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MICROBIOLOGY

Characterization of the antimicrobial activity produced by *Bacillus* sp. isolated from wetland sediment

LUCIANI CAVALINI, PRISCILA JANKOSKI, ANA PAULA F. CORREA, ADRIANO BRANDELLI & AMANDA S. DA MOTTA

Abstract: Bacteria of the genus *Bacillus* sp. present the potential for inhibiting various pathogens, making them a promising starting point in the search for new antimicrobial substances. In this study, bacteria were isolated from sediment samples from humid areas of a Natural Conservation Unit in the state of Rio Grande do Sul, Brazil. The isolate *Bacillus* sp. sed 1.4 was selected for production of antimicrobial activity, and was characterized by MALDI-TOF and 16S rDNA sequencing. Phylogenetic analysis showed that *Bacillus* sed 1.4 was closely related to *Bacillus altitudinis* and *Bacillus pumilus*. The cell-free supernatant was partially purified using ammonium sulfate precipitation, gel filtration chromatography (Sephadex G-200) and an ultrafiltration membrane. Partial purification resulted in specific activity of 769.23 AU/mg, with a molecular mass of approximately 148 kDa. This antimicrobial substance showed stability at 100°C for 5 min, and was inactivated by proteolytic enzymes. An antimicrobial effect against *Listeria* species was observed. Considering the importance of the *Listeria* genus in the area of food safety, this antimicrobial activity should be further explored, specifically in the field of dairy products and with a focus on food biopreservation studies.

Key words: food conservation, *Listeria* sp., wetland sediments, bioactive substances.

INTRODUCTION

In recent decades, new challenges have arisen for the pharmaceutical and food industries regarding the fight against pathogenic microorganisms and spoilage. In the clinical area, the challenges are even greater due to the emergence of antimicrobial resistance, which due to selective pressure, causes microorganisms to no longer respond to the usual antimicrobial drugs (Devatkal et al. 2014, Choi et al. 2019). In this view, exploring new antimicrobial substances has become an object of intensive investigation. The sporulation and rapid growth, which are characteristic of the genus *Bacillus*, mean significant advantages in terms of survival in different habitats. This is also the reason for the research on their ability to produce bioactive substances (Ebrahimipour et al. 2014, Dimkic et al. 2017, Beltran et al. 2018, Zhao et al. 2018). *Bacillus* also has industrial applications due to its easy genetic manipulation, favorable cultivation characteristics on a large scale and the ability to secrete GRAS status proteins ("Generally Recognized As Safe") (Zhang et al. 2020).

Bacillus can produce a diversity of antimicrobial substances, including several antimicrobial peptides (Gebhardt et al. 2002, Stein 2005). Bacteriocins, for example, are antimicrobial peptides that can potentially be used as food preservatives. Nisin, produced

by *Lactococcus lactis,* is a bacteriocin that has granted a safe status and has been approved for use as natural food preservative in several countries (Garsa et al. 2014, Bali et al. 2016).

Other studies have shown that antimicrobial lipopetides produced by *Bacillus* also present relevant inhibitory properties. Surfactin can reduce *Salmonella enterica* and *Legionella pneumophila* biofilm formation in urinary catheters. The extract obtained from the *Bacillus subtilis* I1a strain exhibited an inhibitory effect against planktonic and sessile forms of *E. coli*, *Serratia marcescens*, *Enterobacter cloacae*, *Proteus mirabilis*, *Citrobacter freundii* and *E. faecalis* (Bernat et al. 2016).

It is important to highlight that environments such as soil and sediment, due to the multiplicity of metabolic activities they accommodate, are viable and sustainable paths for the isolation of microorganisms and the obtaining of new antimicrobial compounds (Zhou et al. 2017, Quintero et al. 2018). Thus, the objective of this research was to characterize a substance produced by a bacterium isolated sediment samples from a conservation unit (CU) in southern Brazil in order to verify its antimicrobial activity against clinical and foodborne pathogens.

MATERIALS AND METHODS

Identification of the microorganism

The bacterial isolate was obtained from sediment samples from the wetlands of a Conservation Unit (CU), at a park called the Parque Natural Municipal Imperatriz Leopoldina (S 29º45'374"/ W 51º07'992), located in São Leopoldo, Brazil (unpublished data). For identification, the isolate was subjected to analysis by Matrix Associated Laser Desorption-Ionization – Time of Flight (Bruker MALDI-TOF/MS system), MALDI-TOF Biotyper (software v 4.0). From the identification results, a dendrogram was constructed through Principal Component Analysis (PCA). The identification of the bacteria was also performed through the amplification and sequencing of 16S rDNA, run on a Thermal Cycler model 2720 (Applied Biosystems by Life Technologies ®). Primers FC27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R530 (5'- CCGCGGCTGCTGGCACGTA - 3') were used (Gontang et al. 2007). The PCR was run for 5 min at 94 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 58 °C and 1 min at 72 °C, with a final extension of 10 min at 72 °C. The sequencing of the samples was performed at the ACTGene Análises Moleculares Ltda company (Porto Alegre, Brazil). The AB 3500 Genetic Analyzer automated sequencer (Applied Biosystems) was used. Sequencing data were collected with the Data Collection 2 program (Applied Biosystems). Results were analyzed using the Chrome program version 2.6.4 (Technelysium Pty Ltd) and compared to the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). The construction of the phylogenetic tree was performed using the MEGA X software.

Growth curve and production of the antimicrobial substance

The microorganism was cultivated in tryptic soy broth (Kasvi) at 30 °C for 48 h in an orbital shaking incubator at 50 rpm. Aliquots were taken at regular 2-h intervals and evaluated for bacterial growth, which was expressed as colony-forming units per milliliter (CFU/ml) (Miles et al. 1938). The production of the antimicrobial substance was determined by the serial dilution method to measure activity units per milliliter (AU/ml) (Motta & Brandelli 2002). The pH of the culture was evaluated in triplicate using a pH meter (DM-22A Digimed).

Partial purification of the antimicrobial substance

After cultivation, precipitation with ammonium sulfate at 0-60% was performed according to Scopes (1994). The precipitated material was suspended in 10 mM sodium phosphate buffer pH 6.0, and partially purified by liquid gel-filtration column chromatography (Sephadex G-200) eluted with the same buffer. Fractions were collected and the absorbance at 280 nm was determined using a spectrophotometer. The antimicrobial activity of the collected fractions was assessed according to Motta & Brandelli (2002). The fractions presenting antimicrobial activity were fractionated ultrafiltration system, with a 50 kDa retention membrane (Amicon Ultra). The degree of purification of each step was assessed via denaturing polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) stained with silver nitrate (Heukeshoven & Dernick 1985). The protein concentration was determined through the Folin-Ciocalteu reagent method (Lowry et al. 1951).

Characterization of the partially purified substance

The fractions showing antimicrobial activity were set apart and assessed for maintenance of antimicrobial activity under different conditions. Thermal stability was determined by exposing aliquots in an Eppendorf Microtube with a volume of 1000 µL at 100°C, in a dry bath, for 3, 5 and 10 min. Aliquots were also exposed to 121 °C/105 kPa for 15 min. To evaluate stability at low temperatures, aliquots were refrigerated at 4 °C for 10, 20 and 30 days and frozen at -20 °C for 30 days. To determine sensitivity to the proteolytic enzymes, aliquots were treated at 37 °C for 60 min with trypsin and papain (2 mg/mL), with 10 mM sodium phosphate buffer pH 6.0.

The chemical stability of the antimicrobial activity against organic solvents and detergents was also tested. To this test, 25 µL of the chemicals were added to 50 µL of partially purified substance, and incubated for 60 min at 37 °C. The solvents employed were: acetone (NEON), methanol (DINAMICA), ethanol (NEON), chloroform (ACROS) xylene (Synth), dimethyl sulfoxide (DMSO) (Synth), butanol (NEON) and ethyl ether (ALPHA) at a final concentration of 50% (v/v). The detergents used were Tween 20 and Tween 80 at a final concentration of 10% (v/v) (Motta et al. 2007a). The potential synergistic effects of organic solvents were also investigated in this study. At the end of each treatment, the aliquots were examined to verify antimicrobial activity against the indicator bacteria *Listeria monocytogenes* ATCC 7644 (Motta & Brandelli 2002).

Inhibitory spectrum

The antimicrobial activity was tested against cultures of clinical and food isolates. Suspensions of these indicator cultures were prepared in 0.85% NaCl, corresponding to 0.5 turbidity on the MacFarland scale (approximately 1.5×10^8 CFU/mL), and spread with a swab on Mueller Hinton agar plates. Twenty µL of the partially purified fractions were applied in duplicate. The plates were incubated for 24 h at the optimum temperature for each indicator microorganism. The isolates used in the study were *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Salmonella* Typhimurium ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* ATCC 43861, *Corynebacterium fimi* NCTC 7547, *Corynebacterium striatum* ATCC BAA 1293, *Listeria innocua* (CT) 00354 ATCC 33090, *Listeria ivanovii* 00355 ATCC 19119, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus*

ATCC 25923, *Staphylococcus saprophyticus* ATCC 15305, *Staphylococcus epidermidis* ATCC 35954, *Staphylococcus aureus* ATCC 29213, *Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258. Microorganisms isolated during the routine of a clinical laboratory (Laboratório de Análises Clínicas de Porto Alegre), as well as foodborne samples collected from the Instituto de Ciências Básicas da Saúde (ICBS) laboratory of Universidade Federal do Rio Grande do Sul, were also used in the evaluation of antimicrobial activity. These include isolates of *Escherichia coli, Klebsiella pneumoniae, Enterobacter* sp., *Pseudomonas aeruginosa*, *Salmonella* sp., *Shigella* sp., *Pseudomonas* sp., *Acinetobacter baumanni*, *Staphylococcus* sp., *Enterobacter* sp., and *Listeria* sp. The inhibition zones formed after the incubation period were measured, and expressed in millimeters (mm).

RESULTS

Identification of the selected isolate

The bacterial isolate sed 1.4 was identified at genus level as *Bacillus* sp. through the MALDI-TOF/MS analysis, considering the score result (score 2.048). The data obtained were used to construct a dendrogram, which is arranged according to the levels of similarity among the phenotypic expression of proteins present in the strains (Figure 1). The comparison was carried out with *Bacillus* sp. sed 1.4 and *Bacillus* strains present in the MALDI-TOF database. Based on Figure 1, it could be suggested that the isolate *Bacillus* sp. sed 1.4 is more closely related to *Bacillus altitudinis*, with a distance level near 100, whereas its relationship to *Bacillus pumilus* is more distant, slightly greater than 100. However, both bacteria share an ancestor with common phenotypic characteristics.

The sequence of the 16S rRNA gene was compared with sequences similar to those of reference microorganisms, using the NCBI Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nich-gov/BLAST). The sequences of the following species were obtained from GenBank: *Bacillus pumilus* WAPB4 DQ355508 (AUNPAD & NA-BANGCHANG 2007), *Bacillus altitudinis* D70 MK720001, *Bacillus altitudinis* D164 MK720011, *Bacillus pumilus* SAFR-032, *Bacillus pumilus* WAPB4 DQ355508, *Bacillus pumilus* strain ATCC 7061 NR_043242. *Bacillus* sp. sed 1.4 showed 75% similarity with *Bacillus altitudinis* KJ020347 and *Bacillus pumilus* NR043242 and 97% with *Bacillus* sp. sed 2.2 MH666075. According to the phylogenetic tree of the 16S rDNA (Figure 2) *Bacillus* sp. sed 1.4, *Bacillus altitudinis* and *Bacillus pumilus* isolates shared 100% similarity to the grouped 16S rDNA sequence. The phylogenetic tree was built using the Neighbor-Joining method. The gene sequence of *Bacillus* sp. sed 1.4 was submitted to Standard Nucleotide BLAST with the access code MH666076 (https://blast.ncbi.nih.gov/).

Growth curve and production of the antimicrobial substance

The antimicrobial activity production by *Bacillus* sp. sed 1.4 was observed during the exponential growth phase at 9 h cultivation. Maximum antimicrobial activity of 200 AU/mL was reached in the stationary phase, between 12 and 30 h (Figure 3), followed by a decline to 100 AU/mL after 33 h incubation. The indicator bacterium was *L. monocytogenes* ATCC 7644. After obtaining this result, crude supernatant obtained at 24 h cultivation was sampled and used for purification steps. The pH of the culture remained constant (7.0) throughout the incubation period.

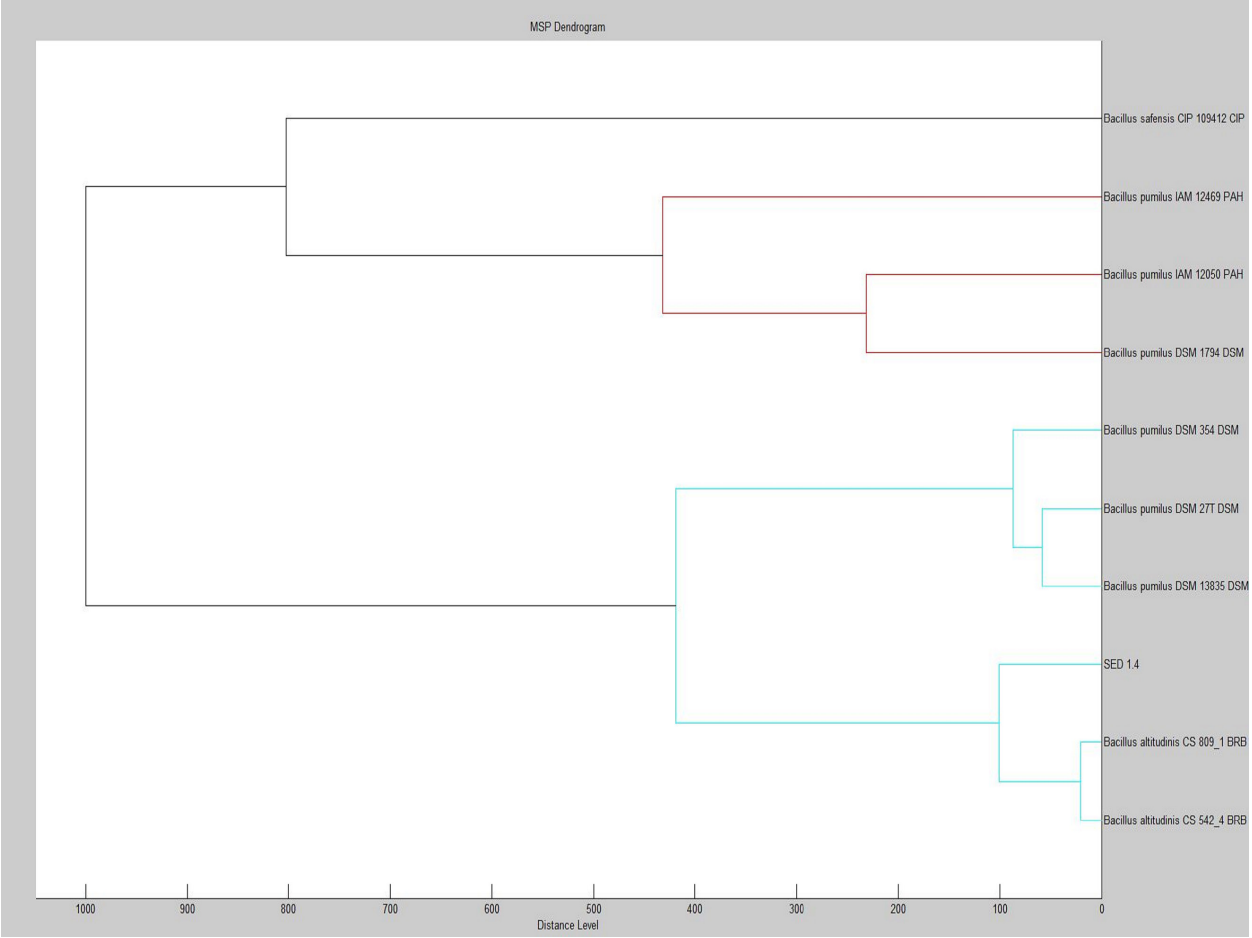

Figure 1. Dendrogram of *Bacillus* species showing the levels of similarity in the phenotypic expression of proteins present in the isolates.

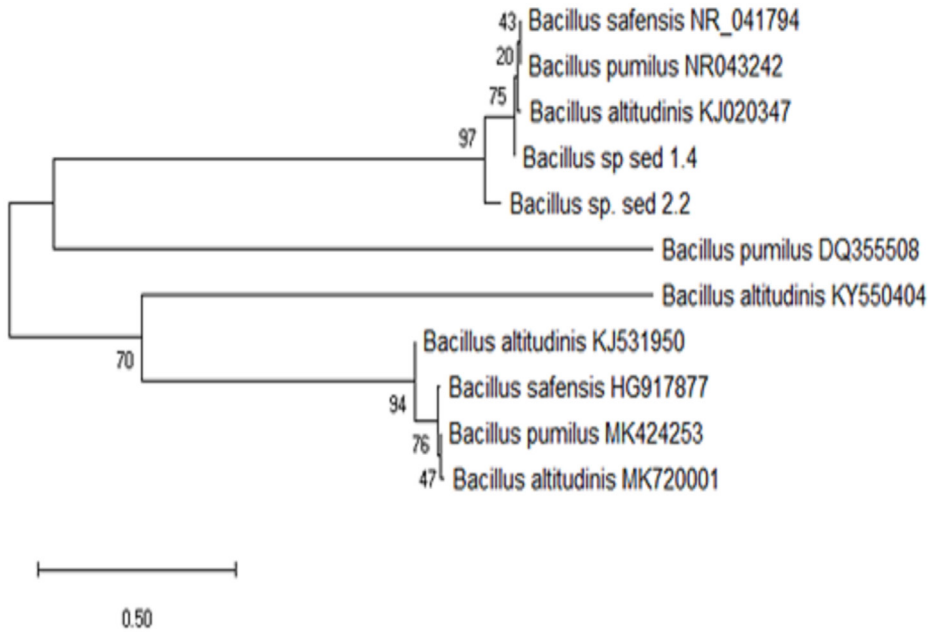

Figure 2. Phylogenetic tree obtained from the 16S rRNA gene of the sequences with closer similarities suggested by BLAST and identified isolates from other studied sediments.

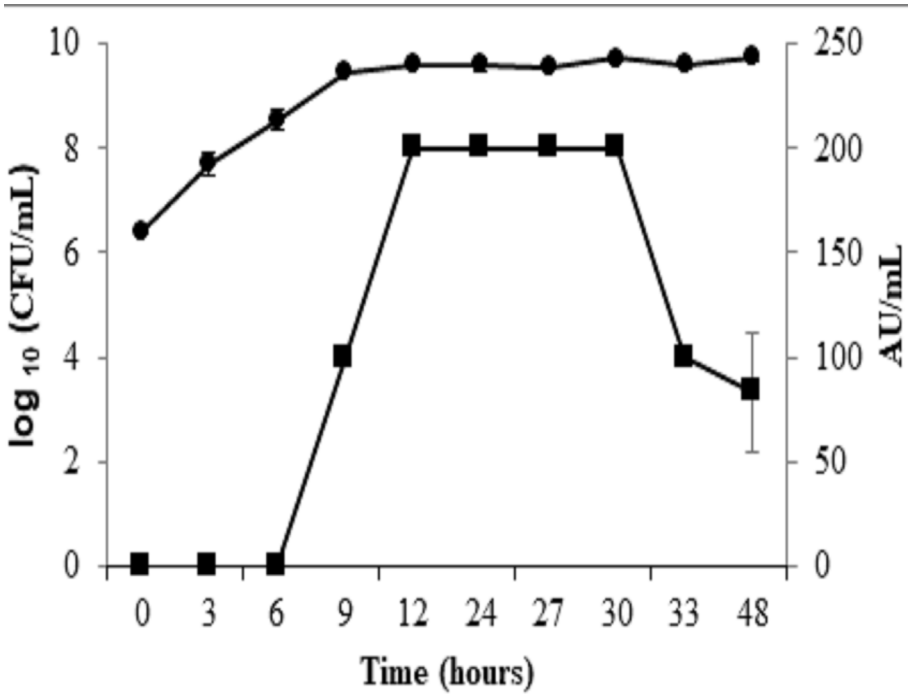

Figure 3. Growth curve of the *Bacillus* sp. sed 1.4 isolate. Growth (•) and antibacterial activity (■) were monitored during growth in TSB at 30 °C. Each point represents the average of three independent experiments. The indicator strain was *Listeria monocytogenes* ATCC 7644. The error bars represent the standard deviation (n=3).

Partial purification of the antimicrobial substance

The precipitation with ammonium sulfate was carried out with the crude supernatant obtained after 24 h culture. It was found that the grouped saturation bands (0 to 60%) reached an activity of 3200 AU/mL when tested against the indicator strain *L. monocytogenes* ATCC 7644.

The pellet resulting from precipitation with ammonium sulfate was resuspended with 10 mM sodium phosphate buffer pH 6.0, and applied to a gel filtration chromatography. Of the 30 fractions collected, fractions 8 and 9 showed antimicrobial activity corresponding to 100 AU/mL each. Three repetitions were performed and the elution profile was the same.

The fractions exhibiting antimicrobial activity were grouped and subjected to ultrafiltration using membranes with a cut-off molecular weight of 50 kDa. In this purification step, activity was observed in the retained fraction, with inhibition zones of 12 mm on average; while the antimicrobial activity of 100 AU/mL was maintained, the filtered fraction did not show antimicrobial activity against the indicator strain *L. monocytogenes* ATCC 7644.

As seen in Table I, it was noted that the antimicrobial substance showed 22.37 AU/mg specific activity in the crude supernatant, while the fractions obtained after gel filtration chromatography presented 769.23 AU/mg, with a final yield of 2%. The protein profiles of the crude supernatant, ammonium sulfate precipitate and the antimicrobial substance resulting from gel filtration chromatography, was evaluated by means of polyacrylamide gel electrophoresis (Figure 4). In the polyacrylamide gel, a main band of the active fractions eluted from the Sephadex G-200 column was observed, with a molecular weight of approximately 148 kDa.

Characterization of the antimicrobial substance

In assessing the stability of the studied antimicrobial substance, it was seen that antimicrobial activity was maintained at 100% when treated at 100 °C for up to 5 min, but complete inactivation occurred after exposure

Table I. Concentration of soluble proteins and antimicrobial activity from the fractions resulting from the purification steps, produced by *Bacillus* sp. sed 1.4.

Fraction	A	Vol	TA	TProt	S.A.	P.F.	Y
Crude	200	299	59.800	8.94	22.37	1	100
PPT	3200	6	19200	6.87	465.79	17.2	32.1
Sephadex	100	12	1200	1.56	769.23	32.9	2.0

PPT- post-precipitation at 0-60%, A, Activity(AU/ml); Vol, Volume (ml); TA, Total Activity (AU); TProt, Total protein (mg/ml); S. A., Specific activity (UA/mg); P.F., Purification factor; Y, Yield (%).

for 10 min at the same temperature, and also at 121 °C/15 min. When refrigerated at 4 °C, it maintained 100% antimicrobial activity for 10 days, and for 30 days when frozen at -20 °C. The antimicrobial substance was completely inactivated when treated with the proteolytic enzymes papain and trypsin.

As for the effects of chemicals and detergents, it can be seen that there was complete inactivation when treated with Tween 80. When antimicrobial stability was tested against acetone, 66% residual activity was observed and above 80% when tested with methanol, ethanol, chloroform, xylene, diethyl ether, dimethyl sulfoxide, butanol and Tween 20, as shown in Table II.

Determination of the antimicrobial activity spectrum

In this evaluation, the antimicrobial substance demonstrated activity against the tested *Listeria* species, with inhibition zones between 10 and 13 mm (Table III). However, there was no inhibition of gram-negative bacteria, yeasts and other gram-positive species.

DISCUSSION

The identification of *Bacillus* sp. sed 1.4 was performed using MALDI-TOF and by sequencing the 16S rRNA gene. However, the methodologies were not sufficient for identification at the species level. Although the 16S rRNA gene is widely used for the identification of bacteria, it is limited for the genus *Bacillus* as it contains several groups of closely related species. This can make identification challenging. *Bacillus altitudinis*, *Bacillus stratosphaericus* e *Bacillus aerophilus* and *Bacillus pumilus,* for example, have very similar sequences of the 16S rRNA gene, making it difficult to identify them based on this type of analysis (Starostin et al. 2015). Elbanna et al. (2014), report that *B. altitudinis* and *B. pumilus* showed 99% similarity to each other after having their 16S rRNA gene sequences analyzed. Thus, techniques such as differential PCR of the genes *rpoB*, *gyrB*, *nifD*, *recA* and *atpD*, have been studied as alternatives in distinguishing among of *Bacillus* species (Ki et al. 2009, Bhandari et al. 2013, Quintero et al. 2018). For instance, the *rpoB* gene, which is homogeneous within cells because it is a single-copy gene, has relatively long sequences (approximately 3.5 kb in *Bacillus*), and many of these sequences are available in public databases (Ki et al. 2009).

The phylogenetic tree revealed that *Bacillus* sp. sed 1.4 is related to *Bacillus altitudinis* KJ020347 and *Bacillus pumilus* NR 043242, by a similarity of 75%. Additionally, there is a 97% similarity with *Bacillus* sp. sed 2.2 MH666075, another isolate from the same Conservation Unit, but collected in a different period. This

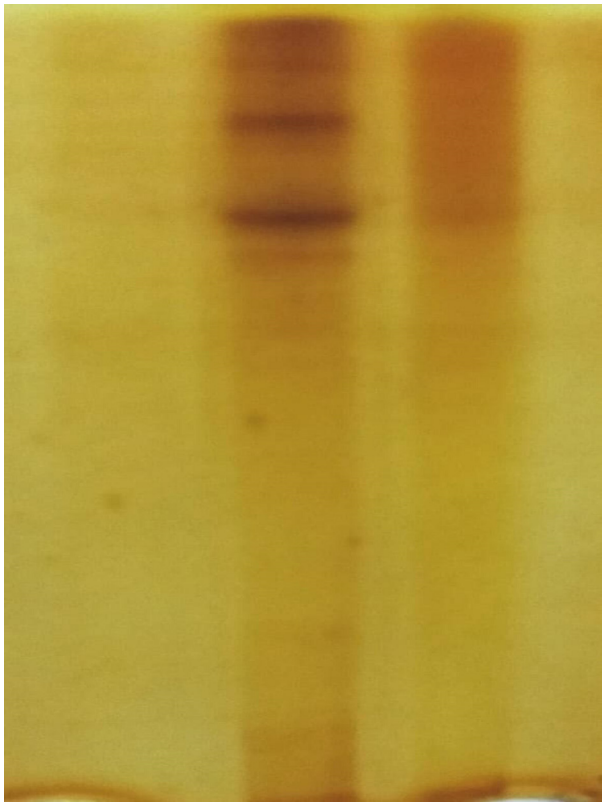

Figure 4. Electrophoresis in polyacrylamide gel stained with silver nitrate showing the protein bands of the stages of partial purification of the antimicrobial substance produced by *Bacillus* sp. sed 1.4. A: G-200, B: precipitated in the 0-60% range, C: crude supernatant, MW: molecular weight marker.

finding confirms the close relationship between species.

Data from MALDI-TOF/MS demonstrate that *Bacillus* sp. sed 1.4 is closely related to *Bacillus altitudinis*, with a level of similarity near to 100. The correlation with the species *Bacillus pumilus* showed a similarity level to the clusters of 400. Based on the reference database obtained in a comparison of MALDI-TOF data, Starostin et al. (2015) identified bacterial isolates such as *Bacillus pumilus* and *Bacillus altitudinis*. The dendrograms also showed that both groups have a similarity level of 400.

Bacillus sp. sed 1.4 was evaluated for its antimicrobial potential. The antimicrobial substance produced by this isolate demonstrated maximum activity in the stationary phase between 12 and 30 h, which is characteristic of a secondary metabolite. In a previous study, Boottanun et al. (2017), investigated the production of metabolites with antimicrobial activity secreted by *Bacillus amyloliquefaciens*. These metabolites were produced early in the incubation period until reaching a steady state between 12 and 72 h. Chalasani et al. (2015) also detected the beginning of antimicrobial substance production at 9 h cultivation, reaching a maximum production at 40 h. The activity of some secondary metabolites decreases when the producing bacteria enter the stationary phase, due to the adsorption of molecules on the surface of the producing cells and proteolytic degradation (Guo et al. 2012, Chikindas et al. 2018).

The antimicrobial substance produced by *Bacillus* sp. sed 1.4 was partially purified using precipitation with ammonium sulfate at 0 - 60% saturation, gel filtration chromatography and an ultrafiltration membrane. The antimicrobial substance has an apparent molecular mass greater than 50 kDa, as it was retained in the ultrafiltration membrane and showed approximately 148 kDa during electrophoresis, which is included in the fractionation range of the Sephadex G-200 resin (5 to 600 kDa). According to the literature, the antimicrobial substances produced by *Bacillus* are generally small, showing molecular weight below 30 kDa (Zhao et al. 2018). Antimicrobial peptides produced by *Bacillus* spp. evaluated by denaturing polyacrylamide gel electrophoresis often shown a molecular weight of approximately 3 kDa or less (Chalasani et al. 2015, Regmi et al. 2017). However, the large molecular weight observed in this study may suggest that the substance is secreted in the form of aggregates. This corroborates findings described by Cladera-Olivera et al. (2004), in which *Bacillus licheniformis* P40 produced

Table II. Effect of different chemicals and organic solvents on the antimicrobial activity of the partially purified crude supernatant produced by *Bacillus* sp. sed 1.4.

Treatment	Concentration	Residual activity (%)	Standard deviation
Acetone	50% (v/v)	66	4
Methanol	50% (v/v)	100	6
Ethanol	50% (v/v)	87.5	2.25
Chloroform	50% (v/v)	87.5	0.5
Xylene	50% (v/v)	100	1
DMSO	50% (v/v)	91.66	1
Ethyl ether	50% (v/v)	87.5	2.81
Butanol	50% (v/v)	100	6
Tween 20	10% (v/v)	141.66	1
Tween 80	10% (v/v)	0	0

Tests were performed by incubating the partially purified crude supernatant and treating it at 37ºC for 1 h. Residual activity was estimated against *Listeria monocytogenes* ATCC 7644.

an antimicrobial substance of approximately 150 kDa when the sample was eluted using Sephadex G-100. These results are also similar to those reported for the bacteriocin Linocin M18 (Valdés-Stauber & Scherer 1994). It has been suggested that the association of molecules in large aggregates is possibly due to the highly hydrophobic nature of the peptides.

Motta et al. (2007b) demonstrate the effects of a peptide produced by *Bacillus* P34 on the growth of *Listeria monocytogenes* and *Bacillus cereus* with antimicrobial activity of 160 AU/mL over a period of 24 h. At the end of the partial purification process, the fraction was purified 32.9 times with a yield of 2% and specific activity of 769.23 AU /mg. A protease produced by *Bacillus cereus* was purified by chromatographic precipitation techniques with ammonium sulfate (50%), ion exchange (DEAE-Cellulose) and filtration gel (Sephadex G-100) by Lakshmi et al. (2018), resulting in a protein with specific activity of 300 AU/mg and recovery of 34.6%. Mothe & Sultanpuram (2016) purified an enzyme produced by a new species *Bacillus caseinilyticus* and it was found that purification using DEAE cellulose column chromatography increased purity by 20.74 times, with specific activity of 89.2 AU/mg.

The effects of proteolytic enzymes, thermal treatments and organic solvents and detergents on the antimicrobial substance produced by *Bacillus* sp. sed 1.4 suggest that the substance has a protein nature (Sharma et al. 2018, Aunpad & Nabangchang 2007) The trypsin cleavage site is highly specific, cleaving the peptide bond after residues with long positively charged side chains, such as arginine and lysine, while papain presents broad specificity, cleaving peptide bonds of basic amino acids, leucine, or glycine (Sangeetha & Abraham 2006, Berg et al. 2014). Antimicrobial peptides that are not sensitive to trypsin probably do not have a lytic site for this protease; nevertheless, inactivation by pancreatic proteases such as trypsin is

Table III. Spectrum of action of the partially purified antimicrobial substance according to the inhibition zone (mm).

Indicator microorganism	Inhibition zone (mm)
Listeria monocytogenes ATCC 7644	12
Listeria monocytogenes 4B (carcass)	11
Listeria innocua L 10 (buffalo milk)	11
Listeria monocytogenes QF Oxford (sliced cheese)	10
Listeria seeligeri BQ Oxford (countertop)	11
Listeria seeligeri BP Palcam – 2 (countertop)	13
Listeria seeligeri PF Oxford (sliced ham)	10
Listeria seeligeri MP Oxford (hands)	11
Listeria seeligeri BP Oxford (countertop)	12

interesting in terms of food safety since it does not have the ability to promote changes in the gastrointestinal tract. Some antimicrobial peptides produced by *Bacillus* spp. are sensitive to trypsin and papain, such as NS02 (Senbagam et al. 2013), trypsin-sensitive N2-4 (Boottanun et al. 2017) and trypsin-sensitive RX7 (Lim et al. 2016). Previous work also described similar results for antimicrobial peptides from *Bacillus* that were sensitive to trypsin and pronase E (Motta et al. 2007a), while Lisboa et al. (2006) verified the sensitivity of the antimicrobial substance produced by *B. amyloliquefaciens* the proteolytic enzymes papain and trypsin.

The stability of the antimicrobial substance to thermal treatments, organic solvents and detergents, resembled those observed for some antimicrobial compounds of peptide nature produced by *Bacillus* spp. (Motta et al. 2007b, Ebrahiumipour 2014, Chalasani et al. 2015, Lee & Chang 2018).

While the studied substance inhibited the growth of different species of *Listeria*, it was not able to inhibit Gram-negative bacteria, yeasts and the other Gram-positive species tested, demonstrating a more restricted spectrum of activity. According to Liu (2015), antimicrobial substances produced by Gram-positive bacteria have less potential to inhibit the growth of gram-negative bacteria. This is due to the fact that Gram-negative bacteria have an outer membrane, which functions as an impermeable barrier for the cell, making it difficult for antimicrobial peptides to reach the cytoplasmic membrane (Garcia-Gutierrez et al. 2019).

The antimicrobial peptide studied by Lee & Chang (2018) was tested against a range of Gram-positive and Gram-negative bacteria, and the results obtained showed that the substance was able to inhibit the growth of Gram-positive bacteria such as *Bacillus cereus* and *Listeria monocytogenes*. Torres et al. (2015) also found antimicrobial activity against strains of *Listeria monocytogenes*. In view of the results presented, it is suggested that the substance may be a class IIa bacteriocin, an anti-Listeria type bacteriocin.

Although foodborne disease associated with *L. monocytogenes* is not as common as those of *Salmonella, Campylobacter* or *Escherichia coli*, the mortality rate can be considered the highest. Approximately 30% of invasive listeriosis cases end in death, and therefore *L. monocytogenes* can be considered a pathogen

of food and public health importance (Haggerty et al. 2018, Gray et al. 2018). Often introduced into the processing environment through raw foods, *Listeria* species can adhere to a variety of surfaces, with some strains persisting for several years and acting as a source of continuous cross-contamination (Colagiorgi et al. 2017, Gray et al. 2018). *L. monocytogenes* is a difficult organism to eradicate and its presence still occurs despite the best management practices, such as cleaning and sanitizing after contact with food and sterilizing non-food contact surfaces, in addition to equipment maintenance (Drew & Clydesdale 2015). Thus, the search for alternative antimicrobial substances to control strains of *L. monocytogenes* is a point of great interest (Leite et al. 2016). Based on the results of this work, the substance produced by *Bacillus* sp. sed 1.4 presented the potential for application in food preservation, as it inhibited all *Listeria* species tested.

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LUCIANI CAVALINI[1]
https://orcid.org/0000-0002-0628-3713

PRISCILA JANKOSKI[1]
https://orcid.org/0000-0003-1204-9939

ANA PAULA F. CORREA[2]
https://orcid.org/0000-0001-7588-5657

ADRIANO BRANDELLI[3]
https://orcid.org/0000-0002-9307-6471

AMANDA S. DA MOTTA[1]
https://orcid.org/0000-0001-8637-3868

[1]Universidade Federal do Rio Grande do Sul, Departamento de Microbiologia, Imunologia e Parasitologia, Instituto das Ciências Básicas da Saúde, Rua Sarmento Leite 500, Sala 216, 90050-170 Porto Alegre, RS, Brazil

[2]Universidade Federal de Roraima, Programa de Pós-graduação em Recursos Naturais-PRONAT, Av. Ene Garcez, 2413, Bairro Aeroporto, 69304-000 Boa Vista, RR, Brazil

[3]Universidade Federal do Rio Grande do Sul, Departamento de Ciência dos Alimentos, Instituto de Ciência e Tecnologia de Alimentos, Av. Bento Gonçalves, 9500, Campus do Vale, Prédio 43.212, 91501-970 Porto Alegre, RS, Brazil

Correspondence to: **Amanda de Souza da Motta**
E-mail: amanda.motta@ufrgs.br

Author contributions

Priscila Ribeiro Jankoski and Luciani Cavalini: Conducted the collection of sediment samples, performed the analyzes and wrote the manuscript. Ana Paula Folmer Correa: Assisted in chromatographic analysis and purification steps. Adriano Brandelli: Revised the manuscript. Amanda de Souza da Motta: conceived the idea; assistance in purification analysis and manuscript review. All authors read and approved the manuscript.