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Postbiotic Activity of *Enterococcus asini* EAs 1/11D27 Strain Originating from the Norik of Muráň Breed

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Abstract

The Norik of Muráň breed is a Slovak horse breed mainly intended for forestry and agriculture-related work. It is also a unique type among the cold-blooded horse breeds. In general, the microbiota influences animal health status. However, limited information regarding the microbiota of this horse species is available. Similarly, few aspects are known about the species *Enterococcus asini* bacteriocin (postbiotic) potential. Therefore, this study investigated the *E. asini* strain EAs 1/11D27, isolated from mucosal samples obtained from horses, along with the evaluation of its molecular characteristics and bacteriocin (postbiotic) activity. Taxonomic allocation of the strain EAs 1/11/D27 was confirmed using the sequencing method, reaching 99.86% similarity (match) with the nucleotide sequence of the strain *E. asini* NR113929.1. *E. asini* EAs 1/11D27 has been assigned the GenBank accession number (AN) MN822908. This strain is hemolysis-negative, deoxyribonuclease-negative, and gelatinase-negative. EAs 1/11D27 lacks genes encoding for virulence factors such as gelatinase, enterococcal surface protein, adhesins, hyaluronidase, and aggregation substance. It does not produce damaging enzymes and is susceptible to antibiotics. Additionally, it produces bacteriocin-like substances with inhibitory activity against 165 out of 170 indicator bacteria tested (97%). The highest inhibitory potential was recorded against staphylococci (88), enterococci (57), lactococci (7), and streptococci (4/8). The growth of 9 out of 10 Gram-negative strains was also inhibited. These results suggest a valuable postbiotic potential of the studied bacteriocin substance, and further studies are needed to establish its applications in horses.

Keywords

Equine; Norik of Muráň breed; postbiotic; antimicrobial effect; *Enterococcus asini*

1. Introduction

The Norik of Muráň breed is a unique cold-blood horse that resulted from original cold-blooded mares of different genetic backgrounds that were bred by Norik stud horses in Slovakia [1]. This breed is primarily used for work in forestry and agriculture due to its good temperament and excellent constitution. Additionally, it is frequently utilized for hippotherapy and recreational riding [2]. The breeding locations for this breed are Veľká Lúka and Dobšiná in the National Park Muráň Plain, located in the central Slovakia region encompassing the districts of Brezno, Revúca, and Rimavská Sobota. The National Park Muráň Plain is a part of the Spiš-Gemer Karst within the Slovenské rudohorie (Slovak Ore Mountains).

Previous studies have indicated the influence of microbiota on the overall health status, particularly in horses, where the

intestinal microbiota significantly impacts equine health and performance [3]. Along with traditionally confirmed common bacteria, gut microbiota dysbiosis is increasingly associated with diseases [4]. Horses are susceptible to various disorders, many of which have a bacterial etiology and manifest as gastrointestinal clinical signs [5]. To optimize the microbial status and preserve normal microbiota in horses, the use of beneficial bacteria-probiotics and their antimicrobial substances-bacteriocins-postbiotics could be an important approach [6,7]. Lactic acid bacteria (LAB) are among the frequently used beneficial bacteria [8]. Enterococci have been allotted as a part of LAB. Nowadays, the genus *Enterococcus* comprises approximately 61 validated species. Individual strain species as representatives of the genus *Enterococcus* have been found to have beneficial/probiotic properties [9] and the ability to produce bacteriocins-enterocins [10–12]. Bacteriocins-enterocins are substances of proteinaceous character produced by mostly enterococcal species with antimicrobial effects against more or less related bacterial species [9]. Production of enterocins by horses strains has been already reported in our previous studies [13,14]. These strains produce enterocins which have demonstrated beneficial effects under *in vivo* conditions in horses [13,14]. They have shown a reduction in Gram-negative bacteria and an increase in non-specific immunity parameters, specifically phagocytic activity [13,14]. These bacteriocin substances align with the concept of postbiotics [7], which refers to non-viable bacterial products produced by individual bacterial strain species that have biological activity in the host [8].

Among enterococci, *E. faecium* strains have been identified as the most frequently enterocin producers [9–11]. However, strains of *E. mundtii* isolated from Norik of Muráň horses [12], as well as strains from the skin mucosa, such as *E. moraviensis* [15], have also been found to produce enterocins. The species strain *E. asini* was first isolated and described from caecal samples obtained from donkeys (*Equus asinus*) [16]. However, the bacteriocin potential of this species has not been previously reported. To the best of our knowledge, this is the first report describing the postbiotic potential based on the inhibitory activity of the species strain *E. asini* EAs 1/11D27, which was isolated from the inner mucosa of the auricle in the Slovak horse breed Norik of Muráň.

2. Materials and Methods

2.1. Strain Isolation

A mucosal swab was taken from the inner part of the auricle of a Norik mare of the Muráň breed and placed in Amies agar gel without charcoal (Copan, Italy) [15]. The swab was then placed in one ml of isotonic saline solution with a pH of 7.0 and processed using the standard microbiological method (ISO, 1:9). An aliquot of 100 µl from the appropriate dilutions was spread onto De Man-Rogosa-Sharpe agar (MRS, pH 6.4; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and cultivated under partially anaerobic conditions (Gas Pak Plus, BBL, Microbiology Systems, Cockeysville, USA) at 37 °C for 48 hours, as previously described by Lauková *et al.* [15]. Morphologically distinct colonies were selected from the individual dilutions under the same growth conditions to obtain a pure culture. Each picked colony was checked using Gram-positive staining. For further testing, the isolate was stored at -70 °C using the Microbank™ system (Pro-Lab Diagnostics, Ontario, Canada) [15].

2.2. Strain Identification Process: DNA Extraction, PCR Amplification, and Sequence Analysis

The genomic DNA was extracted from a pure colony using DNAzol direct (Molecular Research Center Inc., Cincinnati, USA) according to the manufacturer's instruction, as previously reported by Focková *et al.* [12]. The 16S ribosomal RNA (rRNA) gene from the isolate was amplified by PCR using the universal primers: Bac27F(5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-CGGYTACCTTGTTACGACTT-3 (Merck-Sigma Aldrich, Darmstadt, Germany). The PCR reaction was performed in a 50 µl PCR mixture containing 2 µl of DNA shield, 46 µl of a reaction mixture comprising One Taq 2x Master Mix with Standard Buffer (New England Biolabs, 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 protocol conditions (thermocycler- TProfesional Basic, Biometra GmbH, Göttingen, Germany) were as follows: 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, and finally at 72 °C for 10 min as previously reported by Focková *et al.* [12]. The 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). The amplified product was sent (in a low bind tube at a minimal volume of 15 µl) for purification and sequencing in both directions using 1492R and Bac27F primer (Microsynth, Wien, Austria). The obtained 16S rRNA sequence was validated and assembled using Geneious 8.0.5 (Biomatters, Auckland, New Zealand) and subjected to BLASTn analysis (https://BLAST.ncbi.nlm.nih.gov/BLAST.cgi).

2.3. Additional Phenotypic Test (Gram-Positive BBL Crystal)

Additional tests were performed using the Gram-positive ID system (BBL Crystal GP, Becton and Dickinson, Sparks, Maryland, USA). This panel includes 29 enzymatic and biochemical parameters, following the reference strain *E. asini* DSM 11492^T [16]. Among these tests, the preferred ones were carbohydrates fermentation tests, such as arabinose, xylose, lactose, sucrose, mannitol, ribose, as well as the enzymes β-glucuronidase and alkaline phosphatase. The strain was cultivated on M-Enterococcus agar at 37 °C for 48 hours. The picked-up colony was suspended in a labeled tube of inoculum fluid to a turbidity equivalent to a 0.5 McFarland standard. The tube was vortexed for 15 seconds, and the entire content was poured into an appropriately labeled panel base. The inoculum was gently rolled along the tracks of the base to fill the wells. A lid was aligned over the base and snapped into place. The panel was placed in an incubation tray at 37 °C for 24 hours. The results were visually read using the BBL Crystal Panel Viewer. A 10-digit profile number was generated and recorded on a pad listing the results. The profile number and Gram stain reaction were entered into a computer with the BBL Crystal ID System Electronic Codebook installed. The computer program generates a single genus and species identification or several differentiated identifications. The identification of the tested strain was derived from a comparative analysis of the reaction patterns of the tested strain with the reference strain in the database.

2.4. Bacteriocin Activity Testing (Postbiotic Potential)

The inhibitory activity of antimicrobial substance produced by the strain *E. asini* EAs 1/11D27 was checked using the quantitative agar spot method [17]. The 18-hour culture (60 ml, A_{600}=0.797) of EAs 1/11D27 strain in Brain Heart Infusion (BHI broth, pH 7.0, Difco, USA) was centrifuged at 10.000 × g for 30 minutes at 4˚C. The pH was checked and adjusted to 4.5. Then, the cell-free supernatant was treated with EDTA/Chelaton III (Sigma, Germany) and heated at 80˚C for 10 minutes to eliminate the effects of other organic substances. The supernatant was concentrated using Concentrator Plus (Eppendorf, Hamburg, Germany) to obtain a concentrated substance in a final volume of 6 ml. The inhibitory activity (IA) was tested against indicator strains *Enterococcus avium* EA5 (from our laboratory, the principal and most sensitive indicator) and *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 (kindly provided by Dr. Styková, UVMP in Košice) as well. The IA was expressed in arbitrary units per ml (AU/ml). It is defined as the highest dilution of the substance which inhibited the growth of the indicator strain. In addition to those two indicator bacteria, other indicator bacteria were used, including 13 faecal strains of *E. mundtii* (isolated from Norik of Muráň breed, [12]), 7 human strains of *Str. pneumoniae* (Spn58, Spn51, Spn49, Spn57, Spn922), and *Streptococcus pyogenes* (Sp117, Sp113) from *otitis media* [18], 21 human strains of *E. faecium* with high resistance to aminoglycoside antibiotics (kindly provided by Dr. Aleksandra Troscianczyk from the University in Lublin, Poland), 9 faecal poultry-originated strains of *E. faecium*, and 13 faecal *E. faecium* strains from wild-living animals (Dr. Troscianczyk). Lactococci (7 strains) isolated from raw goat milk were also included in the testing. Moreover, staphylococci were used as indicators, including 32 strains of *Staphylococcus pseudintermedius* from canine faeces, 13 *S. chromogenes* from cows, 15 strains of *S. felis* from cats, and *S. aureus* from pigs (28) (kindly provided by Dr. Troscianczyk). Furthermore, 10 faecal Gram-negative species strains isolated from horses and roe deer were used (isolated in our laboratory), such as *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 fonticola* 11/91/1, *Escherichia coli* 12/111/1, *E. coli* 10/116/2, and *E. coli* 10/139/2. In total, 170 indicators were used, including 160 Gram-positive strains (57 enterococci, 7 lactococci, 8 streptococci, and 88 staphylococci), and 10 strains of various Gram-negative species.

2.5. Virulence Factor Checking: Hemolysis, Nuclease, Gelatinase Activity, Genes for Gelatinase, Enterococcal Surface Protein, Adhesins, Hyaluronidase, and Aggregation Substance

Hemolysis activity was analyzed on BH agar (Difco, USA) supplemented with 5% defibrinated sheep blood according to Semedo-Lemsaddek *et al.* [19]. The agar plate was incubated at 37 ºC for 48 h in an incubator. The presence/absence of a cleared zone around the colonies was interpreted as α/β-hemolysis; negative hemolysis is indicated as γ-hemolysis.

Deoxyribonuclease activity was evaluated as previously described by Lauková *et al.* [20]. The strain was inoculated onto the surface of DNase agar (Oxoid, USA) and incubated at 37 ºC for 24 hours. Colonies producing DNase hydrolyze the deoxyribonucleic acid (DNA) within the medium. After flooding and acidifying the medium with 1 N HCl (hydrochloric acid), the DNA precipitated, and the medium became turbid with cleared zones around DNase-positive colonies. *Staphylococcus pseudintermedius* SPs 948 served as a positive control (our strain from ruminant).

The gelatinase phenotype test was analyzed by streaking single colonies onto Todd-Hewitt agar (Difco, USA) supplemented with gelatin (Biomark, 30g/l) and incubating at 37 °C for 48 hours. After flooding the medium with 1.5% $HgCl_2$ in 2.0% HCl, the medium became turbid with cleared zones around gelatinase-positive colonies. *Staphylococcus aureus* ATCC 25923 served as the positive control.

Virulence factor genes tested were *gelE* (gelatinase), *agg* (aggregation substance), *EfaAfm* (adhesin *E. faecium), EfaAfs* (adhesin *E. faecalis)*, *esp* (enterococcal surface protein), and *hylEfm* (hyaluronidase). The PCR product was separated using agarose gel electrophoresis (1.2% w/v, Sigma-Aldrich, Saint Louis, USA) with 1 µl/ml content of ethidium bromide (Sigma-Aldrich) using 0.5 × TAE buffer (Merck, Darmstadt, Germany). The PCR fragment was visualized with UV light. The strain *E. faecalis* 9Tr1 (our strain from beaver) *E. faecium* P36 (Dr. Semedo-Lemsaddek, University of Lisbon, Portugal) were used as positive controls. The PCR was carried out in a 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.* [21]. The PCR conditions for *gelE*, *agg*, *esp*, $EfaA_{fm,}$ and *EfaAfs* were as follows: denaturation at 95 °C for 3 minutes followed by 35 cycles for 30 seconds at 95 °C, 30 seconds at 55 °C, 30 seconds at 72 °C, and 5 minutes at 72 °C. The PCR condition for *hyl* gene was as follows: denaturation at 94 °C for 4 minutes, followed by 30 cycles for 30 seconds at 94 °C, 30 seconds at 50 °C, 30 seconds at 72 °C, and finally for 4 minutes at 72 °C.

2.6. Metabolic Enzyme Activity Testing and Antibiotic E-Test

For this test, the API-ZYM panel system (BioMerieux, Marcy l'Etoile, France) was applied according to the manufacturer's recommendations, as previously reported by Lauková *et al.* [22]. The following enzymes were tested using this panel: 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. A volume of 65 µl of McFarland standard 1 inoculum was transferred into each well of the test panel plate. Incubation was performed at 37 °C for 4 hours. Reagents Zym A and Zym B were added to each well. Enzyme activity was evaluated based on color intensity values ranging from 0 to 5. Then their relevant values in nanomoles (nmol) were assigned for each reaction according to the color chart supplied with the kit.

Antibiotic susceptibility was evaluated using the EUCAST (European Committee on Antimicrobial Susceptibility Testing) [23] E-test strip diffusion process. The minimum inhibitory concentration (MIC) was also established. The antibiotic strips used were as follows: penicillin (0.016-256 µg/

ml), chloramphenicol (0.016-256 µg/ml), gentamicin (0.064-1024 µg/ml), rifampicin (0.032-32 µg/ml), streptomycin (0.064-1024 µg/ml), and erythromycin (0.015-256 µg/ml). Agar plates (Mueller-Hinton agar, BioRad, United Kingdom) were seeded with an overnight broth culture (BHI, Difco) of tested strain (100 µl), and the appropriate antibiotic strips were placed there. *E. faecalis* ATCC29212 was included as the positive control strain.

3. Results

3.1. Identification of EAs 1/11D27 Strain and Assessment of its Characteristics

The pure bacterial isolate was analyzed using sequencing (BLASTn analysis). BLASTn analysis assigned this strain to the species *Enterococcus asini*. The percentage identity (match) of BLASTn 16S rRNA sequence for the strain reached up to 100% (99.86% similarity was detected with the nucleotide sequence of the strain *E. asini* NR113929.1). The strain EAs 1/11D27 has been assigned the GenBank accession number (AN) MN822908.

Phenotypization using the BBL Crystal GP system confirmed individual characteristics typical for this species, such as acid production from lactose and xylose. However, no acid production from arabinose was observed by the EAs 1/11D27 strain. The esculin reaction was positive.

E. asini EAs 1/11D27 is hemolysis-negative (ɣ-hemolysis), deoxyribonuclease-negative, and gelatinase-negative. Moreover, this strain was also absent of genes for virulence factors such as gelatinase, enterococcal surface protein, adhesins, hyaluronidase, and aggregation substance. Evaluation of the API ZYM kit results confirmed that *E. asini* EAs 1/11D27 did not produce damaging enzymes (e.g., β-glucuronidase), but also no production of other enzymes was found. This was a reason to test beneficial property-bacteriocin activity. Following the E-test evaluation, *E. asini* EAs 1/11D27 was found to be susceptible to antibiotics with MIC = 2 µg for chloramphenicol, MIC = 16 µg for gentamicin, MIC = 0.012 µg for rifampicin, MIC = 0.12 for erythromycin, and MIC = 1 µg for penicillin. Resistance to streptomycin was observed (MIC = 192 µg).

3.2. Postbiotic Potential of Concentrated Substance EAs1/11D24

The concentrated substance (CS) of EAs1/11D27 strain was active against the principal indicator strain *E. avium* EA5 (1600 AU/ml). Moreover, the growth of 13 *E. mundtii* strains from the Norik of Muráň horse breed was inhibited **(Table 1)** with inhibitory activity ranging from 200 to 400 AU/ml. *E. faecium* strains from various sources, including 21 human strains with high resistance to glycosamide antibiotics, were inhibited with inhibitory activity of 200-400 AU/ml **(Table 2)**. Similarly, *E. faecium* strains (9) derived from poultry with similar resistance were inhibited with activity ranging from 100 to 400 AU/ml, as well as the growth of *E. faecium* (13) from wild-living animals, which was inhibited with activity 100 AU/ml. Seven strains of lactococci were inhibited with activity of 100 AU/ml **(Table 2)**.

Table 1: Inhibitory activity of concentrated substance EAs 1/11/27D against *Enterococcus avium* EA5 and *E. mundtii* in arbitrary units per ml (AU/ml).

Indicator	Inhibitory activity
EA5	1 600
EM12/1	200
EM13/3	200
EM22/1	200
EM23	200
EM24/1	200
EM29/1	200
EM31/2	200
EM32/3	400
EM34/2	400
EM40/2	200
EM37/1	200
EM38/1	200
EM41/3	400

EM - *Enterococcus mundtii* - faecal strains from the Norik of Muráň breed

Table 2: Inhibitory activity of concentrated substance EAs 1/11/27D against *E. faecium* strains and lactococci in AU/ml.

Indicator	Tested/Inhibited strains	Inhibitory activity
EF human HLAR	21/21	200-400
EF poultry HLAR	9/9	100-400
EF wildlife animals	13/13	100
Lactococci	7/7	100-400

EF human HLAR - high resistance to aminoglycosides, *Enterococcus faecium strains*, human-derived; EF poultry HLAR-high resistance to aminoglycosides, *Enterococcus faecium strains* from poultry; EF wildlife animals, *E. faecium* from wild-living animals; lactococci from raw goat milk (MK2/1-MK2/8)

In terms of species strains, one strain of *Str. pyogenes* (Sp117) was inhibited with inhibitory activity of 100 AU/ml, and two strains of *Str. pneumoniae* (clinical human isolates) were inhibited with activity of 100 AU/ml **(Table 3)**. *Str. equi subsp. zooepidemicus* (from horses) was inhibited at 200 AU/ml. Surprisingly, all tested staphylococci (88) were inhibited regardless of the species, with inhibitory activity of up to 200 AU/ml **(Table 4)**. When Gram-negative indicators of different species (10 strains) were used, the growth of 9 strains was inhibited **(Table 5)**. In total, 170 indicator bacterial strains were involved in testing, and the growth of 165 strains (97%) was inhibited. The principal indicator strain EA5 showed the highest susceptibility among enterococci. Enterococci, in general, were inhibited with almost the same inhibitory activity, as were lactococci (**Table 1** and **Table 2**). All tested enterococci (57) and lactococci (7) were inhibited. Among streptococci, 4 strains were resistant to CS EAs1/11D27, while 4 strains were susceptible, mostly with an activity of 100 AU/ml, and one strain showed an activity of 200 AU/ml **(Table 3)**. Most staphylococci (88) **(Table 4)**, were inhibited at 100 AU/ml. Five strains of *E. coli* were inhibited

at 100 AU/ml, as were *Acinetobacter lwofii*, *Ac. johnsonii*, and *Serratia liquefaciens* **(Table 5)**. However, *Pantoea agglomerans* PATK4/2 exhibited the highest susceptibility among Gram-negative bacteria, with inhibitory activity of 400 AU/ml.

Table 3: Inhibitory activity of concentrated substance EAs 1/11/27D against streptococci in AU/ml.

Indicator	Inhibitory activity
CCM	200
Sp117	100
Sp113	ng
Spn49	100
Spn51	ng
Spn57	100
Spn58	ng
Spn922	ng

CCM 7316 - *Streptococcus equi* subsp. *zooepidemicus*, Sp. - *Streptococcus pyogenes*, Spn - *Str. pneumoniae*, ng-negative - non inhibited

Table 4: Inhibitory activity of concentrated substance EAs 1/11/27D against staphylococci in AU/ml.

Indicator	Tested/Inhibited strains	Inhibitory activity
S. pseudintermedius	32/32	100
S. aureus	28/28	100-200
S. felis	15/15	100-200
S. chromogenes	13/13	100

Staphylococcus pseudintermedius - canine faeces; *S. aureus* - faecal strains of pigs; *S. felis* - faecal strains of cats; *S. chromogenes* - faecal samples of cows

Table 5: Inhibitory activity of concentrated EAs 1/11D27 against Gram-negative bacterial strains from roe dear and horses in AU/ml.

Indicator	Inhibitory activity
Ac.j. K17/PL2	ng
Ac. lwofii K8/3	100
S. lq.K2PL/1	200
Ac. sp.K10PL/2	100
P. aggl.PATK4/2	400
Ec 12/111/2	100
Ec 11/91/1	100
Ec 12/111/1	100
Ec 10/116/2	100
Ec 10/139/2	100

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 fonticola* 11/91/1, *Escherichia coli* 12/111/1, *E. coli* 10/116,/2, *E. coli* 10/139/2

4. Discussion

Only very limited information exists regarding the occurrence of the species *E. asini* **[16]**. This species belongs to the phylum Firmicutes, Family Enterococcaceae, and genus *Enterococcus*. Based on 16S rRNA gene similarity analysis, enterococci are divided into several principal groups. The species *E. asini* was allotted in *E. dispar* group together with the species *E. dispar*, *E. caninintestini*, *E. hermaniensis*, and *E. pallens* **[9]**. Regarding the basic taxonomic allocation of EAs 1/11D27, de Vaux *et al.* **[16]** described similar properties for *E. asini* strain from caecum of donkeys (*Equus asinus*) as formerly mentioned.

Furthermore, knowledge about the use of beneficial bacteria in horses is limited **[6,13,14]**. Our previous studies in horses have documented the use of bacteriocins produced by faecal enterococci **[14,24]**. Enterocin (Ent) M was applied in horses which led to a statistically significant reduction of coliforms and campylobacters ($p < 0.05$), and clostridiae ($p < 0.001$) in faeces. A beneficial effect on non-specific immunity (phagocytic activity values, PA) was also noted ($p < 0.0001$). When Mundticin-like substance EM 41/3 was applied to horses of the Norik of Muráň breed, a decrease in staphylococci was noted in individual horses, along with a statistically significant difference in the decrease of coliforms and pseudomonads. Tendency to increase PA was also noted **[24]**. However, to date, bioactive substances from the species *E. asini* and/or its postbiotic potential have not been mentioned. Although it is necessary to study other properties of a newly discovered active substance, the substance produced by *E. asini* EAs1/11D27 appears to have a broad antimicrobial spectrum.

Lauková *et al.* **[15]** reported the production of a bacteriocin-active substance by the species strain *E. moraviensis* EMo 1-1Nik from the buccal mucosa of Slovak warm-blood horses. The Norik of Muráň breed was found to be a source of bacteriocin-active strains of *E. mundtii* **[12]**. However, that substance showed a bacterial-related inhibitory spectrum. Bacteriocin substances produced by enterococcal species strains mostly belong to thermo-stable bacteriocins-enterocins **[10]**. Some of them exhibited a broad inhibitory activity spectrum, while others demonstrated a limited inhibitory activity spectrum, as also presented by Kubašová *et al.* **[25]** in the case of Ent B from canine *E. faecium* strain. Additional *in vitro* and *in vivo* studies are required to better understand the mechanisms involved.

Currently, the advantages of postbiotic use are frequently discussed. From the general perspective, postbiotics play an important role in the host's health status and their unique advantages include targeted regulatory effects that are not limited to the intestinal tract but also extend to the oral cavity, skin, and other parts **[26]**. Based on this information, it can be supposed that postbiotics also benefit the health status of horses health. Postbiotics are currently recommended as preventive and curative tools **[26]**. Mosca *et al.* **[27]** reviewed the use of postbiotics in clinical treatment. They showed evidence that postbiotics have a more pronounced effect in improving acute/chronic diarrhea, immune function, allergic reactions, and neurodegenerative diseases with good stability and safety. Considering this evidence and our previous conclusions based on postbiotic application (from the species *E. faecium*) in horses, these are the benefits of postbiotics that can be expected regarding the horses' health status.

Recently, for example, a bacteriocin-active substance from the strain species *Lactobacillus plantarum* was reported to be used as a complementary and adjuvant therapy for human colorectal cancer [28].

5. Conclusion

Enterococcus asini EAs 1/11D27, isolated from a mucosal swab of the Slovak breed Norik of Muráň, was taxonomically allocated based on a sequence match of 99.86% with the nucleotide sequence of the strain *Enterococcus asini* NR113929.1 in GenBank. The strain EAs 1/11D27 has been assigned the GenBank accession number MN822908. This strain is hemolysis-negative, deoxyribonuclease-negative, and gelatinase-negative, and it lacks genes for virulence factors such as gelatinase, enterococcal surface protein, adhesins, hyaluronidase, and aggregation substance. It does not produce damaging enzymes and is susceptible to antibiotics. The most important is its bacteriocin-like substance production with a broad antimicrobial spectrum, mostly against staphylococci (88). In total, the growth of 165 out of 170 indicator bacteria was inhibited (97%). This strain exhibits a broad postbiotic potential, and further studies are intended to explore its use in horses. To the best of our knowledge, this is the first report describing the postbiotic potential of species strain *E. asini* isolated from horses.

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Ethical Approval

Ethical approval was not required to conduct this study. However, we obtained kind agreement for sampling from Štátne Lesy a s. Dobšiná (Norik of Muráň breed) - director. Samples from live animals were taken by veterinarians and/or responsible persons involved in the project. They were analyzed at both institutions for diagnostic purposes and approved by the relevant Ethics Committee (permission code: SK U 0716).

Authors' Contributions

A.L. Conceptualization, Investigation, Data Curation, Writing, Project Administration; E.S. Resources, Methodology; V.F. Methodology; M.M. Methodology; A.T. Indicator resources, Methodology.

Data Availability

All data presented are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflicts of interest.

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