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Mundticin KS: Characterization and Production Method

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Abstract The paper aims to develop a method for the single-stage purification of mundticin KS and to study its physicochemical and antimicrobial properties. Mundticin produced by strain *Enterococcus mundtii* B-8398 and belonging to class IIa bacteriocins was obtained by adding 10% of the CM Sephadex C-25 sorbent to the culture medium before cultivation. At the end of cultivation, the sorbent was collected and packed into a column followed by a one-step elution. As a result, a fraction containing mundticin KS was obtained with a purification quality of about 70%. The activity of mundticin KS remained stable over a wide range of pH and temperatures. It is completely inactivated by such enzymes as proteinase K and α-chymotrypsin and partially by trypsin; however, such enzymes as amylase, lipase, and papain do not have any effect on its activity. Mass spectral analysis, electrophoresis, and DNA sequencing showed the high range similarity of mundticin KS produced by strains *E. mundtii* B-8398 to other previously studied mundticins. The antibacterial activity of mundticin KS was recorded ageist all tested strains of *Listeria* spp., *Enterococcus* spp., and *Clostridium perfringens* in nanomolar concentrations. In addition, mundticin KS inhibits the growth of most tested Gram-positive food-borne pathogens. By the sorbent cultivation method, it was possible to increase the yield of mundticin KS by 2,9 times compared to the control.

Keywords: *bacteriocin, Mundticin KS, purification methods, antibacterial spectrum*

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1. Introduction

Mundticins are small heat-stable, non-modified peptides of class IIa (pediocin-like bacteriocins) produced by the species *Enterococcus mundtii*, weakly immunogenic and nontoxigenic for humans and animals [1,2,3,4,5]. Class IIa bacteriocins are synthesized as precursors containing an N-terminal leader peptide, which are processed and post-translationally modified. Subsequently, bacteriocins are transported and site-specific proteolytically cleaved to generate the mature form. Classification of class IIa bacteriocins have been broadly defined first on the basis of their conserved N-terminal region, the "pediocin box" (YGNGX), and then subdivided into 4 subclasses through sequence alignments of the less conserved C-terminal region [6,7,8,9]. The 50 class IIa bacteriocins were classified into eight groups on the basis of their conserved primary structures, 3D structures and mode of action [1]. Mundticin E28 belongs to Group I subgroup I-1 having a flexible hinge at the conserved Asp 17 residue and common consensus motif IGNNxxANxxTGG located at the C-terminal region.

Class IIa bacteriocins kill target cells by forming pores and disrupting the integrity of target cell membranes, causing dissipation of proton motive force, depletion of interacellular ATP and leakage of intracellular material [9,10]. The sugar transporter mannose phosphotransferase system (Man-PTS) serve as target receptors for class IIa bacteriocins on sensitive cells [11,12]. The presence of such specific receptors on the surface of *Listeria* spp. is associated with the anti-listeriosis activity of the bacteriocins under consideration [8,9].

The best-known mundticins are mundticin ATO6 produced by the *E. mundtii* ATO6 strain derived from endive chicory, mundticin KS produced by *E. mundtii* NFRI 7393 isolated from grass silage in Thailand, mundticin CRL35 by *E. mundtii* CRL35 isolated from Argentine cheese, and mundticin QU2 produced by the *E.*

mundtii QU2 strain isolated from soy beans [5,6,11,13]. Despite a variety of isolation sources of producers, the peptides after post-translational modifications are nearly identical. Many articles have described the physicochemical, genetic, and biological properties of mundticins, making it possible to treat them as potential biopreservatives [1,3,4,10,11,13]. However, there are some factors restricting their practical application. It primarily relates to the low-level induction of a target peptide into the culture medium and its substantial loss during purification to remove admixtures.

Earlier, the authors obtained mundticin KS by using the *E. mundtii* strain isolated from sour milk and deposited under the catalog number B-8398 (here and after *E. mundtii* B-8398) into the GCPM-Obolensk collection (Obolensk, Moscow region, Russia). Sequencing of the *E. mundtii* B-8398 genome (NCBI Assembly No. ASM1293551v1.) revealed an operon accountable for the biosynthesis of mundticin KS. It carried three genes: *munA*, *munB* and *munC*. The *munA* gene encoded a precursor of mundticin. Analysis of the mundticin KS amino acid sequence obtained by *in silico* translating the *munA* gene sequence showed its identity to that of mundticin KS (Table 1). However, the authors decided to determine the antibacterial spectrum of this bacteriocin against a wide range of strains from different sources with a focus placed on strains causing spoilage of meat products. We assume that Mundticin KS is a promising for its use as a bio-preservative; therefore, it was necessary to study the maximum possible spectrum of its antimicrobial activity. As the analysis of the literature has shown [1,10,11,13,14,15], the authors, to determine the spectrum of antimicrobial activity, used known museum strains. They focused on the types of bacteria that are dangerous to humans – *Listeria* spp., *Clostridia* spp., bacilli, i.e. those that cause foodborne disease, but the types of microorganisms that actually cause spoilage of meat products were not included in their studies. In our article, we tried to fill this gap by including strains of *Listeria* and *Clostridia* isolated directly from poultry meat processing plants, and foodborne pathogen strains responsible for meat spoilage. Other tasks were to choose appropriate conditions for the fermentative production to increase the yield of the target peptide, as well as to elaborate a technology of its purification that could be suitable for industrial application. It is known that this is one of the main obstacles to the use of bacteriocins. Optimization of cultivation conditions rarely leads to a significant increase in yield. We suggest an original approach to solving this problem, which combines the processes of cultivation and purification, while remaining simple in technology. This paper aims to solve these problems.

2. Materials and Methods

2.1. Bacterial Strain, Storage and Media

E. mundtii B-8398 aliquots were stored in presence of 30% glycerol at -70°C. For each experiment, a new aliquot was prepared and streaked over the nutrient agar (HiMedia Laboratories Pvt. Ltd, Mumbai, Maharashtra, India) to produce individual colonies. To prepare an inoculum and to perform submerged cultivation in a bioreactor, the medium of the following composition: Brucella broth (HiMedia) as a base and 5 g/L of meat extract (HiMedia) was used. The medium was adjusted to pH 7.6 and autoclaved at 121°C for 15 minutes. Glucose and lactose were added simultaneously at the final concentration of 5 g/L each were a source of carbohydrates. Their solutions were prepared and autoclaved separately and added to the medium right before cultivation.

2.2. Indicator Strains

The anti-enterococcus activity of mundticin KS was tested against vancomycin-resistant *Enterococcus* spp (Vancomycin Resistant Enterococci Microbial Panel ATCC® MP-1™), including strains containing the vanA, vanB, and vanC1,2 genes. *Listeria* spp. indicator strains were obtained from the ATCC (n=5) and GCPM-Obolensk (n=54) collections. There were also strains isolated from food and at a poultry slaughterhouse (n=33) identified by MALDI-TOF MS in a Microflex LRF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). To determine the antibacterial activity of mundticin KS in the spot test, 1% glucose Muller-Hinton agar (HiMedia) was used. *Clostridia* spp. strains were obtained from the ATCC collection (n=11), as well as isolated from clinical specimens, avian intestines, and food samples (n=51). In culturing and determining the anti-clostridium activity of mundticin KS, the authors used O. P. S. P. agar (HiMedia), selective additives, and anaerobic incubation by anaerobic jars (Schuett-biotec GmbH D-37079, Goettingen, Germany) with BD Gas-Pak Anaerobe container system (Becton Dickinson and Company, Loveton Circle Sparks, MD, USA). Food-borne pathogenic strains obtained from the DSMZ collection (n=15) were *Lactococcus termiticola* DSMZ 107259, *Lactobacillus sakei* DSMZ 6333, *Carnobacterium inhibens* DSMZ 13024, *Pediococcus claussenii* DSMZ 14800, *Lactobacillus sakei* subsp. *carnosus* DSMZ 15740, *Leuconostoc gelidum* subsp. *gasicomitatum* DSMZ 15947, *Leuconostoc citreum* DSMZ 15948, *Pediococcus cellicola* DSMZ 17757, *Leuconostoc mesenteroides* subsp. *dextranicum* DSMZ 20187, *Weissella confusa* DSMZ 20194, *Oenococcus oeni* DSMZ 20252, *Tetragenococcus halophilus* DSMZ 20337, *Leuconostoc mesenteroides* DSMZ 20343, *Weissella viridescens* DSMZ 20410, and *Carnobacterium antarcticum* DSMZ 103363. They were cultured in A1 Medium 1252, A11, medium 220, medium 92, medium 693 («Pronadisa», Condalab, Madrid Spain), and on MRS agar (HiMedia).

2.3. Identification of Mundticin KS

DNA was extracted from the *E. mundtii* B-8398 bacterial culture grown on the nutrient agar (HiMedia) plates with a simple cell lysis (boiling at 95°C for 15 min, centrifugation at 12.000×g for 2 min). The supernatant was used as a template for PCR without further purification. The mundticin KS producing gene was identified by PCR using specific primers [14,16]. Briefly, a 25-µl reaction mixture contained 5 µl of the strain lysate, PCR mix (20 µl) containing 1 µl of each primer, 1 µl

dNTP (Thermo Scientific, Waltham, MA, USA), 2.5 µl 10x DreamTaq Green Buffer (Thermo Scientific), and 0.2 µl DreamTaq DNA Polymerase (5 U/µl, Thermo Scientific). The reaction was performed by Gradient Palm-Cycler CG1-96 (Corbett Research, Sydney, Australia). An initial activation step at 95°C for 5 minutes was followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, followed by final extension at 72°C for 3 min. Amplicons were detected by visualization in 2% agarose gel electrophoresis in the presence of ethidium bromide (2 µg/mL) using The ChemiDoc XRS+ System (Bio-Rad, Hercules, CA, United States).

2.4. Database Search of DNA and Amino Acid Sequences

Database searches for the amino acid and the DNA sequences obtained were performed using the BLAST program of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). The genome and SRA were deposited in the GenBank/ENA/DDBJ databases under the accession number SAMN14600455.

2.5. Preparation of the Sorbent for Cultivation

A sorbent CM Sephadex C-25 (Sigma, C25120, Sweden) was prepared as follows: dry powder was filled with deionized water and left for full swelling. It was then converted to Na+ through a series of rinses with 6 volumes of 1 M HCL, 10 volumes of deionized water, 6 volumes of 1 M NaOH, and finally with deionized water to neutral pH (5-7) followed by autoclaving at 1 atm for 10 minutes. After that, the sorbent at the volume of 10% was transferred into a bioreactor containing the nutrient medium right before cultivation.

2.6. Inoculum Preparation

E. mundtii B-8398 overnight colonies were transferred into a tube containing 5 ml of broth and cultured at 37°C. Re-culturing was performed every two hours. The culture of the 4th passage was transferred to a flask containing 300 ml of medium and cultured in an incubator IS-971 RF (GMI, Ramsey, MN, USA) at 32°C, 110 rpm. The inoculum was a 4-hour-old culture.

2.7. Submerged Cultivation

Cultivation was performed in an 8-liter bioreactor Sartorius Biostat B plus (Sartorius Lab Instruments GmbH & Co., Goettingen, Germany) at 32°C, 120-160 rpm for 10-14 hours. The pH (Hamilton EasyFerm Plus pH Sensors 225mm) and dO_2 concentrations (Hamilton OxyFerm™ DO Sensor 225mm) were monitored. During cultivation, aliquots were taken every hour to determine OD and antimicrobial activity. At the end of cultivation, 2.5 liters of deionized water and 10 ml of 1 M HCL were added into the bioreactor for mixing 30 min longer. The biomass together with the sorbent was then pumped into abottle and autoclaved at 0.5 atm for 15 minutes. The sorbent was collected by means of a filtration sieve (the authors' own design) by passing the biomass through it.

2.8. Purification of Mundticin KS

The sorbent CM Sephadex C-25 collected in the volume of 800 ml was washed sequentially with 6 volumes of deionized water, 2.5 liters of 0.1 M PBS (pH 7.4) with 10% ethanol and applied to a 50/60 chromatographic column (Cytiva, Uppsala Sweden). Chromatography was performed using a chromatograph AKTA Purifier 100 (Cytiva) with A280 wavelength detection at a speed of 12 ml/min. The sorbent was rinsed with 10% ethanol-added 0.1 M PBS (pH 7.4) until the baseline was achieved and eluted with 100% buffer B (0.5 M NaCL, 35% ethanol, 10% glycerol (PanReac, Barcelona, Spain). The activity of the collected fraction was determined. To determine the purity and yield of mundticin, an aliquot of the active fraction was analyzed by reversed-phase high-performance liquid chromatography (HPLC) using a Partisphere rtf c18, 5µm 150×4.6 column (Hichrom Limited, Reading Berkshire, UK). Elution was performed in the gradient regimen 0→100%. Buffer A: dH2O+0.1% trifluoroacetic acid (TFA), Buffer B: ACN + 0.1% TFA.

Post-separation culture fluid ("residue") was concentrated using a 15 kDa size-limit hollow fiber device AR-0.1 (NPK Biotest, Kirishi, Russia) and UPL-0.6 apparatus (NPK Biotest) to have 1-liter volume. pH was adjusted to 2.5 with concentrated HCl, 250 ml of isopropanol were added followed by centrifugation in a Beckman J2-21 centrifuge (Beckman Coulter, South Kraemer Boulevard Brea, CA, USA). Supernatants were combined and applied to the CM Sephadex C-25 XK50/60 column (Cytiva). Chromatography was performed as described above.

2.9. Production of Mundticin KS with Purity of ≥ 90%

To produce high-purity mundticin KS after one-stage chromatography, the active eluate was adjusted to pH 10.6 with 1 M NaOH solution with mixing followed by centrifugation. The supernatant was brought to pH 7.4 with 1 M HCl. Reverse-phase chromatography was performed using a ReproSil-Pur Basic C18, 5µm 250×4.6 column (Macherey-Nagel GmbH & Co., Duren, Germany). Elution in the gradient of 0→100% was performed. Buffer A: dH_2O+0.1% TFA. Buffer B: ACN + 0.1% TFA. The active fraction was collected, vaporized in a rotary vaporizer Laborota 4000 (Heidolph Instruments GmbH & CO, Schwabach, Germany), and brought to 1/10 of the initial volume with deionized water. Solid-phase extraction was performed using a Strata C18-E column (Phenomenex, Fremont, CA, USA).

The thus obtained purified mundticin at a concentration of 245.8 ng /L was poured into 1 mL vials and lyophilized in a FreeZone Triad Benchtop Freeze Dryer (Labconco Corporation Kansas City, MO Fort Scott, KS, USA). Further, when determining the antibacterial activity as well as to determine the thermostability of mundticin and its relation to proteolytic enzymes, we used a sample purified in this way.

2.10. Determining the Antimicrobial Activity of Mundticin KS in a Spot Test

The method relies on the procedure proposed by Ennahar *et al*. [17]. Briefly: 20 mL of "bottom" agar containing Antibiotic Assay Medium (HiMedia) with BactoAgar added to 1% was poured into Petri dishes. After solidification, *L. monocytogenes* ATCC 19111 test strain suspension was added into a flask containing "top" agar preliminarily melted and cooled to 50°C (semi-solid Mueller-Hinton agar) to a final concentration of 0.5 McFarland units, mixed and layered by 5 mL over the "bottom" agar in Petri dishes. The dishes were left for agar solidification and dried. Ten microliters of twofold dilutions of mundticin KS solution were spotted onto each double-layered agar plate followed by incubation at 37°C. After 16-18 hours of incubation at 37°C, bacterial lawns were checked for inhibition zones. The activity was defined as the reciprocal of the highest dilution causing a clear zone of growth inhibition in the indicator lawn and expressed in activity units (AU, a 2-mm growth-free halo at the highest dilution was taken as one unit) per milliliter of a bacteriocin preparation.

2.11. Converting Activity Units (AU) of Mundticin KS Activity to mg/L

To convert units-expressed activity into concentration units (mg/ mL), a "reference" mundticin KS sample was collected (item 8), and its activity toward "reference" *L. monocytogenes* ATCC 19111 was determined.

The sample concentration (mg/mL) was calculated by the formula:

C= A/ƹl, where C is the concentration, A is the optical density at 280, l is the length of the cuvette optical path (1 cm is ignored), ƹ is the coefficient of extinction, a constant value calculated using the Bactibase database and is 3.27 (14105/4308.55).

Because the obtained a "reference" mundticin KS has a purity higher than 90% (92-93%, see Figure 5, Table 5), and there are no major peptides among ballast peptides, we believe that this error can be neglected when determining the mundicin concentration in mg/L. When converting the activity of the mundticin KS reference sample in the spot test to the concentration of the sample produced, it was determined that 1 AU was equal to 1.25 ng of mundticin E28 for *L. monocytogenes* ATCC 19111.

2.12. Determination of the Physicochemical and Biological Properties of Mundticin KS

To check the thermal stability of Mundticin, we used purified and dried samples with a concentration of 245.8 ng/vial. One mL of DI water was added to each vial with mundticin and placed in a solid-state thermostat "Gnome" (LLC "DNA-Tekhnologiya", Moscow, Russia) at temperatures 65 °C, 85°C, and 95°C for 15 min followed by autoclaving at 0.5 atm/110°C and 1 atm/121°C for 15 min. To determine the pH effect on mundticin, solutions of 1 M NaOH to 8.0, 9.0, 10.0, 10.5, 11.0, and 11.5 were prepared by titrating them to the corresponding pH with 1 M HCl solution. pH of acidic values such as 4.0, 3.0, 2.0, and 1.5 were prepared from 1M HCl by titrating it with 1M NaOH. One ml of each solution was added into a vial with dried mundticin, kept for 60 minutes, and the activity was determined on the *L. monocytogenes* ATCC 19111 by the spot method using serial dilutions. PBS was used as a diluent. Comparison control samples were also titrated in PBS.

Proteolytic activity was determined using the following enzymes: papain (Fluka, Steinheim, Switzerland), trypsin (Merck KGaA, Darmstadt, Germany), α-chymotrypsin (Merck), proteinase K (PanReac), lipase (Merck), amylase (Merck). The enzymes were dissolved in PBS to a final concentration of 1 mg/mL and added ml each into a vial with dry purified mundticin, kept for 30 minutes. The preparations were incubated at 30°C (for trypsin and a-chymotrypsin) or 37°C (for the others) and then autoclaved at 110°C for 10 min to inactivate enzymes. The activity was measured after.

The molecular weight was determined by 16% tricin SDS-PAGE using markers Spectra Multicolor Low Range Protein (Thermo Fisher Scientific 168 Third Avenue Waltham, MA USA) in a Mini Protean Tetra system (Bio-Rad Laboratories, Inc., Alfred Nobel Drive Hercules, CA, USA) [18]. Staining and washing of the gel were performed by conventional methods. The antibacterial activity of the lane containing mundticin KS gel was determined by using *L. monocytogenes* ATCC 19111 agar and incubation at 37°C for 18 hours.

2.13. Mass Spectrometry Protein Analysis

A sample and NSSA matrix (α-cyano-4-hydroxycoric acid in an aqueous solution of acetonitrile and trifluoroacetic acid) in 1:1 v/v were mixed and applied to the target surface (AnchorChip MALDI Target, Bruker Daltonics GmbH, Fällanden, Switzerland). The sample was dried and analyzed by MALDI-TOF MS in a Microflex LRF mass spectrometer (Bruker Daltonics GmbH) in a linear regimen. The produced mass spectra were analyzed with the FlexControl and FlexAnalysis software. Detectable masses ranged between 2 and 6 kDa. An individual mass spectrum was obtained by 40 laser pulses (60 Hz). The total mass spectrum obtained at 10 target points (400 laser pulses) was used for analysis. The laser power depended on the laser status to achieve optimal resolution and signal intensity. [19]

Mass spectrometry strains analysis. Sample preparation was made as described by Hummel [20]. Protein extraction was carried out according to the protocol proposed by Bruker (Bruker Daltonics GmbH). The method of direct application of the culture to the target plate was also used [2]. The results were summarized using the FlexAnalysis program (Bruker Daltonics GmbH).

2.14. Statistical Analysis

The analysis was carried out using the R language version 3.4.4. For all parameters, the following elements of descriptive statistics were calculated: sample size, measures of central tendency, scattering, and accuracy.

The analysis of deep cultivation data was carried out using a two-factor linear model with a pairwise comparison of trends with Tukey correction for multiple comparisons with the logarithm of the source data. The value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of Mundticin Genes

The PCR analysis of the strain revealed munKS genes. The full-length genome sequencing of *E. mundtii* B-8398 also confirmed the identity of the mundticin KS sequence to those of known mundticins (Table 1).

3.2. Submerged Cultivation of *E. mundtii* B-8398 for Producing Mundticin KS

Figure 1 and Figure 2 show curves of pH, activity, and OD_{600} depending on fermentation time (with or without parameter control).

The results of two variants of submerged cultivations of *E. mundtii* B-8398 were compared. The optical density for control cultivation was more than one unit higher compared to "sorbent" fermentation, reaching its maximum by hour 8 (vs hour 6 in "sorbent" cultivation). The activity was three times more because the mundticin in "sorbent" fermentation was sorbed on the sorbent particles. At the same time, the maximum activity was accumulated at hour 10 followed by a slight decrease by the end of cultivation for both variants. The pH level decreased in time during entire "sorbent" fermentation – with the mundticin sorbed, bacterial cells divide more intensively.

3.3. One-Stage Purification of Mundticin KS

After elution, an active fraction was analyzed by HPLC. For comparison with the "sorbent" cultivation, cultivation under the same conditions using the same nutrient medium in the absence of the sorbent (control cultivation) was performed. Figure 3 and Table 2 present the results of sorbent-free reverse-phase chromatography of the culture fluid. Figure 4 and Table 3 provide chromatographic data for an eluate aliquot in the presence of the sorbent.

As can be seen from analytical chromatographic data, the amount of mundticin KS in the culture medium does not exceed 2.45% of the total amount of peptides, whereas the purity of mundticin KS is 71.34% after one-stage purification in the presence of CM Sephadex C-25.

Table 4 presents data on calculating the activity of mundticin KS in comparison with the control sorbent-free culture fluid depending on a purification stage.

Table 1. Alignment mundticin precursors

```
                                    1                                                  58
Mundticin KS, strain B-8398 (1)  MKKLTAKEMSQVVGGKYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS
Mundticin KS                (1)  MKKLTAKEMSQVVGGKYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS
Mundticin AT06              (1)  MKKLTAKEMSQVVGGKYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS
Mundticin CRL35             (1)  MKKLTSKEMAQVVGGKYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS
Mundticin L                 (1)  MKKLTSKEMAQVVGGKYYGNGLSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS
Consensus                   (1)  MKKLTAKEMSQVVGGKYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS
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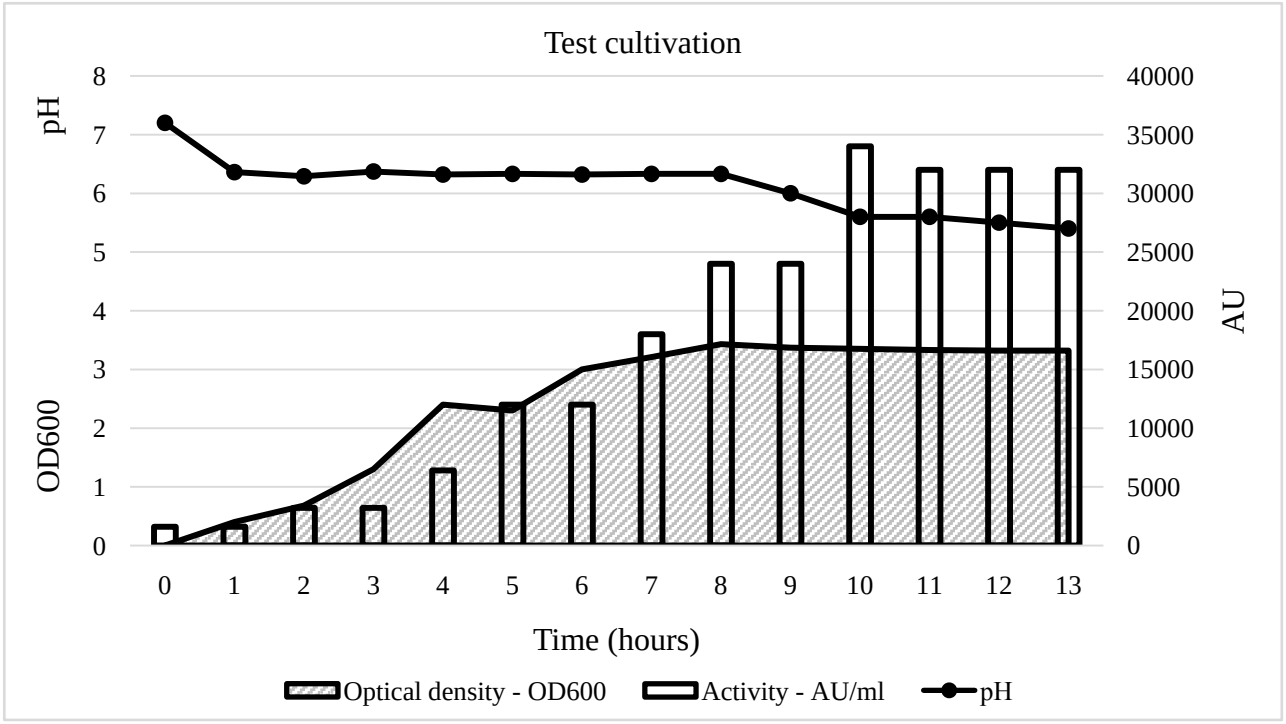

Figure 1. *E. mundtii* B-8398 test cultivation (without sorbent) (*CV of the results of 3-e cultivation)

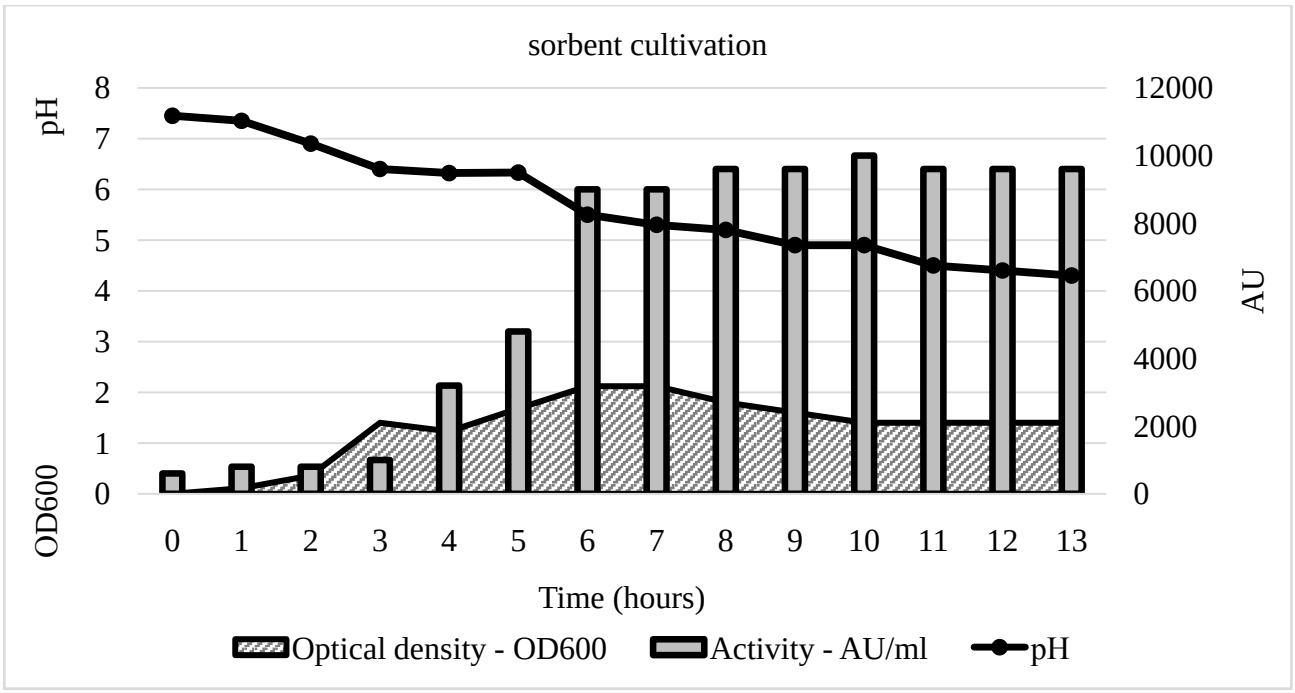

Figure 2. *E. mundtii* B-8398 “sorbent” cultivation (with sorbent) (*CV of the results of 12-e cultivation)

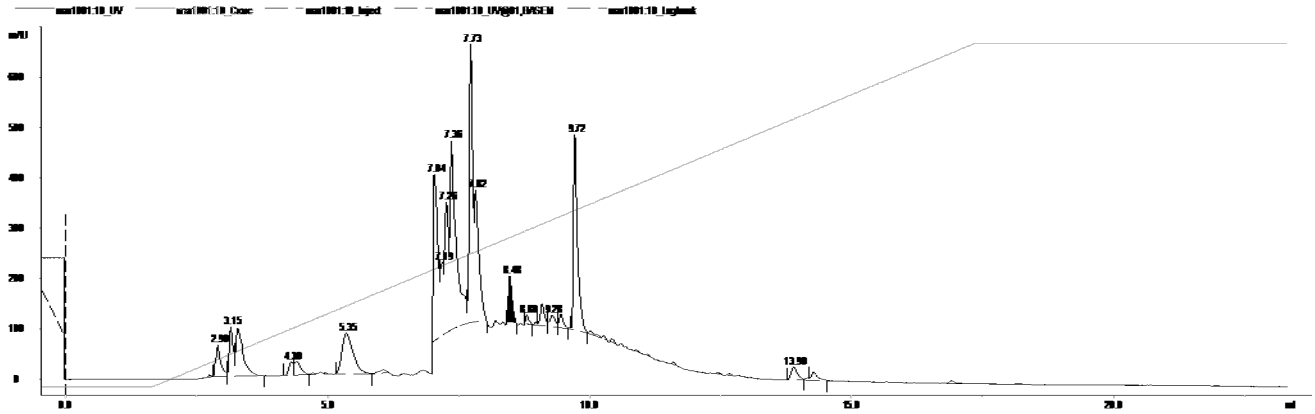

Figure 3. Mundticin KS culture fluid chromatogram (*Mundticin KS peak is highlighted in black)

Table 2. Mundticin KS culture fluid chromatographic data

No	Peak name	Retention (ml)	Area (mAU*ml)	Area/Total area ((volume) %)	Area/Peak area ((volume) %)	Height (mAU)
3		3.28	15.3483	4.03	5.12	95.665
6		5.35	20.1361	5.28	6.71	81.906
7		7.04	32.3306	8.48	10.78	331.011
8		7.19	8.7949	2.31	2.93	145.170
9		7.26	24.0607	6.31	8.02	260.052
10		7.36	51.3368	13.47	17.11	376.522
11		7.73	40.7410	10.69	13.58	553.939
12		7.82	27.0722	7.10	9.02	262.865
13	mundticin KS	8.48	7.2741	1.91	2.42	91.633
17		9.45	2.4861	0.65	0.83	27.243
Total number of detected peaks				268		
Total area (mAU*ml)				381.2468		
Area in evaluated peaks (mAU*ml)				300.0319		
Ratio peak area / total area				0.786976		
Total peak width (ml)				5.06		

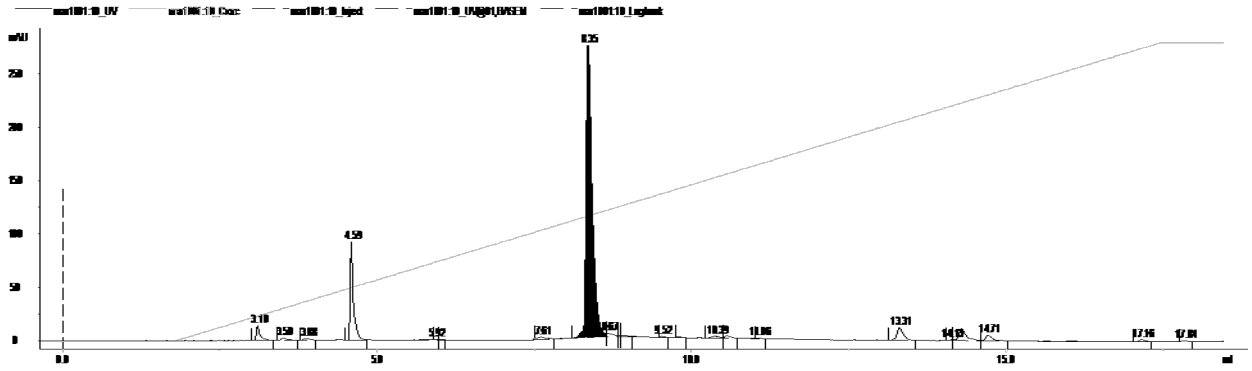

Figure 4. Mundticin KS eluate post-one-stage purification chromatogram (*Mundticin KS peak is highlighted in black)

Table 3. Mundticin KS eluate post-one-stage purification chromatographic data

No	Peak name	Retention (ml)	Area (mAU*ml)	Area/Total area (volume) %)	Area/Peak area (volume) %)	Height (mAU)
4		4.59	5.3779	12.12	12.43	92.163
8	mundticin KS	8.35	30.8030	69.44	71.20	274.214
9		8.67	0.4063	0.92	0.94	3.381
10		8.85	0.0523	0.12	0.12	1.198
Total number of detected peaks				288		
Total area (mAU*ml)				44.3599		
Area in evaluated peaks (mAU*ml)				43.2629		

Table 4. Mundticin KS purification stages

No.	Stage, fraction	Volume, ml	Concentration, mg/L	Activity, AU/ml	Total activity, AU	Total concentration, mg/V	Purity	Yield, %
	Culture fluid control without sorbent	8,000	26	32,000	2.6×10^8	208	0	100
1	Culture fluid + sorbent (initial)	8,000	7.7	9,600	7.7×10^7	61.6	0	29.6
2	Culture fluid without sorbent after heating at 110 °C, 10 min ("residue")	8,000	31	38,400	3.1×10^8	248	0	119.2
3	Concentrate of the "residue" prior to chromatography	1,500	20.7	25,600	3.8×10^7	30.4	10	14.6
4	Eluate	500	1,024	819 200	4.1×10^8	512	71	246.2
6	Eluate of the "residue"	600	160	204 800	1.2×10^8	96	35	46.2

The results allow concluding that after the filtration of the sorbent, the culture medium remained 43, 6% active compared to the control. However, after autoclaving at 0.5 atm, its activity went up and exceeded the control by 74,6%. Unfortunately, processing large volumes (8 liters) using a column is rather difficult. Therefore, the authors applied hollow fiber ultrafiltration to concentrate the material. However, the activity dropped to as little as 21,8% compared to the control sample, and the final yield of purified mundticin KS from the "residue" was 41,9% of the control.

As to the mundticin taken off the sorbent, its yield was 232.7% compared to the control sample, *i.e.* applying the sorbent stimulated 2.3-fold mundticin KS induction. The total amount of the produced mundticin (eluates of sorbent + "residue") was 284,6%, *i.e.* it was a 2,9-fold increase in the total concentration.

3.4. Production of Mundticin KS of ≥90% Purity

For making further experiments to determine the activity and physical and chemical properties of mundticin, it was decided to bring it to an electrophoretically pure state. As a result, a sample producing (see Figure 5, Table 5) single electrophoretic lane with activity preserved was obtained (Figure 6) and analyzed then by MALDI-TOF mass spectrum (Figure 7). The concentration of the sample at A280 and its antimicrobial activity toward *L. monocytogenes* ATCC 19111 (17^{th} dilution in the spot test, 3 AU) were determined. The sample was used as a reference one in converting activity to ng/mL.

3.5. Physical and Chemical Properties of Mundticin KS

Mundticin KS was tested for thermostability, pH, and proteolytic enzyme exposures (Table 6).

It is seen that mundticin KS retains activity within a wide temperature range (up to 120 °C, 1 atm), at pH up to 10.0, and slightly loses it at pH 11.0 within 30 min. It fully inactivates when exposed to proteinase K and α-chymotrypsin and partially to trypsin while retaining activity when treated with amylase, lipase, and papain.

3.6. Spectrum of Mundticin KS Antibacterial Activity

It was decided to divide the collection of *Listeria* spp. strains by isolation places, suggesting that the activity of mundticin may vary depending on the strain specificity. Table 7 presents the spectrum of its antibacterial activity.

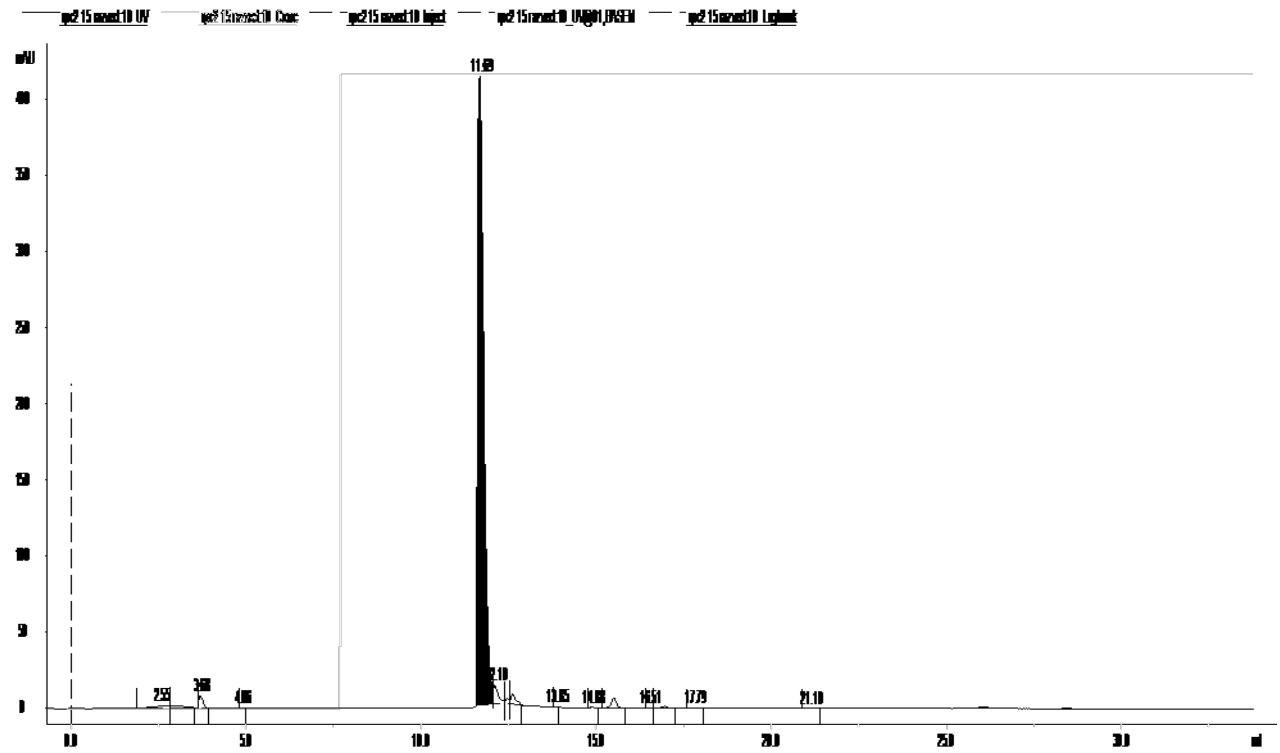

Figure 5. Mundticin KS eluate two-stage purification chromatogram

Table 5. Mundticin KS eluate two-stage purification chromatographic data

No	Peak name	Retention (ml)	Area (mAU*ml)	Area/Total area ((volume) %)	Area/Peak area ((volume) %)	Height (mAU)	Resolution ((alg. 3))
1		2.55	0.5307	0.57	0.58	1.387	
2		3.02	0.6271	0.68	0.68	1.474	0.70
3		3.68	1.0888	1.18	1.19	7.834	1.40
4		4.86	0.0170	0.02	0.02	0.095	4.27
5	mundticin KS	11.69	84.2672	91.17	92.02	413.301	20.88
6		12.10	2.0053	2.17	2.19	12.318	1.44
7		12.46	0.4450	0.48	0.49	3.617	1.47
8		12.61	1.0734	1.16	1.17	6.654	0.64
	Total number of detected peaks			250			
	Total area (mAU*ml)			92.4299			

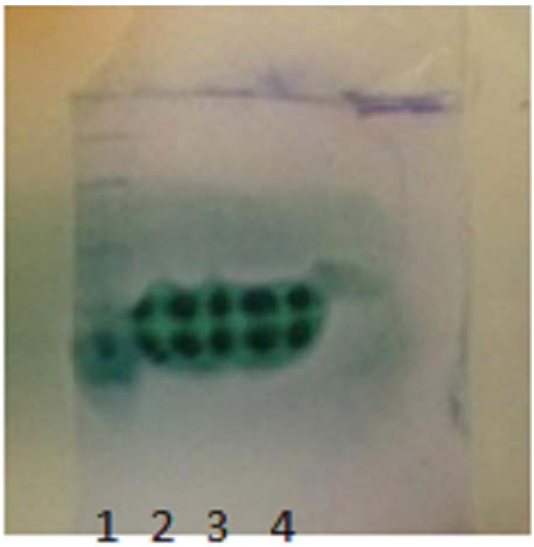

Figure 6. Electrophoretic image of mundticin KS activity toward *L. monocytogenes* ATCC 19111. Lane 1, size markers, downwards (42; 26; 17; 10; 4,6; 1,7 kDa). Lanes 2, 3, 4, mundticin KS

Table 6. Mundticin KS properties

Parameter	Value	E28 activity
Temperature, 15 min heating	60 °C	+
	90 °C	+
	100 °C	+
	110 °C	+
	120 °C	+ (60% of the initial temperature)
pH, 30 min exposure .	1.5	+
	2	+
	3	+
	5	+
	7	+
	9	+
	10	+
	11	+ (80% of the initial pH)
	12	+ (40% of the initial pH)
Enzymes	amylase	+
	trypsin	+/-
	α-chymotrypsin	-
	proteinase K	-
	lipase	+
	papain	+

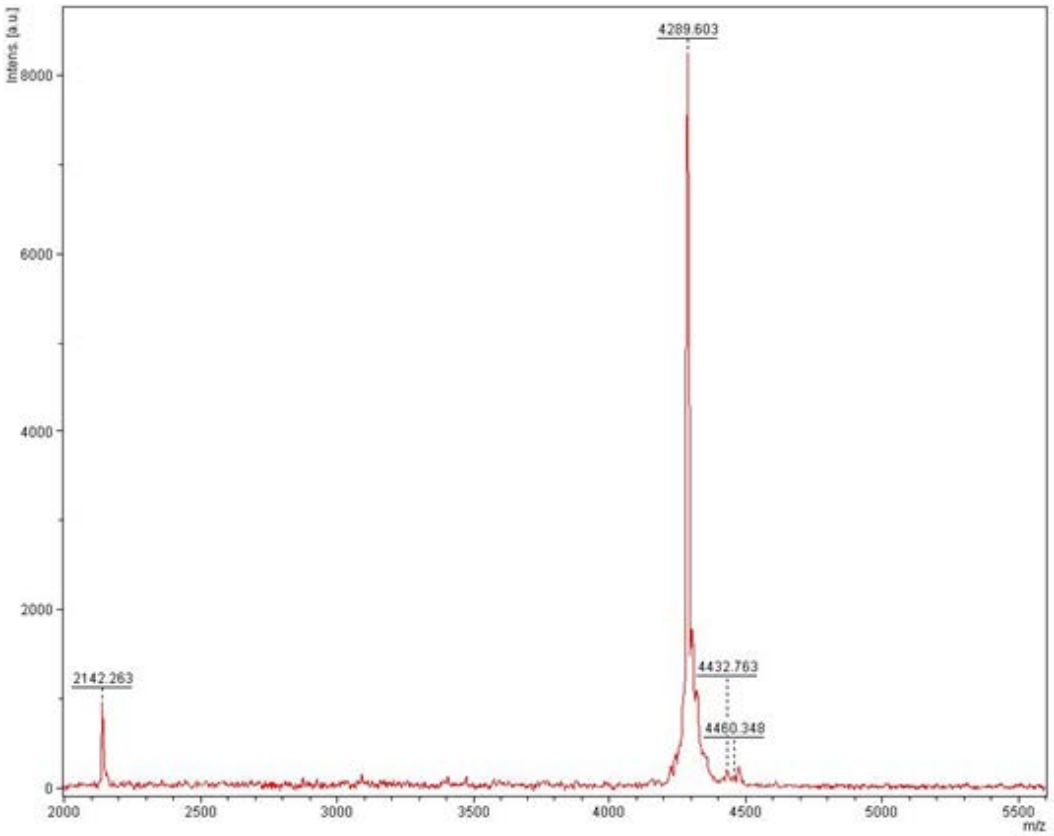

Figure 7. MALDI-TOF mass spectrum of purified Mundticin KS

Table 7. Mundticin KS minimal inhibiting concentrations (MICs) for *Listeria* spp., *Enterococcus* spp. and *Clostridia* spp.

Strain source	n=	MIC, ng/mL	
		Average value /group	MIC range
ATCC *L. monocytogenes*	2	1.99	0.23-3.75
Patients and autopsy material *L. monocytogenes*	25	18.9	0.94-75.4
Food *L. monocytogenes*	29	16.8	0.145-75.4
Environment *L. monocytogenes*	7	23.3	7.5-37.5
L. ivanovii	2	2.8	1.88-3.75
L. innocua	3	2.8	0.94-3.75
L. welschimeri	2	5.2	0.94-9.4
L. seligerii	3	0.23	0.23
L. grayi	3	2.8	0.94-3.75
Poultry slaughterhouse (equipment, poultry carcasses)			
L. monocytogenes	1	13.3	13.3
L. welschimeri	2	0.96	0.67-1.25
L. innocua	13	126.2	13.3-640
Ent. faecium vanA	6	40	30-60
Ent. faecalis vanB	4	24.5	0.47-60
Ent. gallinarum vanC-1	4	13.1	7.5-15
Ent. casseliflavus vanC 2.3	1	10	10
Ent. faecalis Van-sensitive	4	11.3	7.5-20
Enterococcus spp. human clinical material	12	3.3	1-7.5
Enterococcus spp., sick poultry	21	2.5	0.23-8
Ent. faecalis vanB, poultry	1	15	15
Enterococcus spp., the environment and food	9	15	3.3-120.6
Clostridium strains			
Cl. perfringens, ATCC	3	60	60
Cl. perfringens, animals and the environment	31	120.6	15-241.3
Cl. beijerinckii	2	15	15
Cl. novyi	2	-	-
Cl. tertium. Cl. baratii, Cl. sardellii, Cl. bifermentans, Cl. paraputiriticum, Cl. butiricum, Cl. septicum, Cl. histolyticum, Cl. difficile – one for each	9	-	-
Cl. coccoides, Cl. cadaveris, Cl. sporogenes – two for each	6	-	-
Cl. botulinum	5	-	-

The minimal inhibiting concentrations (MICs) of mundticin KS for *Listeria* spp. were compared. Food-isolated strains were shown to be the least resistant while MICs for listerial strains isolated from patients and the environment were 12.5 and 39% higher, respectively. MICs for *Listeria* spp. other than *L. monocytogenes* were markedly less from 0.23 to 5.2 ng/mL. MICs for *Listeria* spp. isolated at a poultry farm slaughterhouse were unusual. If MICs for *L. monocytogenes* and *L. welschimeri* were comparable to those of the collected strains, then *L. innocua* MICs were on average as high as 120.6 ng/mL and reached 640 ng/mL for the most resistant strain. At the same time, MICs varied greatly, approximately by two orders of magnitude, in all groups. Clostridial MICs averaged 120 ng/mL. The other *Clostridia* spp., except *Cl. beijerinckii* (MIC=15 ng/ml), appeared resistant to mundticin KS. MICs for VRE enterococci obtained from ATCC were of special interest. The MICs were clearly dependent on the type of vancomycin resistance, varying from 40 ng/mL in *vanA* strains to 10 and 11.3 ng/mL in *vanC* 2,3 and Van-susceptible strains, respectively. The MIC value for the clinical strain *vanB* isolated from poultry was high, too (15 ng/mL), comparable with that for the collected strains. MICs for *Enterococcus* spp. from sick people and sick birds were markedly less than those for *Enterococcus* Van+ strains. They were 3.3 and 2.5 ng/mL, respectively. MICs for *Enterococcus* spp. isolated from the environment and foodstuff varied greatly between 3.3 and 120.6 ng/mL.

Table 8 shows data on the activity of mundticin KS against microorganisms causing food spoilage.

Table 8. Activity and MICs of mundticin KS against food spoilage microorganisms

Strain	Source	Activity ±	MIC, ng/mL
Lact. termiticola	DSM 107259	+	640
Lact. sakei	DSM 6333	+	12
Carnobacterium inhibens	DSM 13024	+	0.23
Ped. claussenii	DSM 14800	+	320
Lactobacillus sakei subsp. *carnosus*	DSM 15740	+	640
Leuconostoc gelidum subsp. *gasicomitatum*	DSM 15947	+	0.8
Leuconostoc citreum	DSM 15948	+	140
Ped. cellicola	DSM 17757	+	64
Leuc. mesenteroides subsp. *dextranicum*	DSM 20187	+	6
Weissella confusa	DSM 20194	+	640
Oenococcus oeni	DSM 20252		
Tetragenococcus halophilus	DSM 20337	+	0.23
Leuconostoc mesenteroides	DSM 20343	+	6
Weissella viridescens	DSM 20410	+	0.23
Carnobacterium antarcticum	DSM 103363	+	0.23
Lact. saerimneri	Turkey packing plate	+	16
Lact. reuterii	Minced chicken	+	10
Lact. garvieae	Minced chicken	-	-
Lact. kitasotomis	Minced chicken	+	0.8
Lact. agilis	Chicken thighs	-	-
Weissella cibaria	Chicken thigh packing plate	-	-
Carnobacterium spp.	Chicken thigh packing plate	-	-
Weissella cibaria	Minced chicken	+	2.5
Weissella cibaria	Minced chicken	+	2.5
Leuc. lactis	Sausage	+	0.12
Leuc. lactis	Minced chicken	+	0.8
Leuc. lactis	Chicken thigh packing plate	+	0.14
Leuc. lactis	Chicken hearts on the packing plate	+	0.8
Leuc. lactis	Chicken liver on the packing plate	+	1.88
Leuc. mesenteroides	Chicken thighs	+	1.88
Leuc. mesenteroides	Chicken thighs	+	0.8
Leuc.mesenteroides	Minced chicken	+	0.14
Coryne. falsenii	Turkey packing plate	+	0.14
Coryne. falsenii	Chicken thighs	+	1.2
Coryne. falsenii	Chicken hearts on the packing plate	+	3.3
Coryne. falsenii	Chicken hearts on the packing plate	+	3.3
Coryne. falsenii	Chicken heads	+	3.3
Coryne. aurimicosum	Minced chicken	+	0.24
Coryne. aurimicosum	Minced chicken	+	2.6
Coryne.aurimicosum	Chicken legs	+	5
Pediococcus spp.	Minced chicken	+	2.5
Pediococcus spp.	Chicken hearts	-	-
Pediococcus spp.	Chicken hearts on the packing plate	+	10
Pediococcus spp.	Chicken liver	+	4
Candida rugosa	Minced chicken	+	3.3
Candida rugosa	Minced chicken	+	10
Vagococcus fluvialis	Chicken liver	+	0.23
Vagococcus fluvialis	Minced chicken	+	0.23

By data analysis, it can be concluded that all strains responsible for spoilage of meat products obtained from DSMZ were sensitive to mundticin KS. At the same time, the MICs of mundticin KS with respect to some of them (*Lact. termiticola* DSMZ 107259, *Ped. claussenii* DSMZ 14800, *Lact. sakei* subsp. *carnosus* DSMZ 15740, *W. confusa* DSMZ 20194) were high (320-640 ng/mL). For the rest, it was represented in nano-gram concentrations. It should be noted that a number of strains isolated from meat products, such as *Lact. garvieae*, *Lact. agilis*, *Carinobacterium* spp., ¼ of pediococci and 1/3 of *W. cibaria* were resistant to mundticin KS. For the rest, including *Candida rugosa*, MICs were at or below the MIC for *Listeria* spp.

The activity of mundticin KS against the *Staphylococcus* spp. and *Salmonella* spp. strains was additionally determined and found to be ≥ 4 μg/mL.

4. Discussion

The provided data show a possibility to increase the induction of mundticin KS by direct adding of the sorbent CM Sephadex C-25 into the broth during submerged cultivation. As a result, a sharp 25% increase was registered, along with producing 70% purity mundticin by sorbent-based one-stage chromatography. How was the effect obtained? Mundticins are known to be highly charged cationic peptides successfully adsorbing to any matrices with ligated carboxyl groups. In addition to its cation-exchange properties, CM Sephadex C-25 can serve a filter due to its pore sizes, culling peptides of more than 30 kDa, *i.e.* it acts as a sorbent with mixed properties. After attaching the carboxyl group inside a pore, mundticin does not interact with the producing cell, thereby forcing it to synthesize new molecules of mundticin deprived of the ability to adsorb on the cell (at early culture stages). In the authors' view, this effect determines increased synthesis. Moreover, mundticin is preliminarily purified due to the adsorption of admixed proteins with a molecular weight of > 30 kDa on the sorbent.

The literature survey shows that CM Sephadex C-25 is used for purification of various enzymes, but is for few bacteriocins only [15,21]. Mundticins are commonly purified by conventional methods. Farias *et al.* applied successively ammonium sulfate precipitation, Biogel P-6 filtration, cation exchange chromatography and HPLC at the last stage [10]. Kawamoto *et al.* used ammonium sulfate precipitation, SP-Toyopearl cation-exchange chromatography, and C18 SPE reverse-phase chromatography to purify mundticin KS [11]. Zendo *et al*. applied SP Sepharose cation-exchange chromatography, Amberlite XAD-16 hydrophobic chromatography, and reverse-phase chromatography in a PepRPC 5/5 HR column to purify mundticin QU 2 [5]. In the authors' opinion, the application of these procedures to produce preparative amounts of a mundticin is ambiguous. Moreover, the yield of the product does not exceed 25% even in the best applications.

Many researchers overlook the fact that a significant portion of bacteriocins remains on the cell surface, and the culture medium passing through the bacterial filter loses some of the already small yield of the target peptide. Yang *et al.* studied some bacteriocins, whose concentration in the supernatant did not exceed 2% of the total concentration by the end of cultivation [22]. It was a pleasant surprise that the activity significantly increased by heating the culture fluid to 100 °C, possibly, due to the release of cell-adsorbed mundticin KS. Based on the data obtained (Table 4), one can speak about a 20% or more increase in the yield of mundticin KS (unpublished data). The authors have failed to use the entire portion of the product, but plan to solve the problem in the future. Taking into account the already achieved 3.3-fold increase in induction due to the "sorbent" cultivation, one can hope for obtaining the product in the concentration of ≥ 100 mg/L acceptable for industrial application. It is also important that the purification procedure is timesaving: it took no more than 8 hours to process 8 liters of the culture fluid.

To study the potential for the practical application of mundticin KS, the authors carried out research to determine the spectrum of its activity and MICs toward human pathogens and food spoilage microorganisms. Despite a variety of relevant papers [1,4,5,10,11], the authors realized the necessity of much more information. Sonomoto *et al*. tested 26 strains, including *Lactobacillus* spp., (n=9), *Enterococcus* spp. (n=3), *L. lactis* (n=1), *L. mesenteroides* (n=1), *Pediococcus* spp. (n=2), *Bacillus* spp. (n=5), *L. innocua* (n=1), *M. luteus* (n=1), *Staphylococcus* spp. (n=2), and *E. coli* (n=1). The activity varied and was expressed in AU (typical nearly for all known papers). A pilot study of mundticin ATO6 showed that even used in nanomolar concentrations, it inhibited the growth of many Gr+ bacteria, including *L. monocytogenes*, as well as *Cl. botulinum* (inconsistent with the authors' results) [6]. It also inhibited the growth of LAB species (*Lact. salivarius*, *Lact. sake*, *Leuc. paramesenteroides*, *Leuc. mesenteroides*, *C. piscicola*, *Ped. dextrinicus*, *Ped. pentosaceus*, *Ent. faecalis*, *Ent. hirae*, *and L. inoccua*), but was ineffective against Gr- bacteria and fungi. However, there was neither the number of test cultures nor MIC values in the paper.

Certainly, this part of the research is not a key one, focusing mainly on structures, mechanisms of antimicrobial activity, and physicochemical properties of mundticins. So, the authors found it necessary to demonstrate the maximum possible range of activity of mundticin KS against strains isolated from foodstuff (poultry products) directly at poultry processing workshops, and commonly spread poultry meat spoilage pathogens (Gr+ bacteria).

According to the results obtained by the authors, mundticin KS was highly active against all laboratory and clinical strains of *Listeria* spp. (n=92), *Enterococcus* spp. (n=62), and *Cl. perfringens* (n=34). Their susceptibility varied from a tenth of a nanogram to tens of nanograms, *i.e.* the bacteriocin was 10-100-fold more active than antibiotics used to combat bacteria of these groups.

Mundticin KS MICs varied significantly in *Listeria* spp. test strains. Of all *L. monocytogenes* strains, those isolated from environmental objects and from sick people and people who died from listeriosis were the most resistant to mundticin KS, with MICs being 23.3 and 18.9 ng/mL, respectively (see Table 7). MICs for human non-pathogenic *Listeria (L. innocua, L. ivanovii, L. welschimeri, L. grayi, L. selegeri)* were markedly less than MICs for

L. monocytogenes. They averaged from 0.23 to 5.2 ng/mL. At the same time, rather high MICs (13.3 ng/mL to 640 ng/mL) for *L. innocua* isolated from poultry carcasses and processing equipment of a slaughterhouse were of particular interest.

All 62 *Enterococcus* spp. strains were susceptible to mundticin KS, with MICs varying between 0.23 and 120.6 ng/mL. *Ent. faecium* vanA strains (n=6) carrying genes determining high vancomycin resistance (≥100 μg/mL) appeared the most resistant to mundticin KS. MICs for this Enterococcal group ranged from 30 to 60 ng/mL. There was a similar level of resistance in some *Ent. faecalis* vanB and Enterococcal strains from food and the environment (Table 7). *Enterococcus* spp. isolated from human clinical materials and poultry were the most susceptible, with MICs ranging from 0.23 to 15 ng/mL.

Of 16 *Clostridium* species, only *Cl. perfringens* and *Cl. beijerinckii* were sensitive to mundticin KS, including *Cl. botulinum* strains (n=4). This observation went against results presented by Wells-Bennik *et al.* [2]. MICs varied from 15 to 241.3 ng/mL for those strains.

The authors have tested 48 strains involved in meat products spoilage. The growth of strains of the genera *Leuconostoc* spp. (n=12), *Vagococcus* spp. (n=2), *Corynebacterium* spp. (n=8) and species of *T. halophilus* (n=1), *L. termiticola* (n=1), and *C. rugosa* (n=2) was completely inhibited by mundticin KS, whereas its action against the other microorganisms differed. Thus, 7/9 strains of lactobacilli (resistant *L. garvieae* and *L. agilis*), 2/3 corynebacteria, 2/3 *W. cibaria*, and 4/5 *Pediococcus* spp. were susceptible to mundticin KS. It should be noted that the obtained information partially agrees with the data of Kawamoto *et al.*: the anti-*Weissella viridescens* activity of mundticin KS was documented (*vs* inactive mundticin KS) [11].

It is obvious that the research is incomplete and should progress, particularly for understanding differences in inhibitory properties depending on a *Lactobacilli* species (that is what the authors are doing now). Nevertheless, the authors hope that the data presented will be useful for other researchers of bacteriocins.

5. Conclusions

As a result of the work, a one-stage purification method for obtaining mundticin KS with a purity of about 70% was developed. Besides, the addition of CM Sephadex C-25 sorbent directly into the nutrient medium before cultivation increased the yield of bacteriocin by 2,9 times compared to the control. The antibacterial activity of the obtained mundticin was demonstrated in nanomolar ranges against all strains of *Listeria* spp., *Enterococcus* spp., *Cl. perfringens*, and most Gram-positive microorganisms responsible for meat spoilage.

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Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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