Isolation and characterization of bacteriocin-like substances from Bacillus paranthracis strain MHSD3, a potential probiotic

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Research Article

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Isolation and characterization of bacteriocin-like substances from *Bacillus paranthracis* strain MHSD3, a potential probiotic

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Abstract

Bacteriocins have gained attention as alternative therapeutic agents in pharmaceuticals and are also used as preservatives in the food industry. This study focused on the characterization of bacteriocin-like inhibitory substances (BLISs) produced by a potential probiotic strain *Bacillus paranthracis* strain MHSD3 and evaluations of their antibacterial activity against Gram-positive and Gram-negative pathogenic strains using disc diffusion method. The inhibitory substances were characterized based on their sensitivity to heat, pH, enzymes, and treatment with organic solvents. Inhibitory activity was observed against *Escherichia coli, Staphylococcus aureus, S. saprophyticus* and *S. epidermidis.* The BLIS remained active over different ranges of temperature, pH, enzymes, and solvents, but was sensitive to chloroform. Furthermore, proteomic analysis using liquid chromatography–mass spectrometry (LC–MS) revealed the presence of peptides with potential bacteriocin-like characteristics. These results indicates that the BLIS may be a potential candidate to be used as a therapeutic agent.

Keywords: *Bacillus paranthracis* strain MHSD3; bacteriocin-like inhibitory substances; bacteriocin; liquid chromatography mass spectrometry; proteomics

1. Introduction

Bacteriocins are ribosomally synthesized peptides with antimicrobial properties that often kill or inhibit closely related bacterial species without affecting the secreting strain [1]. Some bacteriocins may have a broad spectrum of activity on various bacterial species without limitation to closely related species [2]. Bacteriocins are categorized into different classes based on their molecular weight, physicochemical and structural properties. Class I are small peptides that undergo extensive posttranslational modification to produce active peptide. Class II bacteriocins are heat and pH stable, low-molecular weight (0.77-10kDa), membrane-active peptides. Class III are large heat-labile proteins and class IV encompasses cyclic peptides [3,4]. These peptides show great potential as antimicrobial compounds that can be vital in pharmaceuticals, agriculture, food industry and biochemical engineering [5]. Bacteriocins have received attention due to their therapeutic effects in treating various bacterial infections such as methicillin-resistant *Staphylococcus aureus* [6]; penicillin-resistant *Streptococcus pneumoniae* and *S. epidermidis* [7,8]. In addition, bacteriocins have been used for inhibition of other antibiotic-resistant strains such *Mycobacterium tuberculosis, and Escherichia coli* [9] and *Pseudomonas aeruginosa* [8]. Bacteriocins secreted by probiotic bacteria can balance the gut microbiota and inhibit the invasion of foreign pathogenic bacteria in the gut [2,9]. Furthermore, bacteriocins are known to inhibit various cancerous cells [10, 11] and have immune-modulatory effects [9] as well as anti-inflammatory properties in human intestinal epithelial cells *in vitro* [12]. Bacteriocins produced by lactic acid bacteria have been extensively studied due to their generally recognized as safe (GRAS) status [13]. Similarly, several studies have reported on bacteriocins secreted by *Bacillus* species such as *Bacillus subtilis*, *B. coagulans* and *B. licheniformis* which have the generally recognized as safe status [14,15]. *Bacillus* species are known to produce numerous antimicrobial compounds such as peptides and lipopeptide antibiotics, bacteriocins, and bacteriocin-like inhibitory substances (BLIS) [3,16]. In addition, some of the *Bacillus* species secrete non-ribosomal peptides such as iturin [17]. Production of bacteriocins or BLIS have been identified in *B. subtilis*, *B. megaterium, B. cereus*, *B. stearothermophilus*, and other *Bacillus* species [3]. Bacteriocins extracted from *Bacillus* species have a broad spectrum of activity and are stable over wide pH ranges compared to LAB [18]. Thus, they are of great interest in evaluating their functions and elucidating their structures for use in food and pharmaceutical industries, to name a few. The aim of the present study was to characterize and evaluate the antimicrobial activity of a bacteriocin-like substance secreted by a potential probiotic *Bacillus paranthracis* strain MHSD3 isolated from medicinal plant, *Pellaea calomelanos.*

2. Method and Material

2.1 Bacterial strain isolation

Bacillus paranthracis strain MHSD3 was isolated from sterilized leaves of the medicinal plant *Pellaea calomelanos*, as described by Mahlangu and Serepa-Dlamini [19] and maintained at -80 ºC in 30% (v/v) glycerol. It was initially identified through partial 16S rRNA gene sequence and later confirmed to be a strain of *Bacillus paranthracis* through whole genome sequencing [20].

2.2 Production and partial purification of the BLIS

The method from Sharma *et al.* [21], was adopted for the production and partial purification of the BLIS. Briefly, *Bacillus paranthracis* strain MHSD3 was grown in 30 mL of tryptic soy broth (TSB) for 18 hours (h) at 30 ºC with gentle agitation at 120 rpm as pre-culture. Five milliliters of the pre-culture were inoculated into 500 mL of tryptic soy broth and incubated at 30 ºC, 120 rpm for 24 h. The cell culture was centrifuged at 9000 rpm at 4 ºC for 15 minutes (min). The pH of the cell free supernatant (CFS) was adjusted to pH 6.5 with 1M NaOH and the supernatant was filtered using a 0.45 μm micro-filter. The CFS was gradually precipitated to 80 % ammonium sulphate saturation with continuous stirring at 4 ºC for 8 h. The BLIS precipitate was obtained by centrifugation at 9000 rpm at 4 °C for 15 min. The pellet was dissolved in phosphate buffer saline (PBS), pH 6.5 and the concentration of the bacteriocin-like substance (BLIS) was obtained using a nanophotometer N60/N50 (Implen, Germany). The BLIS was stored at -20 ºC until further use.

2.3 Antibacterial activity using disc diffusion method

The antibacterial activity of the BLIS was tested against several Gram-positive and Gram-negative indicator strains, including *Bacillus cereus* (ATCC 10876), *Escherichia coli (ATCC 10536), Mycobacterium smegmatis* (ATCC 21293), *Pseudomonas aeruginosa* (NCTC 10662), *Klebsiella pneumonia* (ATCC 10031), *K. oxytoca* (ATCC 13182), *Veillonella parvula* (ATCC 10790), *Enterococcus faecium* (ATCC 13048), *Staphylococcus aureus* (ATCC 25923), *S. saprophyticus* (ATCC 15305) *and S. epidermidis* (ATCC 14990) using disc diffusion method described by Bauer *et al*. [22]. Pathogenic strains were grown in Muller-Hinton broth (MHB) overnight and incubated at 37 ºC, agitating at 200 rpm. Briefly, 100 µL of the pathogenic strains (adjusted to match 0.5 McFarland standard) were spread on Muller Hinton agar (MHA) and allowed to dry for 10 min. Sterile paper discs (Whatman No.1) impregnated with 40 µL of BLIS extract, placed onto the surface of the MHA and incubated at 37 °C for 24 h. After incubation, the diameter (mm) of inhibition zones were measured.

2.4 Minimum inhibition concentration

Minimum inhibition concentration (MIC) of the BLIS was determined using microdilution method described in the guideline of Clinical and Laboratory Standards Institute [CLSI], [23]. The MIC of the

BLIS was tested against *Staphylococcus aureus* (ATCC 25923), *S. saprophyticus* (ATCC 15305), *S. epidermidis* (ATCC 14990) and *Escherichia coli (ATCC 10536)*. Overnight cultures of pathogenic strains in MHB were diluted in fresh MHB to obtain a 0.5 MacFarland standard. Two-fold serial dilutions were performed from the crude BLIS (14 mg/mL to 0.5 mg/mL). Equal volumes (100 µL) of pathogenic strains and serially diluted BLIS were mixed into the wells of 96-well microtiter plates. The MHB with pathogenic strain was used as positive control and the BLIS with MHB was used as negative control. The plates were incubated at 37 ºC for 24 h and optical density (OD) was measured at 595 nm using a Bio-Rad microplate reader (Bio-Rad, South Africa). The total inhibition percentage of bacterial growth was calculated using the following formula: [Control OD − Sample OD/Control OD × 100] [21].

2.5 Effect of temperature, pH, enzyme activity, and solvent on BLIS antibacterial activity

To evaluate thermal stability, the crude BLIS was heated at 40, 80, 100 ºC for 30 min. All samples were cooled to room temperature before analysis of residual activity. To test the effect of pH on the BLIS, the crude BLIS was adjusted to pH level ranging from 2 to 10 using either 1M HCI or 1M NaOH and incubated at 30 ºC for 1 h. To analyze sensitivity to various enzymes, the crude BLIS solution was treated with protease K, lysozyme, and α-amylase (Sigma-Aldrich) to a final concentration of 1 mg/mL at 30 ºC for 1 h and heated at 80 ºC for 10 min to inactivate the enzymes. The crude BLIS was also treated with 50 % organic solvents, which included chloroform, ethyl acetate and methanol. The BLIS residual activity of all samples were examined by microtiter plate assay using *Staphylococcus aureus* ATCC 25923 as an indicator organism [24].

2.6 Preparation of protein extract

Bacterial cells were grown in TSB for 24 h at 30 ºC agitating at 140 rpm. The cells were harvested by centrifugation at 5000 rpm for 10 minutes and resuspended in 200 µL lysis buffer (1% SDS in 50 mM Tris HCl pH8). The bacterial cells were lysed by sonication on ice using a probe sonicator (9 pulses; 10 sec per pulse with 10 sec intermissions between pulses; 50% power setting). Two microliter of RNase was added to the cell lysate and incubated at 37 ºC for 30 min. The sample was centrifuged at 11 000 rpm for 10 minutes to remove cell debris. Aliquots of the CFS sample were stored at -20 ºC until further use.

2.7 Molecular weight determination of bacteriocin

The molecular mass of the CFS protein sample was analyzed by running glycine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) with 12% separating gel and 4% stacking gel. Twenty-five microliters of the BLIS lysate (1:1 sample buffer: BLIS extract) were loaded into the gel and separated using constant voltage (200 V) for 55 minutes. A BLUeye pre-stained protein ladder was

158 used with a range of 11 to 180 kDa (Sigma-Aldrich). The gel was stained with Coomassie blue for 30
159 min and de-stained with 40% methanol, 10% acetic acid and water.

160 **2.8 In-gel digestion**

161 Proteins were digested from gel fractions according to Shevchenko *et al*. [25]. Each gel lane was
162 prepared in 6 separate gel fractions (according to molecular weight ranges). Briefly, the proteins were
163 reduced in gel with 10 mM Dithiothreitol (DTT) in 25 mM NH_4HCO_3 for 1 h at 60 °C. Samples were
164 cooled to room temperature, then 100 % acetonitrile was added and incubated for 10 min. The
165 supernatant was discarded and 55 mM iodoacetamide (IAA) in 25 mM ammonium bicarbonate was
166 added to the gel pieces. The reaction proceeded in the dark for 20 min at room temperature. The
167 supernatant was discarded, and gels were dehydrated with 25 mM NH_4HCO_3 in 50 % acetonitrile,
168 vortexed and the supernatant was removed. The gel pieces were dried completely, and freshly prepared
169 trypsin was added. Protein digestion was allowed to proceed overnight at 37 °C. The digestion was
170 quenched by adding final 0.1 % formic acid and the samples were dried under vacuum. Dried samples
171 were re-suspended in 2 % acetonitrile and 0.2 % formic acid for mass spectrometry analysis.

172 **2.9 Liquid chromatography–mass spectrometry (LC–MS) analysis**

173 Tryptic peptides from each gel fraction were analyzed using a Dionex Ultimate 3000 RSLC system
174 coupled to an AB Sciex 6600 TripleTOF mass spectrometer. Injected peptides were inline de-salted
175 using an Acclaim PepMap C18 trap column (75 µm × 2 cm; 2 min at 5 $\mu L.min^{-1}$ using 2 % ACN/0.2 %
176 FA). Trapped peptides were gradient eluted and separated on a Waters nanoEase CSH C18 column (75
177 µm × 25 cm, 1.7 µm particle size) at a flowrate of 0.3 $\mu L.min^{-1}$ with a gradient of 10-55 % B over 10
178 min (A: 0.1 % FA; B: 80 % ACN/0.1 % FA). The 6600 TripleTOF mass spectrometer was operated in
179 positive ion mode. Data-dependent acquisition (DDA) was employed; precursor (MS) scans were
180 acquired from *m/z* 400-1500 (2^+-5^+ charge states) using an accumulation time of 100 ms followed by 40
181 fragment ion (MS/MS) scans, acquired from *m/z* 100-1800 with 20 ms accumulation time each.

182 **2.10 Data analysis**

183 Raw data files were searched with Protein Pilot V5.0 software (SCIEX), using a database containing
184 sequences from *Bacillus subtilis* downloaded from UniProt (Swiss-Prot and TrEMBL on 20 August
185 2021) and common contaminants. Trypsin was set as the digestion enzyme, cysteine alkylation
186 (iodoacetamide) was allowed as a fixed modification and biological modifications allowed in the search
187 parameters. A 1 % false discovery rate filter was applied at the protein level for refinement of
188 identifications. Molecular function data were acquired from the UniProKB (www.uniprot.org) database
189 [26].

190

3. Results and Discussion

3.1 Antimicrobial activity of the BLIS

The antimicrobial spectrum of the BLIS was determined on Gram-negative and Gram-positive pathogens. The BLIS showed complete inhibition against *E. coli, S. aureus,* and partial inhibition against *S. epidermis* and *S. saprophyticus* (**Table 1**). Inhibitory activity against Gram-negative bacteria was lower compared with Gram-positive bacteria. Bacteriocins produced by Gram-positive tend to be more inhibitory towards Gram-positive pathogens and less effective towards Gram-negative bacteria [1,27]. The reason being bacteriocins inhibit similar or closely related species [28]. The results correlate with other studies where the BLIS secreted by *B. amyloliquefaciens* An6 exhibited antibacterial activity against *S. aureus* and *E. coli* [29], and bacteriocin isolated from *B. subtilis* GAS101 exhibited inhibition against *E. coli* and *S. epidermis* [21]. Sonorensin isolated from *Bacillus sonorensis* MT93 had inhibition against *E. coli* and *S. epidermis* [4].

Table 1| Antimicrobial spectrum of the bacteriocin-like inhibitory substance produced by *Bacillus paranthracis* strain MSHD3.

Bacterial indicator	Zone of inhibition	Zone of inhibition (mm)
Bacillus cereus ATCC 10876	-*	0
Enterococcus faecium ATCC 13048	-	0
Escherichia coli ATCC 10536	++*	10
Klebsiella pneumonia ATCC 10031	-	0
Klebsiella oxytoca ATCC 13182	-	0
Mycobacterium smegmatis ATCC 21293	-	0
Staphylococcus auerus ATCC 25923	++	18
Staphylococcus epidermis ATCC 14990	+*	15
Staphylococcus saprophyticus ATCC 15305	+	9
Veillonella purvula ATCC *10790*	-	0

*(-) no inhibition; (+): partial inhibition; (++): complete inhibition

3.2 Minimum inhibition concentration of BLIS

It is essential to perform minimum inhibition concentration of bacteriocin that exhibit antibacterial activity against clinically relevant pathogenic strains to determine the required dosage concentration [30]. The BLIS inhibited 88 and 90 % growth of *S. aureus* at concentration of 14 mg/mL and 7 mg/mL, respectively, and inhibited the growth of *E. coli* with an inhibition percentage of 73 % at 14 mg/mL and 69 % at 1.76 mg/mL. The BLIS inhibited less than 59 % growth of S. *epidermis* and S. *saprophyticus* (**Fig. 1**). The minimum inhibition concentrations of the BLIS secreted by *B. paranthracis* strain MHSD3 were higher compared to those reported by Ramachandran et al. [16] and Epparti et al [31]. An antimicrobial peptide secreted by *Bacillus subtilis* RLID 12.1 exhibited inhibition against *E. coli* with an MIC of 0.32 mg/mL [16]. Furthermore, bacteriocin secreted by *Bacillus subtilis* SC3.7 exhibited inhibition against *S. aureus* with and MIC of 0.012 mg/mL [31].

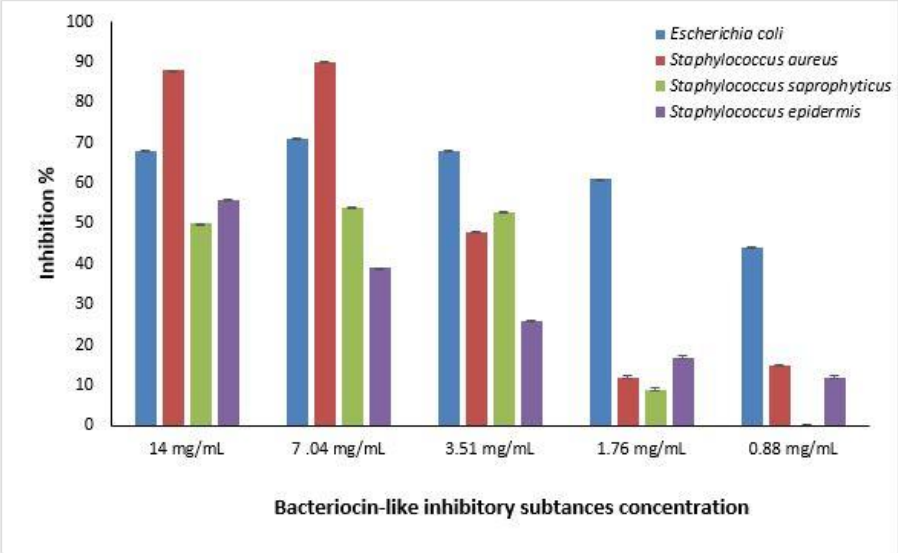

Fig. 1| Minimum inhibition concentration of the bacteriocin-like inhibitory substances against 4 test strains.

3.3 Effect of temperature, pH, enzyme action, and solvent on crude bacteriocin activity

Table 2 summarizes the effects of various treatments and conditions on the activity of bacteriocin-like substance isolated from *B. paranthracis* strain MHSD3. The BLIS retained 90 % of the activity at 40 ^{o}C and lost 32 % of activity at 80 and 100 °C for 30 minutes. Most bacteriocin and BLIS produced by *Bacillus* spp. are heat resistant but their optimal temperature with highest antimicrobial activity differs depending on species [3,32]. Bacteriocin or BLIS isolated from *Bacillus cereus* 8A [33], *B. subtilis* GAS101 [21], *B. amyloliquefaciens* An6 [29], *B. subtilis* BS15 [34] and *B. subtilis* NCIMB 3610 [35], were relatively heat stable with bacteriocin activity ranging from 95-100 % at temperatures from 30 to

100 °C for 30 min. The bacteriocin after exposure of varying pH, maintained 98 % activity at pH 8 but decreased to 90 % at pH 2 and 4. Bacteriocins which have pH stability to both acidic and alkaline environment, have a better advantage for treatment of gastrointestinal infection where drugs are administered orally [32]. Therefore, bacteriocin producing strain could potentially be used in pharmaceuticals as a probiotic for humans and animals [36].

The susceptibility of the bacteriocins to specific enzymes is based mainly on peptide formation and its amino acid sequence [32]. The BLIS exhibited activity against *S. auerus* after treatment with 1mg/mL proteinase K, lysozyme, and α-amylase. Lajis [32] reported that bacteriocins that are resistant towards enzymes found in the digestive system such as pepsin, amylase, and trypsin, are ideal candidates to inhibit infectious pathogens in the gut. Bacteriocin-producing strains can be used as probiotics due to their ability to inhibit pathogenic bacteria in the gut [11,37]. *Bacillus paranthracis* strain MHSD3 can survive in harsh gastrointestinal conditions such as acidic pH and bile salts, possess genes that play a role in acid and bile salt tolerance, adhesion, and as well as production of antimicrobial compounds [20]. Thus, *B. paranthracis* strain MHSD3 could be used as a potential probiotic strain with added benefit as a bacteriocin-like inhibitory substance producing strain.

The BLIS displayed decreasing activity when treated with ethyl acetate and methanol with approximately 72 and 76% respectively. Sixty percent of antibacterial activity was lost when BLIS was treated with chloroform. Solvents are used in oral drug formulation and medicinal cutaneal creams. Therefore, stability of bacteriocin activity in solvents indicates that such solvents can be suitable for antimicrobial drug formulation [32,30]. Similar results were reported where bacteriocins secreted by *B. amyloliquefaciens* RX7 was stable in the presence of methanol with 80% activity [1]. However, other bacteriocins secreted by *B. subtilis* BS15 and *B. amyloliquefaciens* RX7 were stable in the presence of methanol with bacteriocin activity of 100% and 80%, respectively [1,34], which may indicate that these bacteriocins do not contain lipids in their structure [32].

265 **Table 2| Heat, pH, enzyme, and solvent stability tests of the bacteriocin-like substance.**

Stability	**Bacteriocin activity (%)**
Control	100
Temperature	
40 ºC	90
80 ºC	68
100 ºC	68
pH	
2	91
4	90
6	90
8	98
10	91
Enzyme (1 mg/mL)	
Proteinase K	91
Lysozyme	81
α-amylase	73

Solvents	
Chloroform	40
Ethyl acetate	72
Methanol	76

Bacteriocin activity compared with bacteriocin activity before the treatment.

3.4 LCMS analysis for bacteriocin/bacteriocin-like peptides

The LC-MS analysis resulted in the identification of more than 60 protein peptides which were grouped based on their respective global pathways such as purine and pyrimidine biosynthesis, carbohydrate metabolism, amino acid metabolism, biosynthesis of secondary metabolites, cell wall and cellular defense, DNA replication and repair and antibacterial and peptidase act (**Table 3**). The additional protein table is represented in Supplementary data Table S1. The results revealed a bacteriocin-like inhibitory substance (biofilm matrix protein *TasA*) with molecular weight of approximately 29 kDa (**Fig. 2**). Biofilm matrix protein *TasA* has been reported to exhibit antibacterial activity against a variety of Gram-positive and Gram-negative bacteria which include *Agrobacterium tumefaciens* GV3101, *Pseudomonas aureofaciens*, *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *S. epidermis* ATCC 12228, *Micrococcus luteus* ATCC 4698 and *Enterococcus faecalis* ATCC 29212 [38]. Among the proteins produced, two proteins (Acetoin:2,6-dichlorophenolindophenol oxidoreductase subunit beta and 1,4-dihydroxy-2-naphthoyl-CoA synthase) were associated with secondary metabolite biosynthesis. Biosynthesis pathway of secondary metabolites involves genes that cluster together in a genetic package which are referred as biosynthetic gene clusters (BGCs) [39]. Biosynthetic gene clusters contain genes encoding all key enzymes that catalyze the production of secondary metabolites such as non-ribosomal peptide synthase (NRPS), polyketide synthase (PKS), and ribosomally synthesized and post-translationally modified peptide (RiPP) family clusters [40,41]. The BGCs also consists of essential genes involved in regulation, transportation of secondary metabolites, precursor biosynthesis enzymes and genes encoding for resistance gene [39,42].

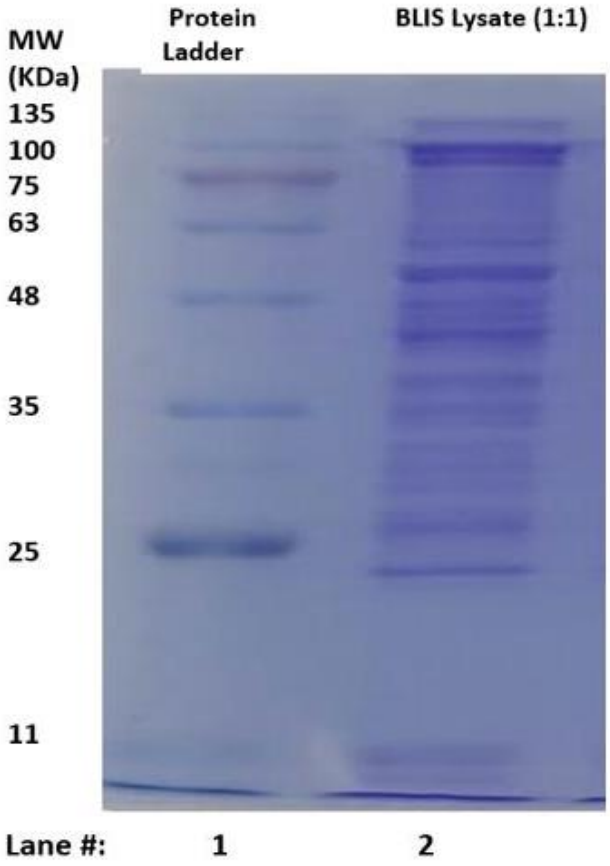

Fig. 2| SDS page gel of dialyzed bacteriocin-like inhibitory substance extract. Lane 1: pre-stained low-range molecular marker (11- 180 kDa); lane 2: BLIS lysate (1:1; sample buffer: BLIS extract).

Three heat shock proteins were also detected (ATP-dependent Clp protease ATP-binding subunit *ClpX*, 60 kDa chaperonin and Chaperone protein *DnaK*). Heat shock proteins include chaperones and proteases that are essential for protecting the bacterial cell under abnormal conditions such protein denaturation [43]. In addition, chaperones are responsible for assisting in the folding of a newly synthesized protein, preventing immature folding proteins, aggregation of protein under stressful conditions, and recovering proteins that have been partially or completely unfolded by stresses such as unexpected increased temperature [44]. A Previous study indicated that the production of chaperones which are used to repair and stabilize proteins can aid probiotic bacteria to acquire tolerance or adapt to acidic environments [45]. Four proteins were associated with cell wall and cellular defenses and two proteins associated with DNA replication and repair. Cellular defense protein, Alkyl hydroperoxide reductase subunit F plays a role in protecting the cell against oxidative stress by detoxifying peroxides [46]. DNA damage may occur due to exposure to ultraviolet light, chemical mutagens or ionizing radiation, oxidative compounds, and some antibiotics [47]. In such instances, cell growth is halted as

DNA replication becomes blocked, which induces SOS response (*LexA* and *RecA* genes), which regulates DNA damage repair [47,48].

Metabolism related proteins

Several other proteins related to various metabolic pathways were also detected from *B. paranthracis* strain MHSD3 BLIS which includes amino acid biosynthesis (S-adenosylmethionine synthase, 1-pyrroline-5-carboxylate dehydrogenase and glutamine synthetase) lipid metabolism (alpha-ketoacid dehydrogenase subunit beta and enoyl-[acyl-carrier-protein] reductase [NADH]) carbohydrate metabolism (ATP synthase subunit alpha, aconitate hydratase and acetate kinase), purine and pyrimidine biosynthesis (CTP synthase, inosine-5'-monophosphate dehydrogenase and pyrimidine-nucleoside phosphorylase) and protein biosynthesis and modification (protein translocase subunit *SecA and* cell shape-determining protein *MreB)*. Enzymes that are involved in the biosynthesis of amino acids are essential for the growth and survival of bacteria [49]. Carbohydrates serve as major energy source for bacteria, and are involved in a variety of cellular processes, such as cell wall biosynthesis [50], and cellular respiration [51]. Purines and pyrimidines are constituents of various coenzymes that are involved in energy carrying reactions, in the transfer of organic molecules and in oxidation-reduction reactions [52]. In addition, purine and pyrimidine biosynthesis emerge as key pathways responsible for antibiotic effectiveness [53]. According to Sharma et al. [54] pyrimidines possess a broad spectrum of biological activities such as antitumor, antibacterial, and anti-HIV.

Peptides identified that are involved in protein biosynthesis include cell shape-determining protein *MreB,* alkyl hydroperoxide reductase subunit F and thioredoxin reductase. Protein synthesis play a pivotal role during extended periods of growth as the bacterial cells needs to prioritize and conserve their energy towards biological processes that are essential in sustaining growth and survival during nutrient starvation and harsh stress conditions [55].

Siderophore biosynthesis

Only one protein (Ferric iron uptake transcriptional regulator) involved in siderophore biosynthesis was observed. Siderophore biosynthetic genes in bacteria involves the universal repressor ferric uptake regulator (Fur), which acts together with iron as a negative regulator. Ferric uptake regulator is a transcription factor which utilizes Fe2+ as a corepressor and represses siderophore in pathogens [56]. The siderophore biosynthesis pathway is another attractive target for antimicrobial therapeutics [57].

337 **Table 3| Expressed proteins identified from *Bacillus paranthracis* strain MHSD3 BLIS.**

Accession number	Protein Id	Function	References	Pathway
A0A6M3Z9J0	Inosine-5'-monophosphate dehydrogenase	Catalyzes the conversion of inosine 5'-phosphate (IMP) to xanthosine 5'-phosphate (XMP). This subpathway is part of the pathway XMP biosynthesis via de novo pathway, which is itself part of Purine metabolism.	[58]	**Purine and Pyrimidine biosynthesis**
A0A6M4JQR2	Pyrimidine-nucleoside phosphorylase	Involved in Pyrimidine metabolism	[59]	
A0A6M4JMM6	S-adenosylmethionine synthase	This protein is involved in step 1 of the subpathway that synthesizes S-adenosyl-L-methionine from L-methionine. This subpathway is part of the pathway S-adenosyl-L-methionine biosynthesis, which is itself part of Amino-acid biosynthesis.	[60]	**Amino acid Biosynthesis**
A0A6M3ZH77	1-pyrroline-5-carboxylate dehydrogenase	This protein is involved in step 2 of the subpathway that synthesizes L-glutamate from L-proline. This subpathway is part of the pathway L-proline degradation into L-glutamate, which is itself part of Amino-acid degradation.	[61]	
A0A6M3ZB07	Glutamine synthetase	Glutamine synthetase that catalyzes the ATP-dependent conversion of glutamate and ammonia to glutamine	[62]	

A0A6M4JKG0	ATP-dependent Clp protease ATP-binding subunit *ClpX*	ATP-dependent specificity component of the Clp protease. Uses cycles of ATP binding and hydrolysis to unfold proteins and translocate them to the *ClpP* protease.	[63]	**Heat shock proteins**
A0A6M4JJA3	Chaperone protein *DnaK*	The Hsp70/*DnaK* chaperone participates in the folding of newly synthesized proteins, transport of proteins across membranes, reactivation of misfolded proteins, disaggregation of aggregated proteins, and control of activity of regulatory proteins	[64]	
A0A6M3Z7Y6	60 kDa chaperonin	The 60kDa Chaperonin are heat shock proteins which are important for protein folding under both normal and stressful conditions	[65]	
A0A6M3ZFG4	Acetate kinase	This protein is involved in step 1 of the subpathway that synthesizes acetyl-CoA from acetate. This subpathway is part of the pathway acetyl-CoA biosynthesis, which is itself part of Metabolic intermediate biosynthesis.	[66]	**Carbohydrate metabolism**
A0A6M3ZBI8	Aconitate hydratase	Catalyzes the isomerization of citrate to isocitrate via cis-aconitate. This protein is involved in step 2 of the subpathway that synthesizes isocitrate from oxaloacetate. This subpathway is part of the pathway tricarboxylic acid cycle, which is itself part of Carbohydrate metabolism.	[67]	

A0A6M3ZE63	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (flavodoxin)	involved in step 5 of the subpathway that synthesizes isopentenyl diphosphate from 1-deoxy-D-xylulose 5-phosphate. This subpathway is part of the pathway isopentenyl diphosphate biosynthesis via DXP pathway, which is itself part of Isoprenoid biosynthesis.	[68]	**Secondary metabolite biosynthesis**
A0A6M3ZIS3	1,4-dihydroxy-2-naphthoyl-CoA synthase	This protein is involved in step 6 of the subpathway that synthesizes 1,4-dihydroxy-2-naphthoate from chorismate. This subpathway is part of the pathway 1,4-dihydroxy-2-naphthoate biosynthesis, which is itself part of Quinol/quinone metabolism.	[69]	
A0A6M4JNV2	Cell shape-determining protein *MreB*	Forms membrane-associated dynamic filaments that are essential for cell shape determination	[70]	**Protein biosynthesis and modification**

338

4. Conclusion

The bacteriocin-like inhibitory substances produced by *Bacillus paranthracis* strain MHSD3 exhibited antibacterial activity against *Escherichia coli, Staphylococcus aureus, S. saprophyticus and S. epidermidis.* The BLIS showed good pH, heat, and enzyme and solvent stability except for chloroform. The BLIS showed potential to be used as an antimicrobial agent in pharmaceuticals. Thus, we recommend further studies for the purification and further characterization of the BLIS, and their mechanism of action on pathogenic strains.

Authors contributions

MHSD and AA designed the study. MOD performed the experiments and drafted the manuscript. MOD and AA analyzed the data. MHSD and AA reviewed the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no competing interests.

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