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Research Article

Safety Assessment of Fecal, Bacteriocin-Producing Strains *Enterococcus mundtii* from Horses

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Abstract

Backround: Knowing and optimizing the host microbiota is important regarding the maintenance of horse`s health. For this approach beneficial bacteria have been usually used. However, to be the most effective in use, their safety, tolerability and efficacy needs to be assessed. Therefore, fecal strains *Enterococcus mundtii* from horses with promising bacteriocinogenic potential have to be evaluated for their safety.

Methodology: *E. mundtii* strains were isolated from feces of 47 horses (n=47; 40 mares and seven stallions), the Norik breed from Muráň in eastern Slovakia. MALDTI-TOF spectrometry and sequencing were used. Phenotypic characteristics were assessed in accordance with those for the reference strain *E. mundtii* ATCC43186. Biofilm-forming ability was tested using plate assay. For enzyme production commercial tests were applied and virulence factor genes were tested using PCR and primers. Antibiotic profile was tested with diffusion method. *In vivo* safety was tested using Balb/c breed mice.

Results: *E. mundtii* strains did not produce the enzyme β-glucuronidase; however, most *E. mundtii* strains produced β-galactosidase. The strains were absent of virulence factors such as gelatinase, aggregation substance, cytolysin A, enterococcal superficial protein, adhesins, hyaluronidase and IS16 element, except *efa*Afs gene in the strain EMKD 24/1. Six strains were found with low-grade ($0.1 \leq A_{570} < 1$) biofilm - forming ability. *E. mundtii* were mostly susceptible to antibiotics. Bacteriocinogenic strain EMKD41/3 reached high counts in feces (5.12 ± 0.26 CFU/g log 10) of Balb/c mice during its 30 days application. No mortality of mice was noted during whole period of EMKD41/3 strain application.

Conclusion: Bacteriocin-producing strains *E. mundtii* should not threaten horses because they were mostly susceptible to antibiotics, they were virulence factor genes absent and with low-grade biofilm formation ability. Bacteriocin-producing strain EMKD41/3 even indicates its sufficient implementation in host organism with any side effect.

Keywords: Safety; *E. mundtii;* Virulence factor; Biofilm

Introduction

The gastrointestinal microbiota play an important role in intestinal and extraintestinal health and disease [1]. In human, e.g. role of the microbiome is well studied [2]; however, much less is known about the microbiome and its role in the different equine species. Therefore, knowing and optimizing the host microbiota is important regarding the maintenance of horse`s health status. This approach can be fulfilled with use of beneficial/probiotic bacteria [1].

In general, beneficial bacteria have been used widely as nutritional supplements in animals; however, there are limited and conflicting information with their use in horses [1]. To be the most effective in their use, their safety, tolerability and efficacy needs to be assessed. The representatives of lactic acid bacteria (LAB) are the most frequently used probiotic bacteria in animals [3-6]. In our previous studies non-autochthonous and also autochthonous beneficial bacteriocin-producing *Enterococcus faecium* strains and/or their bacteriocins were successfully applied in horses [5-7]. When non-autochthonous *E. faecium* strain AL41=CCM8558 was applied in warm-blooded horses, its inhibitory activity was demonstrated against Gram-negative aeromonads ($p<0.001$). A tendency of increased phagocytic activity (PA) was measured in horses and also hydrolytic enzymes activities were significantly increased ($p<0.01$). Biochemical parameters were influenced in physiological range. When autochthonous *E. faecium* EF412 was applied in warm-blooded exercising horses, the total enterococcal and LAB counts were significantly increased ($p<0.001$). The phylum Firmicutes was one of dominated. Phagocytic activity showed an increasing tendency. Administration of Ent M produced by *E. faecium* CCM8558 lead to reduction of coliforms, campylobacters and clostridiae ($p<0.05$, $p < 0.001$) and also increase in PA was noted ($p <0.001$) [5]. So, the species *E. faecium* seems to be very promising following this aim. However, we also detected promising autochthonous fecal *E. mundtii* strains (from horses) which showed bacteriocinogenic potential [8]. As formerly indicated, before their application in horses, their safety should be evaluated.

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Therefore, the aim of this study was to test safety aspects (genes for virulence factors, enzymes production involving undesirable enzymes, biofilm formation ability, antibiotic resistance profile) of 14 fecal *E. mundtii* isolated from horses, bacteriocinogenic potential of which has been already reported [8]. Finally, *in vivo* safety of the most bioactive strain *E. mundtii* EM41/3 was tested using mice model.

Materials and Methods

Sampling and Strains Characterization

Rectal removal (feces) from 47 horses (n=47; 40 mares and seven stallions), the Norik breed from Muráň (eastern Slovakia) were sampled during November 2019 year. Detail description of sampling and characterization of horses has already been indicated in our previous study [8]. Age of horses ranged from 5 months up to 23 years. Horses were grazed on pasture or fed hay and oats. Animals did not be on antibiotic therapy and they did not show any clinical symptoms. In stall, they were placed on straw. Feces were sampled immediately after each horse`s defecation.Treatment of samples and isolation of enterococci detaily reported previously Focková et al. [8]. Fourteen (14) strains were taxonomically allotted to the species *Enterococcus mundtii* based on the MALDI-TOF MS identification system and also using sequencing (BLASTn analysis) reaching percentage identity BLASTn 16S rRNA sequence in all strains up to 100% (99.17-99.91) as previously reported by Focková et al.[8]. Identified strains were stored using the Micro bank system (Pro-Lab Diagnostic, Richmond, BC, Canada) for the next analyses.

Additionally, the strains were phenotyped using commercial identification system BBL Crystal Gram-positive ID System kit (Becton and Dickinson, Cockeysville, USA). This kit includes tests for hydrolysis of urea, esculin, and arginine, hydrolysis of enzymes and fermentation/utilization of carbohydrates (trehalose, lactose, sucrose, mannitol, fructose, arabinose, etc.). This system uses chromogenic and fluorochrome-linked substrates to detect metabolic enzymes. Briefly, isolated strains were cultivated on M-*Enterococcus* agar at 37°C for 48 h. Individual colonies were suspended in a labeled tube of inoculum fluid to a turbidity equivalent to a 0.5 Mc Farland standard. Each tube was vortexed for 15 seconds, and the entire contents were poured into an appropriately labeled panel base. The inoculum was then gently rolled along the tracks of the base to fulfill wells. A lid was aligned over each base and snapped into place. The inoculated panels were placed in incubation trays and incubated for 24 h at 37°C. They were read with the BBL Crystal Panel Viewer and a 10-digit profile number was generated and recorded on a pad listing results. The profile number and spot biochemical test results and Gram stain reaction were entered into a computer on which the BBL Crystal ID System Electronic Codebook had been installed. The computer program generates a single genus and species identification or several differentiate identifications. Identification of tested organisms was derived from a comparative analysis of the reaction patterns of the tested isolates with the reference strains in the database.

Enzyme Activity Measured Using API-ZYM System

Metabolic enzyme activity is a parameter evaluated for both beneficial and damaging enzymes. The API-ZYM panel system (BioMérieux, Marcy l`Etoile, France) was used according to the manufacturer's recommendation as previously described by Lauková et al. [6]. This panel involves the following enzymes: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Briefly, an amount of 65 µl McFarland standard 1 inoculum was transferred into each well of the test panel plate. Incubation was performed for 4h at 37°C. Then, the reagents Zym A and Zym B were added and enzyme activity was evaluated. Color intensity values from 0-5 and their relevant value in nanomoles (nmol) were assigned for each reaction according to the color chart supplied with the kit.

Detection of Genes Encoding Virulence Factors

Based on the previous results [9] demonstrating the most frequently detected genes encoding virulence factors in different enterococci, the presence of the following genes for virulence factors was tested: *gelE* (gelatinase), *esp* (enterococcal surface protein), *efaAfm* (adhesin *E.faecium*), *cylA* (cytolysin A), *hylEfm* (hyaluronidase), *agg* (aggregation substance) and *IS16 element* (IS 16). The PCR products were separated by means of agarose gel electrophoresis (1.2 % w/v, Sigma-Aldrich, Saint Louis, USA) with 1µl/ml content of ethidium bromide (Sigma-Aldrich) using 0.5 x TAE buffer (Merck, Darmstadt, Germany). The PCR fragments were visualized with UV light. The strains *E.faecalis* 9Tr1 (our strain, [10]), *E. faecium* P36 (Dr. Semedo-Lemsaddek, University Lisbon, Portugal) were positive controls. The PCRs were carried out in 25 µl volume, with a mixture consisting of 1x reaction buffer, 0.2 mmol/l of deoxynucleoside triphosphate, 3 mmol $MgCl_2$, 1 µmol/l of each primer, 1 U of Taq DNA polymerase, and 1.5 µl of DNA template with the cycling conditions as previously reported by Kubašová et al. [9] and Lauková et al. [11]. The PCR conditions (for *gelE, agg, cylA, esp, efaAfs, efaAfm*) were as follows: denaturation at 95°C for 3 min followed by 35 cycles for 30 s at 95°C, 30 s at 55°C, 30 s at 72°C and 5 min at 72°C. The PCR conditions for *hyl* and *IS16* genes were as follows: denaturation at 94°C for 4 min, followed by 30 cycles for 30 s at 94°C, 30 s at 50°C, 30 s at 72°C and finally for 4 min at 72°C.

Biofilm Formation Ability Testing

The ability of the identified *E. mundtii* strains to form biofilm was checked using the qualitative method on Congo red agar [12] and using the quantitative plate assay [13,14]. The Congo red agar plates were inoculated with the tested *E. mundtii* strains. They were incubated at 37°C overnight and biofilm formation was assessed through the presence of black colonies with dry crystalline consistency. The agar plates were then maintained at laboratory temperature and checked again at 48 and 72 h. Strains which did not form biofilm remained pink. *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 was used as positive control (kindly provided by Dr. Eva Styková, University of Veterinary Medicine and Pharmacy in Košice, Slovakia).

In the case of the quantitative plate assay according to Chaieb et al. [13] and Slížová et al. [14], one colony of the tested *E. mundtii* strains grown on M-*Enterococcus* agar (Difco, NJ, USA) overnight at 37°C was transferred into 5 ml of Ringer solution (pH 7.0) to reach the suspension corresponding to 1 McFarland standard and corresponding to 1.0 x 10^8CFU/ml. A 100 µl volume from that diluted suspension was transferred into 10 ml of Brain heart infusion

(BHI, Difco, USA). A 200 µl volume of dilution was transferred into microtiter plate wells (Greiner ELISA 12 Well Strips, 350 µl, flat bottom, Frickenhausen GmbH, Germany). The plate was incubated for 24 h at 37°C. The biofilm formed in the microtiter plate wells was washed twice with 200 µl of deionized water and then dried at 25°C for 40 min. The attached bacteria were stained for 30 min at 25°C with 200 µl of 0.1 % (*w/v*) crystal violet in deionized water. The dye solution was aspirated away, and the wells in the microtiter plate were washed twice with 200 µl of deionized water. After water removal, the plate was dried for 30 min at 25°C. The dye bound to the adherent biofilm was extracted with 200 µl of 95% ethanol and stirred. A 150 µl volume was transferred from each well into a new microplate well to measure absorbance (A_{570}) in nm. This measurement was performed using an Apollo 11 Absorbance Microplate reader LB 913 (Apollo, Berthold Technologies, Oak Ridge, TN, USA). Testing of each *E.mundtii* strain was repeated in two independent runs with 12 replicates. Sterile BHI was used in each analysis, serving as negative control. *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 was used as positive control. Biofilm formation was classified as highly- positive ($A_{570} \geq 1$), low-grade positive ($0.1 \leq A_{570} < 1$) or negative ($A_{570} < 0.1$; [13,14].

Antibiotic Phenotype Using the Agar Disk Test Diffusion Method

The antibiotic phenotype was tested by the agar disk diffusion method [15] against antibiotics (13) recommended for enterococci. Strains were cultivated in BHI broth (Difco, MD, USA) at 37°C overnight. A 100 µl volume of tested strain *E. mundtii* was spread on Mueller-Hinton agar (Difco, USA) and the appropriate antibiotic disks were applied. Plates were incubated at 37 °C overnight and evaluated as susceptible or resistant according to the recommendation provided by the antibiotic disc suppliers. The inhibitory zone was expressed in millimeter. The following antibiotics were tested: clindamycin (DA, 2 µg), novobiocin, (5 µg), ampicillin, gentamicin (Amp, CN 10 µg), penicillin G (10 IU), azithromycin, erythromycin (Azm, E 15 µg), streptomycin (25 µg), chloramphenicol, rifampicin, tetracycline, kanamycin, vancomycin (C, RD, T, KAN, VAN 30 µg). Antibiotic disks suppliers were Oxoid and for VAN and KAN, Lach-Ner (Czech Republic).The general positive control was *E. faecalis* ATCC 29212.

In vivo Safety Control of *Enterococcus mundtii* EMKD41/3 Using Balb/c Mice Model

For *in vitro* safety control of *E. mundtii* EMKD41/3 strain, pathogen-free aged eight weeks Balb/c mice, both sexes (VELAZ Prague, Czech Republic) were used. Their weight was around 18-20g. Mice maintenance conditions are the same as previously reported by Vargová et al. [16]. Mice were kept under a 12-h light/dark regimen at temperature 22-24°C with humidity 56%. They were on commercial diet and water available without restriction. Mice were divided randomly into 2 groups: Control (n=15) and Group EM (n=15). The experimental protocol was approved by Slovak Veterinary and Food Administration (Ro 7413/2021-220) and also approved by Ethic Commision of Parasitological Institute of the Slovak Academy of Sciences in Košice- where it was experimented-, SK CH 21016). To differ EMKD41/3 strain from other enterococci, its rifampicin resistant variant was prepared [17]. *E.mundtii* EMKD41/3 was administered *per os* daily at a dose 10^9 CFU/ml in a total dose 100 µl. Counts of EMKD41/3 as well as other enterococci were enumerated after standard microbiological dilution of feces, jejunum and liver (hepar); jejunum and liver were homogenized in Ringer solution using Masticator (Spain) and the appropriate dilutions were plated on BHI agar enriched with rifampicin (100 µg), M-*Enterococcus* agar (Difco, USA) and lactic acid bacteria were cultivated on De Man-Rogose-Sharpe agar (MRS, Merck, Darmstadt, Germany).The total bacterial counts were expressed in CFU/g ± SD. Sampling of feces was performed at the start of experiment (n=30), and also at the end of application (at day 30).

Statistical Evaluation

Statistical evaluation was performed using one-way analysis of variance (ANOVA), followed by Tukey post test. The results are quoted as means ± SD and were compared among groups within the same days of samples collection.Statistically significant differences were considered at $P < 0.05$. All statistical analyses were performed using GraphPad prism statistical software (GraphPad Prism version 6.0, GraphPad Software, San Diego, California, USA).

Results

E. mundtii Strains Phenotypization and Enzyme Activity Evaluation

Phenotypic properties included in panel kit were compared with those for reference strain *E. mundtii* ATCC 43186 [18,19]. The strains fermented arabinose, fructose, cellulobiose, lactose, maltose, sucrose, trehalose, glycerol and arginine.Also esculin hydrolysis reaction was positive as well as Voges-Proskauer test (VP), while urea showed negative reaction.

Regarding enzyme activity, the majority of *E. mundtii* strains showed production of alkaline phosphatase (up to 10 nmol) except the strains EMKD5/1, EMKD13/3, EMKD24/1, and EMKD43/1. All strains were β-glucuronidase negative and most *E. mundtii* strains produced β-galactosidase (except the strains EMK5/1, KD12/1, KD24/1, and KD41/3). Leucine arylamidase was produced only by the strain EMKD31/2 (5 nmol) and EMKD40/2 (10 nmol). The

Table 1: Biofilm formation ability testing by qualitative and quantitative method, and detection of virulence factor genes.

Strain	Congo red /72h	Plate assay	EfaAfs
EMK2/1	ng	0.079 ± 0.004	ng
EMK5/1	ng	0.082 ± 0.005	ng
EMKD12/1	d	0.098 ± 0.005	ng
EMKD13/3	ng	0.121 ± 0.021	ng
EMKD22/1	d	0.100 ± 0.029	ng
EMKD24/1	+	0.106 ± 0.035	+
EMKD29/1	d	0.082 ± 0.003	ng
EMKD31/2	+	0.113 ± 0.022	ng
EMKD32/3	d	0.086 ± 0.005	ng
EMKD34/2	d	0.105 ± 0.010	ng
EMKD37/1	ng	0.097 ± 0.004	ng
EMKD38/1	ng	0.092 ± 0.007	ng
EMKD40/2	ng	0.092 ± 0.027	ng
EMKD41/3	d	0.115 ± 0.015	ng

Note: EM-*Enterococcus mundtii*; ng-negative (strains did not form biofilm on Congo red agar); d-dubious (not clear reaction; + (positive, forming biofilm or detected gene); Strains were absent of other tested virulence factor genes.

Table 2: Antibiotic phenotype in *Enterococcus mundtii* strains tested using agar diffusion method (inhibitory zones are expressed in mm).

Strain	AMP	DA	KAN	CN	NB
EMK2/1	14^S	R	R	R	R
EMK5/1	15^S	18^S	13^S	11^S	14^S
EMKD12/1	R	R	11^S	11^S	13^S
EMKD13/3	14^S	R	R	10^S	12^S
EMKD22/1	10^S	19^S	11^S	11^S	14^S
EMKD24/1	R	R	R	10^S	14^S
EMKD29/1	15^S	R	R	11^S	R
EMKD31/2	14^S	R	R	R	13^S
EMKD32/3	R	R	12^S	11^S	12^S
EMKD34/2	16^S	R	11^S	12^S	14^S
EMKD37/1	18^S	R	12^S	14^S	13^S
EMKD38/1	16^S	R	12^S	11^S	18^S
EMKD40/2	12^S	12^S	12^S	11^S	11^S
EMKD41/3	14^S	R	R	13^S	11^S

Note: EM-*Enterococcus mundtii*; AMP-ampicillin (10μg), DA (clindamycin (2μg), KAN-kanamycin (30μg), CN-gentamicin (10 μg), NB –novobiocin (5μg), R-resistant, 14^S-susceptible with inhibitory zone in size 14 mm; Strains were susceptible to azithromycin, chloramphenicol, erythromycin, penicillin, rifampicin, tetracycline, and vancomycin and they were resistant to streptomycin.

rest of strains had this reaction negative. Similarly, the strains did not produce valine arylamidase and cystine arylamidase, except 5 nmol for valine arylamidase in EMKD40/2 strain. Trypsin, and α-chymotrypsin were not produced by tested *E. mundtii* strains. Production of β-glucosidase was measured in all *E. mundtii* strains in range from 5 up to 30 nmol. Naphtol-AS-BI-phosphohydrolase was measured in amount 5 nmol; in the strain EMKD40/2 it was 20 nmol. Lipase, α-glucosidase, α-mannosidase, and α-fucosidase tests were mostly negative (0 nmol). However, α- fucosidase in strains EMKD38/1, EMKD 37/1, EMKD 32/3, EMKD 31/2 and EMKD 22/1 reached 5 nmol. Acid phosphatase reached mostly 5 nmol in tested *E. mundtii;* only in the strain EMKD 38/1 was measured 10 nmol, and the strains EMKD41/3, EMKD 24/1, and EMKD 22/1 did not produce this enzyme. All strains produced esterase and esterase lipase (5-20 nmol), except EMK5/1 (0). EM38/1 produced high amount of those mentioned enzymes (30 mmol). N-acetyl-β-glucosaminidase and α-galactosidase were not produced or only in low amount (5 nmol).

Detection of Genes Encoding Virulence Factors, Biofilm Formation Ability and Antibiotic Phenotype Profile

E. mundtii strains were absent of tested genes for virulence factors (*gelE, agg, cylA, esp, efaAfm, hyl* and *IS16,* Table 1), except gene for *efaAfs* production which was found only in EMKD24/1. Using the qualitative test, biofilm production was confirmed in two strains (EMKD24/1 and EMKD31/2); six strains showed dubious (not clear reaction) and the rest strains were negative. Only six strains (Table 1) were found with biofilm formation ability which was classified as low-grade ($0.1 \leq A_{570}<1$) with the highest amount in EMKD13/3 (0.121). Eight strains did not form biofilm testing by plate assay.

E. mundtii strains were mostly susceptible to antibiotics. The strains (14) were susceptible to rifampicin (RD), tetracycline (T), vancomycin (VAN), azithromycin (AZM), erythromycin (E), chloramphenicol (C), and penicillin (P) meaning to seven out of 13 antibiotics tested. Most *E. mundtii* strains were also susceptible to novobiocin (NB) and gentamicin (CN) meaning 12 out of 14 strains (Table 2), except two strains EMKD31/2 and EMK2/1 (Table 2). *E. mundtii* EMK5/1 was susceptible to all antibiotics. However, enterococci are known to be chromosomally resistant to some antibiotics such as kanamycin, streptomycin (KAN,S). In case of KAN it was seen in six *E. mundtii* strains (Table 2, EMKD31/2, EMKD29/1, EMKD24/1, EMKD13/3, EMKD41/3, and EMK2/1); 12 out of 14 strains *E. mundtii* strains were resistant against streptomycin (S). Ten out of 14 *E. mundtii* strains were clindamycin (DA) resistant. The most resistant was found EMK2/1 strain, resistant against five antibiotics involving also KAN and S. EMKD22/1 was mono-resistant (S). Five strains were resistant against two antibiotics and three strains (EMKD 31/2, EMKD 29/1 and EMKD 24/1) were resistant to 4 antibiotics involving chromosomal resistance to S and KAN. The strains EMKD13/3 and EMKD41/3 were resistant to three ATB; however, two of those resistances were against KAN and S. Regarding our previous study associated with beneficial character of those *E. mundtii* strains [8], *E. mundtii* EMKD41/3 produces antimicrobial substance of proteinaceous character, mundticin-like, and therefore its safety was also assessed using *in vivo* model experiment with hybrid mice Balb/c.

Table 3: *In vivo* safety testing of selected bacteriocin-producing *E. mundtii* strain EMKD41/3 in mice Balb/c.

n=30 Feces	BHI+R	Enterococcci	LAB
0/1	nt	5.05 ± 0.24	5.48 ± 0.34
Control 30	nt	5.87 ± 0.42	6.47 ± 0.54
EM41/3	5.12 ± 0.26	6.10 ± 0.00	6.10 ± 0.00
n=5			
Jejunum			
Control 30	nt	5.63 ± 0.37	6.73 ± 0.45
EM41/3	1.65 ± 0.28	5.70 ± 0.39	6.97 ± 0.64
n=3			
Liver			
Control 30	nt	2.29 ± 0.51	4.25 ± 0.06
EM41/3	1.13 ± 0.00	3.82 ± 0.95	4.03 ± 0.07

Note: 30 mice Balb/c (n=8, mixtures feces), n=5, mixtures of jeunum and n=3, mixtures of liver; 30 means 30 days application, nt-not tested; BHI+R, Brain heart agar enriched with rifampicin to enumerate EMKD41/3 strain. Bacterial count was expressed in colony forming unit per gram (CFU/g) log 10 ± SD, LAB-lactic acid bacteria.

In vivo Safety Control of *Enterococcus mundtii* EM41/3 Using Balb/c Mice Model

No mortality was noted during whole 30 days application of *E. mundtii* EMKD41/3 strain in mice. Its count was high (Table 3) reaching (log 10) 5.12 ± 0.26 CFU/g. Also the total enterococcal count was high (6.10 ± 0.0) CFU/g and lactic acid bacteria count as well (Table 3). Jejunal counts and liver count of strain was lower in comparison with feces, but the most important is that its application did not cause mortality.

Discussion

Enterococci belong to a group of lactic acid bacteria (LAB) with both advantages and harmful aspects. Their advantages are

associated with their beneficial/probiotic character and bacteriocin production [20,7]. Oppositelly, harmful aspects are represented by antibiotic resistance, undesirable enzymes production, biogenic amines production or/and virulence factor genes detection [21]. Enterococci have evolved over ages as vastly amended members of the intestinal microbiota with a wide range of hosts [22]. Among enterococci, also a ubiquitous bacterial species *E. mundtii* has been taxonomically allotted, assigned as a member of the *Enterococcus faecium* group based on homology in 16S rDNA sequence [19,8]. Enterococci belong to the phylum Firmicutes which was found as the major bacterium phylum populating intestine in healthy horses [23]. In spite of the fact, that limited information exists regarding enterococcal community in horses, as mostly detected species were reported *Enterococcus faecium* and *E. faecalis* [24]. However, in our recent study fecal strains of the species *E. mundtii* were detected as dominated and confirmed using next-generation sequencing analysis [8].The species *E. mundtii* has been associated with raw milk, plants, intestinal tract of humans and dairy cattle [22]. In general, this bacterium species has low GC content ranging between 38 and 39%, lack of catalase and cytochrome-C-oxidase enzymes, but it can contribute in carbohydrates fermentation to produce lactic acid [25], and also bacteriocin production (antimicrobial proteinaceous substances) by *E.mundtii* strains species was reported [26,8].

Enterococci can cause problem due to the acquisition of antibiotic resistance determinants. Acquired antibiotic resistance, combined with natural resistance to several classes of antibiotics can increase their pathogenicity [27]. However, *E. mundtii* strains studied were mostly susceptible to antibitotics also regarding their natural (chromosomally coded determinants). Following safety aspects, in our study tested *E. mundtii* showed vancomycin susceptible phenotype, although *van* gene was not analyzed. E. g. Moura et al. [28] detected in Luisitano horses *E. faecium* strains with *van A* gene. Also using different antibiotic resistance testing method [8], fecal *E. mundtii* from horses were mostly susceptible to commercial antibiotics.

Regarding pathogenicity, virulence factors are mostly encoded by, or linked with, transposable genomic elements such as plasmids, IS elements, transposons or phages. Large of these elements are present within the Pathogenicity Associated Islands (PAI) [29]. Biofilm formation ability has recently been suggested to be an important factor. And e.g. gelatinase has been suggested to being involved in the process of biofilm formation, by mediating signals arriving through the quorum sensing *fsr* system [30]. However, our *E. mundtii* were *gelE* gene absent. Biofilm formation and *esp* gene may also exist as single entities as they do in the strain species *E. faecalis*, but they were simultaneously present only in clinical strains *E. faecium*; however, fecal strains *E. mundtii* from horses are absent of this gene. The aggregation substance (agg) on the surface of the host organism has been shown *in vivo* to form large aggregates and hence may contribute to pathogenesis. However, no *agg* gene was detected in tested *E. mundtii* [31]. *Esp* gene is thought to promote adhesion, colonization and evasion of the immune system, and to play some role in antibiotic resistance [27]. Again *E. mundtii* strains were *esp* gene absent, which also indicated their most susceptibility to antibiotics. Hyaluronidase acts on hyaluronic acid and is a degradative enzyme which is associated with tissue damage. This enzyme is encoded by the chromosomal *hyl* gene, of which *E. mundtii* strains tested were absent. Vankerckhoven et al. [32] reported presence of *hyl* gene in only 14% of fecal isolates. Moreover, our *E. mundtii* strains were hemolysis negative [8].

The enzyme β-galactosidase is important because of its activity in the mucosa of the small intestine. Some strains of *E. mundtii* were found to produce this enzyme. On the other hand, β-glucuronidase is enzyme which is associated with some diseases, e.g. as a cancer marker [33]. So, no production by *E. mundtii* strains is also one of parameters avoiding their pathogenicity. This fact was also confirmed by sufficient colonization of bacteriocin-producing EMKD41/3 strain in mice model without no mortality during 30 days application.

Conclusion

Biofilm-forming, multiresistant EMKD 24/1 containing virulence factor gene was found to be the less safe. However, it can be concluded that bacteriocin-producing strains *E. mundtii* could not threaten animals because they were mostly susceptible to antibiotics, virulence factor genes absent, no producing damaging enzymes, and with no or only low-grade biofilm formation ability. Moreover, EMKD 41/3 strain in model experiment with Balb/c mice did not cause mortality and its counts in feces were high to allow its beneficial effect; its count in jejunum and liver reached up to 10^2 CFU/g which can indicate its sufficient implementation in host organism with any side effect, e.g. as diet supplement or post biotic.

Availability of Data and Materials

The data sets used during the study are available from the corresponding author on request.

Ethical Approval

Sampling was approved by the Ethical Commission of University of Veterinary Medicine and Pharmacy (APVV 16-0203) and by a veterinarian. Animal handling follows the Clinical Guidelines for Veterinarians Treating the Performance Horse (2017) by American Association of Equine Practise.

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Authors`Contributions

V.F. Investigation, Methodology, Draft Writing; E.S. Resources; MP.S.methodology; M.V. Methodology; E.D. Methodology;A.L.Conceptualization, Investigation, Data Curation, Writing, project administration. All authors have agreed with the final version of the manuscript and its submission.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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