JSM Veterinary Medicine and Research

Research Article

Enterococcus saccharolyticus Es 3/11 D27 Isolated from Horses and its Postbiotic Activity

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Abstract

The species *Enterococcus saccharolyticus* is rarely occurred and identified. No information exists about this species strain bacteriocin/bioactive/postbiotic potential which could be utilized e.g. for bacterial prevention/protection. Standard microbiological methods as well as PCR analyses were used to basic characterization the strain 3/11D27. To test bacteriocin/postbiotic potential, agar spot test was conducted and 158 indicator bacteria (147 Gram-positive and 11 Gram-negative). The strain *E. saccharolyticus* Es 3/11D27 was isolated from the inner part of the auricle mucosa of clinically healthy mare (Slovak breed Norik from Muráň) using sequence analysis. After sequencing, the strain was involved in GenBank with accession number MN822909. This strain has produced bacteriocin substance with inhibitory potential against Gram-positive and Gram-negative indicator bacteria non- depending on the indicator bacteria species. The inhibitory activity reached 100 up to 800 AU/ml. Es 3/11D27 has been also susceptible to antibiotics tested. It has been hemolysis-, deoxyribonuclease- negative with absence of virulence factors genes. Low-grade biofilm-forming ability using the plate assay was found in Es 3/11D27. It is first time presenting horses-derived species strain *E. saccharolyticus* with bacteriocin activity. Additional studies will be processed to determine detail characteristic of bacteriocin substance produced by the strain Es 3/11D27 with the aim for its further application potential in horses breeding.

Keyworks: Horse; Mucosa; *Enterococcus*; Postbiotic; Activity

Introduction

The genus *Enterococcus* belongs to the family Enterococcacae, order Bacilli, class Lactobacilalles, and phylum Firmicutes [1]. Up to now, about 61 enterococcal species have been validly described and/or validated [2-4]. *Enterococcus saccharolyticus* has been detected previously in various animals and environmental samples [5]. The type strain *E. saccharolyticus*812T was isolated firstly from fresh broccoli. It was identified based on 16S rRNA gene sequences [6]. Moreover, two subspecies were distinguished, *E. saccharolyticus* subsp. *saccharolyticus* and *E. saccharolyticus* subsp. *taiwansis*.

The intestinal microbiota has enormous impact on the health and performance of horses; there are also the other parts of horses inhabiting with enterococci which are in quite limited information regarding it. In literature can be found information associated with mucosal/skin microbiota in horses. More recently, skin microbiota of horses have been investigated in various contexts, using Next-Generation DNA Sequencing (NGS) [7-9]. Kamus LJ, et al. [7] and Ross AA, et al. [8] reported bacteria of the phylum Acidobacteria and the genus *Corynebacterium* (phylum Actinobacteria), respectively, as the most common bacteria found on healthy skin of horses. Furthermore, the species *Staphylococcus aureus* belonging in the phylum Firmicutes is also a part of the normal skin microbiota, but probably occurs in low numbers [10]. Regarding the individual bacterial strains and their properties, most information associated with cutaneous aspect of horses can be found about staphylococci [11]. In some horses-derived enterococci, bacteriocin-producing potential was found, meaning production of antimicrobial proteinaceous substances with inhibitory activity against less or more related bacteria [12-14]. Regarding the new horizon of bioactivity, bacteriocins have been belonged among postbiotics [15].They can be defined as non-viable bacterial products or metabolic products from microorganisms that have biological activity in the host [16]. In this study, *in vitro* antimicrobial potential of bacteriocin substance produced by the strain *E. saccharolyticus* Es 3/11 D27 has been studied. The strain was isolated from the inner part of the auricle mucosa of clinically healthy mare -Slovak Norik from Muráň breed, cold-blood breed of horse. It is first time to study bacteriocin activity from horses-derived species strain *E. saccharolyticus.*

Prior bacteriocin activity control in Es 3/11 D27 strain, its basic characteristics were assessed by testing hemolysis, deoxyribonuclease and gelatinase activity, enzymatic characteristics, virulence factor genes detection, biofilm-forming ability, and antagonistic activity. After these analyses, inhibitory postbiotic potential of Es 3/11D27 was assayed for further possible bacteria reducing potential in horses, staphylococcal cases involving.

Materials and Methods

Handling with animals was approved by veterinarian following the Clinical Guidlines for Veterinarians treating the Performance Horse [17] by American Association of Equine Practise. To isolate individual bacteria, skin and mucosal swab was sampled (Amies agar gel without charcoal, Copan, Italy) from the inner part of the auricle mucosa of clinically healthy mare of Norik from Muráň breed. Animal was kept 24/7 on the pasture in the National Park Muráňska planina or fed hay or oats. The swab

Submitted: 30 January 2024 | **Accepted:** 14 February 2024 | **Published:** 17 February 2024

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Citation: Lauková A, Styková E,Focková V, Maďar M (2024) *Enterococcus saccharolyticus* Es 3/11 D27 Isolated from Horses and its Postbiotic Activity. JSM Vet Med Res 3: 6.

was placed into one ml of Ringer solution (pH 7.0, Merck, Darmstadt, Germany). Sample was treated according to the standard microbiological method (ISO, dilutions 1:9). The aliquot (0.1 ml) from the appropriate dilution was spread onto De Man-Rogosa-Sharpe agar (MRS, pH 6.4; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) to select lactic acid bacteria. The agar plate was cultivated under partially anaerobic condition (Gas Pak Plus, BBL, Microbiology Systems, Cockeysville, USA) at 37 °C for 48 h. The morphologically different colonies were differed based on Gram-positive staining and coccoid shape. Isolate was stored using the system Microbank™ (Pro-Lab Diagnostics, Ontario, Canada) at −70 °C for next analysing.

The genomic DNA was extracted from pure colony by using DNAzol direct (Molecular Research Center Inc., Cincinnati, USA) according to the manufacturer's recommendation as previously reported by Focková V, et al. [14]. The 16S ribosomal RNA (rRNA) gene from isolated colony was amplified by PCR using the universal primers as follows: Bac27F(5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-CGGYTACCTTGTTACGACTT-3 (Merck-Sigma Aldrich, Darmstadt, Germany). PCR reaction was carried out in a 50 µl PCR mixture containing 2 µl of DNA shield and 46 µl of a reaction mixture comprising One Taq 2x Master Mix with Standard Buffer (New England Biolabs, the United Kingdom), diluted with water for molecular biology (PanReac AppliChem, Darmstadt, Germany) to 1x concentration and 1 µl of each primer (concentration 33 µm). The following PCR condition (thermocycler-TProfesional Basic, Biometra GmbH, Göttingen, Germany) was used: 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and primer extension at 72°C for 3 min. Then final processing at 72°C for 10 min. Aliquot PCR product was separated by horizontal 3% (w/v) agarose gel electrophoresis in Tris-acetate-EDTA buffer (pH 7.8) and visualized with GelRed (Biotium, Inc., Hayward, CA, USA). Amplified product was supplied in low bind tube at minimal volume 15 µl for purification and sequencing in both directions using 1492R and Bac27F primers (Microsynth, Wien, Austria). The detected 16S rRNA sequence was validated, assembled by Geneious 8.0.5 (Biomatters, Auckland, New Zealand), and subjected to BLASTn analysis (https://BLAST.ncbi.nlm.nih.gov/BLAST.cgi).

To evaluate susceptibility to antibiotics of the strain Es 3/11D27 strain, disk diffusion method was used according to CLSI [18]. EUCAST evaluation was provided using E strips with concentration of antibiotics ranging from 0.015 µg/ml up to 256 µg/ml, and 0.064-1024 µg/ml for streptomycin and gentamicin. E strips with following antibiotics were tested: chloramphenicol, gentamicin, rifampicin, erythromycin, penicillin, and streptomycin. Strips were applied on Mueller-Hinton agar seeded with 100 µl of overnight broth culture (in Brain heart infusion/broth, Difco, USA) of tested strain. After 18h of cultivation MIC-minimal inhibitory concentration was evaluated (µg). Disk diffusion method was performed with the use of Mueller-Hinton agar and the appropriate disks application such as ampicillin, tobramycin, neomycin (each 10µg), penicillin (10IU), erythromycin, azithromycin (each 15 µg), streptomycin (25µg), tetracycline, chloramphenicol, rifampicin, kanamycin, vancomycin (30µg), and gentamicin (120µg). Antibiotic disks were selected according to the EUCAST panel recommendation. *E. faecium* CCM 4231 was positive control [19].

To assess character of the strain, basic parameters were tested such as hemolysis, gelatinase, and deoxyribonuclease activity as previously reported by Focková et al. [14], Semedo-Lemsaddek et al. [20] using Brain heart agar (Oxoid, Basingstoke, the United Kingdom) with defibrinated sheep blood, DNase agar and TH agar (Difco, Detroit, USA) supplemented with 30 g of gelatin (Oxoid). The positive control strain *S. pseudintermedius* SPs 948 was applied.

The strain *E. saccharolyticus* Es 3/11D27 was controlled for its enzymatic characteristics by using the commercial API-ZYM kit (BioMerieux, France) according to the manufacturers instruction. The enzymes involved in the kit were alkalic phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucin arylamidase, valin arylamidase, cystin arylamidase, trypsin, α-chymotrypsin, acidic phosphatase, naftol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Bacterial cultures (65 µl) with concentration of recommended appropriate McFarland standard were pipetted into each well of kit. Enzymatic profile was evaluated after 4 h of incubation at 37 ºC after the supplementation of ZYM A and ZYM B reagents. Color intensity values from 0 to 5 and their relevant value in nanomoles were assigned for each reaction according to the color chart supplied with the kit [14].

The ability of *E. saccharolyticus* Es 3/11D27 strain to form biofilm was tested by the qualitative and quantitative methods. The qualitative method lies in the strain growth characterization on Congo red agar according to Freeman DJ, et al. [21]. The basic medium was composed of Brain-heart infusion (Difco, Michigan, USA, 37 g/l) supplemented with sucrose (36 g/l), pure agar (30 g/l) and Congo red dye (0.8 g/l, Merck, Germany). The tested Es 3/11D27 strain was inoculated on Congo red agar and incubated at 37°C for 24 hours. Biofilm-forming growth was demonstrated by black colonies with a dry crystalline consistency. Non-biofilm forming strain growth usually remained pink. The colour was repeatedly checked after 48 and 72 hours.

The quantitative method lies in the use of a microtiter plate according to Chaieb K, et al. [22] and/or Slížová M, et al. [23]. The appropriate amount-loop of the tested strain grown overnight at 37°C on Brain heart agar (BHA, Becton and Dickinson, USA) was transferred into 5 ml of Ringer solution (pH 7.0, 0.75% w/v) to reach the McFarland standard 0.5 suspension. The concentration of suspension corresponds with 1 x 10^8 CFU/ml. A volume 100 µl was transferred into 10 ml of BHI broth. The standardized culture (200 µl) was inoculated in a polystyrene microtiter plate well (Greiner ELISA 12 Well Strips, 350 µl, flat bottom, Frickenhausen GmbH, Germany) and incubated at 37°C for 24 h. The biofilm formed in the well of the microtiter plate was washed twice with 200 µl of deionized water and dried at laboratory temperature for 30 min. The remaining attached bacteria were stained at 25°C for half hour with 200 µl of 0.1 % (m/v) crystal violet in deionized water. The dye solution was aspirated away. The wells were washed twice with 200 µl of deionized water and kept to dry at laboratory temperature for half hour. The dye bound to the adherent biofilm was extracted with 200 µl of 95% ethanol and stirred. A 150 µl aliquot was transferred from each well and placed in a new microtiter plate well for absorbance (A570 nm) determination using a Synergy TM4 Multi Mode Microplate reader (Biotek USA). Es 3/11D27 strain and condition was tested in two independent tests with 12 replicates. Sterile medium was included in analysis as negative control. *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). Biofilm formation was classified as highly- positive (A570 ≥1), low-grade positive (0.1 ≤ A570 < 0.1) and negative (A570 < 0.1; Chaieb K, et al. [22] and/or Slížová M, et al. [23].

DNA from *E. saccharolyticus* Es 3/11D27 was extracted using the rapid alkaline lysis method [24]. Analysis was performed using the individual primers [25] and thermocycler C1000™ (BioRad Laboratories, Hercules, USA). The presence of 8 (*gelE* -gelatinase, *esp* -enterococcal surface protein, *efaAfm* -*E. faecium* adhesin, *cylA*-cytolysin A, *hylEfm*-hyaluronidase, *agg*-aggregating substance, *efaAfs* -cell wall adhesins, and element IS16) virulence factor genes was analyzed using the PCR method as previously described by Kubašová I, et al. [25]. The PCR products were separated by electrophoresis on an agarose gel (1.2% w/v Sigma-Aldrich, Saint Louis, USA) containing 1 µl/ml ethidium bromide (Sigma-Aldrich, Germany) using 0.5 x TAE buffer (Merck, Darmstadt, Germany) as previously reported by Kubašová I, et al. [25]. The PCR fragments were visualized with UV light. The PCRs were carried

out in a volume of 25 µl with a mixture consisting of 1x reaction buffer, 0.2 mmol/l deoxynucleoside triphosphate, 3 mmol MgC_{12}, 1 µmol/l of each primer, 1 U of Taq DNA polymerase, and 1.5 µl DNA template [25]. The cycling conditions were as follows (except for IS16 and hylEfm): initial step of 95°C for 3 min, 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, followed by an additional step at 72°C for 5 min. Condition for amplification of IS16 was as follows: 94°C for 4 min, 30 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C, followed by 72°C 4 min [25]. For the *hylEfm* gene, the following condition was applied: 94°C for 4 min; 94°C for 30 s, 50°C for 30 s, 72°C for 30 s (30 cycles), and 72°C for 4 min. The strains *E. faecalis* P36 and *E. faecium* F10 (provided by Dr. T. Semedo-Lemsaddek, University of Lisbon, Portugal) and *E. faecium* UW 9086 (provided by Dr. I. Klare, Robert Koch Institute in Germany) were used as positive control in genes detection. *E. faecium* UW 9086 was control in case of hyaluronidase gene, F10 strain for *IS16* gene and the strain P36 was control for the other tested genes. Oligonucleotides used in this study to amplify virulence factor genes in the strain *E. saccharolyticus* Es 3/11D27 were used as reported previously by Kubašová I, et al. [25] with the procedures according to Eaton TJ, et al. [26], Semedo LT, et al. [20], Werner G, et al. [27], and Klare I, et al. [28].

Antagonistic activity analysis was performed using disk diffusion method and inhibitory zones were expressed in mm. Supernatant of the strain Es 3/11D27 **(**GenBank accession number MN822909) was applied on disk which was placed on the surface of agar (1.5 = w/v). Then it was overlaid with 0.7% agar seeded with indicator strain (200 µl). The clear inhibitory zones were measured (in average) and expressed in mm. The involved indicators as the frequent contaminant species in horses were supplied by the culture collection: *E. coli* O149 F4, *Proteus mirabilis* CCM 7188, *Bacillus cereus*, and *Staphylococcus aureus*. The principal indicator strain (faecal strain from piglet, our laboratory) was tested (*Enterococcus avium* EA5). Testing was provided in duplicate.

Concentrated bacteriocin substance (CBS) was prepared from 18 h culture of Es 3/11D27 **(**GenBank accession number MN822909 strain) (60 ml, A_{600}=0.889) in Brain heart infusion (BHI) (Difco). Broth culture was centrifuged at 10,000 x g at 4°C for 30 min. The pH was adjusted to be 4.5. The cell-free supernatant was treated by addition of EDTA/Chelaton III (Sigma, Germany) and heated at 80°C for 10 min to eliminate effect of other organic substances, e.g. lactic acid). Concentrated substance (final volume 6 ml) was prepared by the supernatant (pH 4.5) to concentrate it by the use of Concentrator Plus (Eppendorf, Hamburg, Germany). The inhibitory activity of CBS was expressed in arbitrary unit per ml (AU/ml). It was analyzed using agar spot test [29] against the target of 158 indicator bacterial species strains (147 Gram-positive bacteria and 11 Gram-negative bacteria, table 1-4). Gram-positive indicator bacterial species involved enterococci (60 strains), staphylococci (64 strains), streptococci (9), lactococci (7), and lactobacilli (7) as follows: the principle (the most susceptible) indicator *Enterococcus avium* EA5 (our strain), 13 faecal horses strains *E. mundtii* (isolated from horses of Norik from Muráň breed, table 1), faecal strains *E. faecium* from poultry-resistant to aminoglycosides, *E. faecalis* isolated from canine faeces (11) and *E. faecalis* from faeces of cat (26, kindly supplied by Dr. Aleksandra Troscianczyk from University of Life Sciences in Lublin, Poland), 6 clinical strains of *Streptococcus pneumoniae* and 2 strains of *Str. pyogenes* from children *otitis media* [30], *Str. equi* subsp. *zooepidemicus* CCM 7316 (kindly provided by Dr. Eva Styková, UVMP in Košice), ruminal strain *Staphylococcus pseudintermedius* SPs 948 (our strain), 16 faecal strains of *Staphylococcus felis* from cat, methicillin-resistant human faecal strain of *S. aureus* from pigs (39, provided by Dr. Troscianczyk, Lublin, Poland), *S. schleiferi* KNSP3 isolated from the meat product (our strain) and *S. arlettae* SArl 44 from raw goat milk (our strain). The other staphylococcal species such as S. *arlettae* AB354, *S. sciuri* AB385, *S. caninintestini* AB372, *S. vitulinus* AB381, *S. simulans* AB383 and *S. haemolyticus* AB376 isolated from cow faeces were again from the collection of Dr. A. Troscianczyk. Moreover, 7 strains of *Lactococcus* spp. and 7 strains of *Lactobacillus* spp. from raw goat milk (our strains) were used as indicators. As formerly mentioned, in addition, 11 faecal Gram-negative species strains isolated from horses and roe deer were involved such as *Acinetobacter johnsonii* K17/PL2, *Ac. lwofii* ACl K8/3, *Acinetobacter* sp. K10PL/2, *Serratia liquefaciens* K2PL/1, *Citrobacter freundii* K10PL/2, *Pantoea agglomerans* PATK4/2, *Yersinia enterocolytica* 12/111/2, and 5 strains of *Escherichia coli* (Ec 12/111/1, Ec11/91/1, Ec 12/111/1, Ec 10/116/, and Ec10/139/2). Stability of concentrated substance (CBS) was tested against EA5 strain after 3 and half monthes storage at -20°C.

Table 1: Inhibitory activity of concentrated bacteriocin substance of *E. saccharolyticus* Es 3/11 D27 against enterococci (expressed in Arbitrary units per ml, AU/ml).

Indicator	T/I	Inhibitory Activity
E. avium EA5	1/1	800 AU/ml
E. mundtii	13/13	200-400 AU/ml
E. faecalis, cats	26/26	100-200 AU/ml
E. faecalis, dogs	11/11	100 AU/ml
E. faecium, poultry	9/9	100-200 AU/ml

EA5-*Enterococcus avium*, the prinicipal- the most susceptible indicator (isolated from piglets faeces, our strain; EM-*E. mundtii* (isolated from faeces of horses, our strains, Focková V, et al. [14] *E. faecalis,* cats; *E. faecalis*, dogs, *E. faecium* poultry, strains kindly supplied from Dr. Aleksandra Troscianczyk, University of Life Sciences in Lublin, Poland, T/I, number of tested strains/number of inhibited strains.

Results

Pure enterococcal isolate (controlled by Gram staining) was analyzed by using BLASTn analysis. Based on this analysis, pure strain was allotted to the species *E. saccharolyticus* subsp. *saccharolyticus*. The percentage identity of BLASTn 16S rRNA sequence for the strain reached up to 100% (99.86% similarity was detected with the strain *E. saccharolyticus* subsp. *saccharolyticus* MK330588.1). The strain Es 3/11D27 has been provided with GenBank accession number (AN) MN822909. This strain is deoxyribonuclease- negative, with negative hemolysis test (ϫ- hemolysis). It showed gelatinase negative phenotype.

E. saccharolyticus Es 3/11D27 (AN) MN822909 was susceptible to antibiotics tested using disk diffusion method. Evaluated diameter of inhibitory zone for ampicillin reached 14 mm; 15 mm zone sized was measured for tobramycin, and neomycin. Inhibitory size zone for penicillin measured 23 mm in diameter, for erythromycin and streptomycin 25 mm; 21 mm for azithromycin. In case of tetracycline was evaluated size zone 19 mm and 20 mm for chloramphenicol and rifampicin. The 15 mm susceptibility inhibitory zone was measured for kanamycin, 19 mm for vancomycin, and 23 mm for gentamicin. This means that Es 3/11D27 strain was susceptible to antibiotics mentioned. E test-strip method also evaluated Es 3/11D27 strain as susceptible reaching MIC (minimal inhibitory concentration) 3 µg for chloramphenicol. MIC for gentamicin was 2 µg, for rifampicin 0.064 µg, for erythromycin 0.12 µg, and for penicillin as well. MIC = 24 µg was measured in case of streptomycin which indicates upper limit. However, E strip method again confirmed susceptibility to antibiotics of Es 3/11D27.

In case of enzymatic activity using API ZYM kit in Es 3/11D27 strain 5 nmol was measured for lipase, acidic phosphatase and β-galactosidase. The other enzymes activities were negative. Using the qualitative method to test biofilm-forming ability of Es 3/11D27 strain (AN MN822909) on Congo red agar, its growth was evaluated as crystalline black colonies forming after 24 h, 48 h and 72 h meaning the strain was biofilm-forming. It was also confirmed comparing with positive control strain *Str. equi* subsp. *zooepidemicus* CCM 7316. Moreover, the quantitative plate assay

assessed Es 3/11D27 strain as low-grade biofilm-forming (0.410 ± 0.64). Positive control strain reached 0.260 ± 0.70. *E. saccharolyticus* Es 3/11D27 was virulence factor genes absent.

Evaluating antagonistic acitivity, growth of indicator strain *E. coli* 0149 F4 was inhibited with inhibitory zone in diameter size 13 mm ± 2.0 mm. Almost the same diameter size was measured in case of indicator strain *Proteus mirabilis* CCM 7188 (13.33 ± 3.77 mm) and *Bacillus cereus* (13.67 ± 1.15 mm). The growth of *Staphylococcus aureus* CCM 4223 was not inhibited. However, the strain *Enterococcus avium* EA5 was inhibited with diameter size inhibitory zone 11 mm. Summarizing by this test, the growth of 2 Gram-negative strains was inhibited and growth of 2 out of 3 Gram-positive strains was inhibited. One strain was resistant.

However, concentrated bacteriocin substance (CBS) was active against *E. avium* EA5 strain reaching inhibitory activity 800 AU/ml (table 1). The growth of 13 faecal horses strains *E. mundtii* was inhibited with inhibitory activity ranging from 200 to 400 AU/ml (table 1). As could be seen from table 1, also growth of the other enterococcal strains was inhibited after treatment with CBs ES 3/11D27, meaning that faecal indicator *E. faecalis* from cats and dogs and *E. faecium* from poultry as well, alltogether 60 strains (100%). Inhibitory activity reached up to 400 AU/ml (except EA5 strain, when 800 AU/ml was reached). Among 64 staphylococcal strains, growth of 62 was inhibited (table 2) which means 96.8% although reaching only 100 AU/ml. Moreover, growth of streptococci tested was also inhibited (table 2) with inhibitory activity up to 400 AU/ml. The growth of indicator lactococci was inhibited with activity up to 400 AU/ml and lactobacilli were inhibited with the activity up to 400 AU/ml as well (table 3). Moreover, 11 Gram-negative species strains were also inhibited with the inhibitory activity mostly 100 AU/ml (table 4). *E. coli* were also inhibited (100 AU/ml). Among Gram-negative indicators, the strain *Pantoea agglomerans* PATK4/2 was the most susceptible (400 AU/ml). The CBS was active with the same activity against EA5 strain after 3 and half monthes storage at -20°C (800 AU/ml).

Table 2: Inhibitory activity of concentrated bacteriocin substance of *E. saccharolyticus* Es 3/11 D27 against streptococci and staphylococci (expressed in Arbitrary units per ml, AU/ml).

Indicator	T/I	Inhibitory Activity
CCM 7316	1/1	200 AU/ml
Str. pneumoniae	6/6	100-400 AU/ml
Str. pyogenes	2/2	200-400 AU/ml
S. arlettae	2/2	100 AU/ml
S. schleiferi	1/1	100 AU/ml
S. pseudintermedius	1/1	100 AU/ml
S. sciuri	1/1	100 AU/ml
S. caninintestini	1/1	100 AU/ml
S. vitulinus	1/1	100 AU/ml
S. simulans	1/1	100 AU/ml
S. haemolyticus	1/1	100 AU/ml
S. felis	16/15	100 AU/ml
S. aureus	39/38	100 AU/ml

CCM, Czech Culture Collection in Brno, Czech Republik- *Streptococcus equi* subsp. *zooepidemicus*, provided by Dr. Styková Eva from University of Veterinary Medicine and Pharmacy in Košice, Slovakia; Sp., *Streptococcus pyogenes*, Spn, *S. pneumoniae*, (Kandričáková A, et al.) [36] from children *otitis media; S. arlettae* SArl 44 from raw goat milk; *S. schleiferi* KNSP3 from the product Spišské párky; ruminal strain *S. pseudintermedius* SPs 948; The other stapyhlococci were kindly supplied by Dr. Aleksandra Troscianczyk from University of Life Sciences in Lublin, Poland, T/I, number of tested strains/number of inhibited strains.

Table 3: Inhibitory activity of concentrated bacteriocin substance of *E. saccharolyticus* Es 3/11 D27 against lactococci and lactobacilli (expressed in Arbitrary units per ml, AU/ml).

Indicator	Inhibitory Activity
Lactococcus spp.	
MK2/1/23	400 AU/ml
MK2/2/23	400 AU/ml
MK2/3/23	400 AU/ml
MK2/4/23	100 AU/ml
MK2/6/23	400 AU/ml
MK2/7/23	400 AU/ml
MK2/8/23	400 AU/ml
Lactobacillus spp.	
MK2/9/23	200 AU/ml
MK2/10/23	200 AU/ml
MK2/11/23	400 AU/ml
MK2/12/23	400 AU/ml
MK2/13/23	200 AU/ml
MK2/14/23	200 AU/ml
MK2/ 16/23	100 AU/ml

Lactococci and lactobacilli from raw goat milk.

Table 4: Inhibitory activity of concentrated substance produced by *E. saccharolyticus* Es 3/11 D27 against faecal Gram-negative bacterial species strains from roe dear and horses (expressed in arbitrary unit per milliliter, AU/ml).

Indicator	Inhibitory Activity
Ac.j. K17/PL2	100 AU/ml
Ac. lwofii K8/3	200 AU/ml
*S. lq.*K2PL/1	200 AU/ml
Ac. sp.K10PL/2	100 AU/ml
*Pnt. aggl.*PATK4/2	400 AU/ml
Ec12/111/2	100 AU/ml
Ec 11/91/1	100 AU/ml
Ec 12/111/1	100 AU/ml
Ec 10/116/2	100 AU/ml
Ec 10/139/2	100 AU/ml
*Y.ent.*12/111/2	100 AU/ml

Acinetobacter johnsonii K17/PL2, *Ac. lwofii* ACl K8/3, *Serratia liquefaciens* K2PL/1, *Citrobacter freundii* K10PL/2, *Pantoea agglomerans* PATK4/2, *Yersinia enterocolytica* 12/111/2, *Serratia font* 11/91/1, *Escherichia coli* 12/111/1, *E. coli* 10/116,/2, *E. coli* 10/139/2.

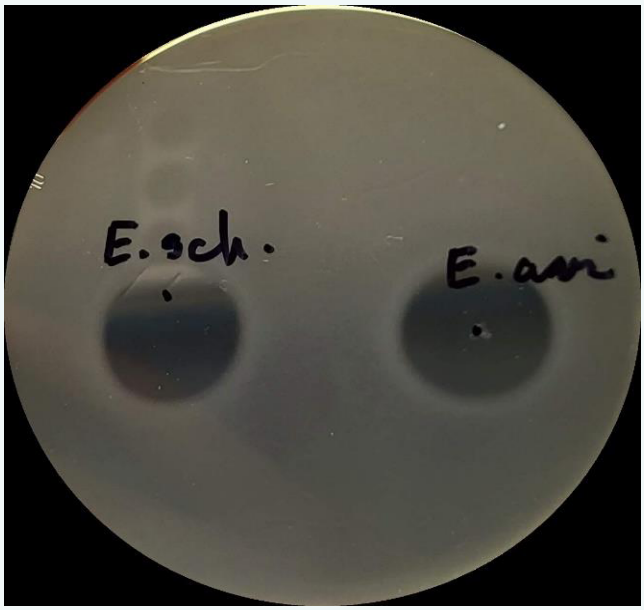

Figure 1: Basic testing (not diluted) of concentrated bacteriocin substance produced by *Enterococcus saccharolyticus* Es 3/11D27 against indicator *Enterococcus avium* EA5. This basic (not diluted substance inhibition) was 100 AU/ml

Discussion

The species *E. saccharolyticus* was isolated from various animal and environmental samples [5]. However, as reported by Layton BA, et al. [5], the species *E. saccharolyticus* was not detected in horses samples but it was found in sewage samples. It could be supposed source of contamination. Enterococci have a genome plasticity and metabolic versatility. These properties enable them to thrive in many divers environments [31].

Es 3/11D27strain was susceptible to antibiotics. Zaheener R, et al. [32] reported in case of One- Health investigation of antimicrobial resistance in *Enterococcus* spp. from various sources prevalent tetracycline and erythromycin resistance. Because of antibiotic susceptibility of Es 3/11D27 it indicates that it should not threaten host organism. Oppositely, its ability to produce inhibitory/bacteriocin activity, indicates its further possible application and/or its bacteriocin substance in horses/animals. In this species, especially in horses-derived strain, bacteriocin activity has not been described yet. Moreover, CBS-concentrated substance of Es 3/11D27 showed a broad antimicrobial spectrum inhibiting the growth of Gram-positive bacteria and Gram-negative contaminants as well. It seems that this a broad antimicrobial spectrum of CBS produced by *E. saccharolyticus* Es3/11D27 could be usefully applied e.g. against non requested microbiome representatives in horses as they were detected by sequencing in aged horses [33]. There were detected genera as *Escherichia-Shigella*, *Mogibacterium* or *Blautia* and others. Previously, more beneficial/probiotic strains were reported to be effective in GIT of horses [34-36]. However, especially those beneficial strains producing bacteriocins [35-37]. Lauková A, et al. [35] reported effect of non-autochthonous strain *E. faecium* AL41=CCM8558 which produces Ent M to demonstrate inhibitory activity against aeromonads by their siginificant reduction ($p < 0.001$). Increased tendency in phagocytic activity (PA) was also noted and hydrolytic activity was significantly increased ($p < 0.001$). Biochemical parameters were influenced in physiological range. When autochthonous *E. faecium* EF 412 was applied in horses in freeze dried form [36], using NGS the phyla Bacteroidetes and Firmicutes were dominated. PA was increased and biochemical profile similarly as in previous case was influenced in physiological range. Microbiota were not reduced. However, when Ent M was applied (produced by AL41=CCM8558 strain) at day 21, coliforms and campylobacters were significantly reduced ($p < 0.05$) and also clostridiae ($p < 0.001$). Moreover, again increase of PA was noted [13] without negative effect on biochemical parameters and hydrolytic activity. In addition, Lauková A, et al. [38,39] reported *in vitro* inhibitory activity of enterocins (postbiotics) against *S. xylosus* from horses as well as against Gram-negative bacteria isolated from horses. It shows importance and promising potential of bacteriocins, their producers in this aspect. Although CBS from Es 3/11D27 has not been detaily studied up to now; in general, bacteriocins produced by enterococci can be classified as thermo-stable substances with a broad inhibitory/antimicrobial spectrum remaining their activity for long storage period [12].

Results from basic characteristics evaluated (virulence factor genes absence, hemolysis negative, etc.) indicate that Es 3/11D27 has been assessed as safe strain. Bacteriocin activity of Es 3/11D27 represents its beneficial property which could be used in reducing potential against other especially to antibiotic resistant bacterial strains. As formerly indicated, in spite of the several enterococcal probiotic/beneficial and bacteriocins application studies in horses, there is still limited information about this benefit among public. Moreover, it is first time to indicate this benefit in *E. saccharolyticus* species strain.

Conclusion

It can be concluded, the strain *E. saccharolyticus* Es 3/11D27 (AN MN822909) isolated from the inner part of the auricle mucosa of clinically healthy mare (Slovak Norik from Muráň breed) showed bacteriocin/postbiotic potential against Gram-positive and Gram-negative bacteria non-depending on the indicators species. CBS remained its postbiotic potential also after storage at -20°C. It is first time presenting horses-derived *E. saccharolyticus* species strain with bacteriocin/postbiotic activity. Additional studies will be processed to determine detail characteristic of the bacteriocin substance and also its application potential in horses breeding.

Acknowledgment

The authors would like to thank Mrs. Dana Melišová for her laboratory skills. This study was supported by the Slovak Academy of Sciences Agency, Doktogrant APP0253 (2022), Slovak-Polish joint project SK-PL (2019-2022), and the Slovak Research and Development Agency under the contract No. APVV-16-0203.

Author Contributions

A.L.Conceptualization, Investigation, Data Curation, Writing; Project Administration; E.S. Resources,Project Administration, Methodology; V.F. Methodology; M.M. Methodology; All authors have approved the final manuscript.

Ethics Approval and Consent to Participate

Samples were collected from live animals by veterinarians and/or responsible persons involving in the project. They were analyzed at both institutions also for diagnostic purposes and they were consistent with procedures approved by the relevant Ethics Committee (Project Number APVV-16-0203).

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