animal defense capacity (by stimulating phagocytosis) with no negative effect on other parameters that contribute to the total health profile of bred animals as indicated in the use of some enterocins [9,27,30,31,33].

5. Conclusions

Administration of Mundticin-like substance (MLS) EM 41/3 produced by a non-autochthonous strain *Enterococcus mundtii* EM 41/3 lead to a significant increase in non-specific parameter immunity (phagocytic activity), but did not influence microbiota composition and had no influence on blood biochemistry. Higher values of glutathione-peroxidase were measured in the CG than in the EG, meaning no oxidative stress was noted. Moreover, higher weight gain was noted in EG rabbits. MLS EM 41/3 stimulated fecal hydrolytic activity and no negative influence on rabbit meat quality was noted. Using postbiotic MLS EM 41/3 in the feeding strategy can be a contributing factor to improve animal health.

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