Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Bacillus cereus MHI 86 and Bacillus thuringiensis 5-29 both produce the antilisterial bacteriocin thuricin S – analysis of antimicrobial spectrum, stability, and producer strain suitability, including cytotoxicity evaluation

Saime Gülsüm Batman

Saime.Guelsuem.Batman@tiho-hannover.de

University of Veterinary Medicine Hannover

Sophie Kittler

University of Veterinary Medicine Hannover

Madeleine Plötz

University of Veterinary Medicine Hannover

Gerald Dräger

Leibniz University Hannover

Nadja Jessberger

University of Veterinary Medicine Hannover

Research Article

Keywords: antibacterial activity, antimicrobial peptides, bacteriocin, thuricin, food safety, Listeria monocytogenes

Posted Date: November 5th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11442499/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

Thuricin-like bacteriocins are known for their potent antibacterial properties. This study characterizes thuricin S from *Bacillus (B.) cereus* MHI 86 and *B. thuringiensis* 5–29 previously isolated from foods. Cell-free supernatants and partially purified thuricin S exhibited antibacterial activity against Gram-positive species, including *Listeria* and *Bacillus*. Thuricin S production started at mid exponential growth phase. Minimum inhibitory concentrations against *L. monocytogenes* were 5.73 µg/mL (MHI 86) and 10.77 µg/mL (5–29). Both genomes harbor thuricin S-encoding open reading frames. From 8 h of growth, transcription of the bacteriocin gene cluster was consistently detected with most prominent bands at 120 bp. Accordingly, antibacterially active peptides were found at ~ 3.1 kDa, consistent with the size of thuricin S. Ultra-performance liquid chromatography-electrospray ionization mass spectrometry confirmed the molecular mass of the partially purified peptides as 3137.6 Da. The bacteriocins were active at neutral and acidic pH (3–7) and up to 80°C. In conclusion, this study is the first to provide a comparative characterisation of thuricin S in two different *Bacillus* spp. It sheds light on the genetic and transcriptional basis of thuricin S production, as well as its growth and production kinetics, antimicrobial activity, molecular weight, stability, and associated toxin production and cytotoxicity. Notably, thuricin S can be used as a natural agent against *L. monocytogenes* to control post-pasteurisation contamination.

1. Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides (RSPs) that are produced by various bacterial species. These peptides exhibit broad-spectrum antibacterial activity, particularly against closely related microorganisms, and have emerged as promising candidates for alternative antimicrobial strategies in response to the growing global challenge of antimicrobial resistance (Simons et al., 2020; Almeida-Santos et al., 2021; Gradisteanu Pircalabioru et al., 2021). Typical mechanisms of action involve disrupting bacterial membrane integrity or inhibiting fundamental cellular processes. This makes bacteriocins an effective tool for targeting pathogenic bacteria without causing significant harm to host cells or inducing cross-resistance to traditional antimicrobials (Basi-Chipalu et al., 2022). However, bacteriocins can exhibit cytotoxicity under certain conditions. This effect depends on the type of bacteriocin, its concentration, and the characteristics of the target cells. For instance, Todorov et al. (2022) emphasized the highly specific and concentration-dependent nature of cytotoxicity against Caco-2 cells, a widely used intestinal epithelial cell line, and Murinda et al. (2003) demonstrated the dose-dependent cytotoxicity of bacteriocins such as nisin, pediocin and ColE6 at concentrations exceeding their effective antimicrobial doses (170–700 activity units per ml). Beyond their therapeutic potential, bacteriocins are increasingly recognized for food preservation and safety applications (Molujin et al., 2022; Todorov et al., 2022). This dual utility in both medical and food safety applications underlines the importance of further studies on their potential antimicrobial activity against pathogenic and spoilage microorganisms, as well as their occurrence in various bacterial species. Furthermore, there is currently little information available on the genetic prerequisites and the mechanisms involved in bacteriocin production.

Bacillus spp. are well-known producers of bacteriocins, making them potential candidates for application in the food industry (Basi-Chipalu et al., 2022; Mercado and Olmos, 2022). Bacteriocins produced by *Bacillus* species can be grouped into three classes: I. small (≤ 5 kDa), post-translationally modified polycyclic peptides, II. small (0.77-10 kDa), non-modifed peptides, and III. large (≥ 30 kDa), heat-labile proteins (Abriouel et al., 2011; Basi-Chipalu et al., 2022; Mercado and Olmos, 2022). The most investigated species in terms of bacteriocin production is *Bacillus subtilis*, followed by others such as *B. amyloliquefaciens*, *B. brevis*, *B. licheniformis*, and *B. velezensis* (Scholz et al., 2014; Sharma et al., 2018; Caulier et al., 2019; Halami, 2019; Basi-Chipalu et al., 2022, Puan et al., 2023). The present study focuses on *B. cereus*, primarily known for causing gastrointestinal diseases (Dietrich et al., 2021; Jessberger et al., 2023), and on the closely related *B. thuringiensis*, which is widely used as a biological insecticide due to its ability to produce large protein crystals with insecticidal properties (Palma et al., 2014; Biggel et al., 2022). To date, more than 50 bacteriocins, belonging to class I or II, have been described to be synthesized by *B. cereus* and *B. thuringiensis* (Morandini et al., 2024).

The thuricin family of bacteriocins, which includes thuricin S, thuricin 17, bacthuringin F4 and thurincin H, are antimicrobial peptides produced by various strains of *B. thuringiensis*. Following ribosomal synthesis, they undergo extensive post-translational modification (Gray et al., 2006; Chehimi et al., 2007; Lee et al., 2009a). Their most distinctive feature is the DWTXWSXL sequence at the amino terminus. It has been shown that the 'X' positions correspond to cysteine residues and that thurincin H and thuricin 17 have an identical mature peptide sequence (DWTCWSCLVCAACSVELLNLVTAATGASTAS) at full length. However, as only the N-terminal region has been characterized for thuricin S and bacthuricin F4, it is unclear whether these peptides have a fully identical sequence (Lee et al., 2009b; Sit et al., 2011; Ruiz-de-Anda et al., 2025). Of this thuricin group, thurincin H is the best studied regarding its biosynthesis (Lee et al., 2009a; Wieckowski et al., 2015; Ruiz-de-Anda et al., 2025).

However, especially in the case of *B. cereus* and *B. thuringiensis*, it is equally important to determine their cytotoxic activity. Key toxins in *B. cereus* include the heat-stable emetic toxin cereulide and the diarrheal enterotoxins CytK, Nhe, and Hbl. Additional enzymes and proteases may also contribute to virulence (Frees et al., 2013). Assessing these factors ensures the selection of safe strains for bacteriocin-based food preservation (Parada Fabián et al., 2025). The present study aimed to characterize a collection of *B. cereus* and *B. thuringiensis* isolates, mainly from food samples, for production of antimicrobial, mainly antilisterial compounds. The most effective isolates, *B. cereus* MHI 86 and *B. thuringiensis* 5–29, were compared across genetic and transcriptional bacteriocin profiles, growth and bacteriocin production kinetics, antimicrobial activity, mass-spectrometry-based molecular weight determination, stability (enzyme, pH, heat) and further toxin production to evaluate both functional equivalence and safety implications.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

A panel of 135 *Bacillus* isolates, mainly from food samples, was used in this study. It comprised 94 *B. cereus* and 41 *B. thuringiensis* isolates. Several of these were kindly provided by Prof. Erwin Märtlbauer, Ludwig-Maximilians University Munich, Germany and Dr. Mareike Wenning, Bavarian Health and Food Safety Authority, Oberschleißheim, Germany. The following strains were used as indicators for antibacterial activity: *B. cereus* DSM 4222, *Campylobacter jejuni* BfR C19285, *Escherichia coli* DSM 1103, *Enterococcus faecalis* (own field isolate), *Enterococcus hirae* (own field isolate), *Lactobacillus casei* DSM 20011, *L. innocua* (own field isolate), *L. monocytogenes* DSM 19094 and DSM 20600, *Salmonella* Typhimurium DSM 1020, and *Staphylococcus aureus* ATCC 29213. Antimicrobial activity against *Candida albicans* DSM 1386 was also tested. All bacteria were maintained as stock cultures in 20% glycerol at -80°C and initially spread on sheep blood agar (Oxoid GmbH, Wesel, Germany). The indicator strains were then grown in LB broth (Luria/Miller; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) overnight at 37°C. For the antimicrobial activity test, *Lact. casei* DSM 20011 was grown on de Man, Rogosa, and Sharpe (MRS) agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) under anaerobic conditions, while *Cand. albicans* DSM 1386 was cultured on yeast peptone dextrose (YPD) agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Brain Heart Infusion (BHI) broth was used in minimum inhibitory concentration (MIC) analysis. To test for bacteriocin production, the *Bacillus* candidate strains were grown in 50 mL tryptic soy broth (TSB) (R-Biopharm AG, Darmstadt, Germany) for 24 h at 37°C in a shaker rotating at 130 rpm.

2.2. Evaluation of antimicrobial activity in agar well diffusion assays

Antimicrobial activity of the isolates was tested using the agar well diffusion method according to Batdorj et al. (2006) with some modifications. After cultivation in TSB for 24 h at 37°C and 130 rpm, cell-free supernatants (CFSs) were prepared by centrifugation (8000 ×g, 4°C, 20 min) and filtration through a 0.22 µm filter membrane (Sarstedt AG & Co. KG, Nümbrecht, Germany). A volume of 200 µL of overnight cultures (~ 10^8 CFU/mL) of the indicator strains was used for inoculation to produce a thin lawn on the agar. Subsequently, 5 mm diameter holes in the agar plates were filled with 60 µL of the CFSs. Plates were left for two h to allow the supernatant to diffuse into the agar before they were incubated for 24 h at 37°C. After incubation, the diameters of the resulting growth inhibition zones were determined.

2.3. Growth and bacteriocin production kinetics

To evaluate time-dependent growth and bacteriocin production, bacteria were cultured in triplicate in 150 mL TSB at 37°C under continuous shaking at 130 rpm. Two mL samples were collected and optical density at 600 nm (OD_{600}) was determined every 2 h. Subsequently, samples were centrifuged (8000 ×g, 4°C, 20 min) and cell pellets were used to extract total ribonucleic acid (RNA) (see 2.7) to investigate bacteriocin gene expression. Supernatants were filtered through a 0.22 µm filter membrane (Sarstedt AG & Co. KG, Nümbrecht, Germany) and subsequently applied to agar well diffusion assays as described in 2.2.

2.4. Determination of bacteriocin titers and minimal inhibitory concentration (MIC)

For quantifying the produced bacteriocin titers, a 1:2 (v/v) serial dilution assay was performed following the method described by Mayr-Harting et al. (1972). In brief, CFSs were serially diluted two-fold in 10 mM phosphate-buffered saline (PBS, pH 7) to determine bacteriocin concentrations at varying dilutions. Aliquots of 30 µL from each dilution were applied to agar well diffusion assays (compare 2.2). After 24 h incubation at 37°C, the plates were evaluated for the presence and size of inhibition zones. Bacteriocin activity was quantified in arbitrary units per milliliter (AU/mL). Arbitrary units (AU/mL) were determined as the highest dilution factor that gave a minimal visible inhibition zone against indicator strains (D) and the volume of bacteriocin used (V, in mL), multiplied by 1000. In the study, ampicillin (25 µg/mL, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was used as the positive control, and the CFS of *B. cereus* DSM 4222 was used as the negative control.

MIC was measured using a microplate dilution method, based on established methodologies (Aka-Gbezo et al., 2014; Balouiri et al., 2016; Cao et al., 2019; Fugaban et al., 2021), with modifications. Twofold serial dilutions of partially purified thuricin S from *B. cereus* MHI 86 and *B. thuringiensis* 5–29, with initial stock solutions, were prepared in sterile phosphate-buffered saline (PBS, pH 7.0). A fixed volume (100.0 µL) of each dilution was combined with 100.0 µL of BHI broth containing approximately 10^6 CFU mL^{-1} of the *L. monocytogenes* DSM 20600 indicator strain in microplate wells. For comparison purposes, ampicillin was similarly diluted twofold and tested under identical conditions. The microplates were incubated at 37°C for 24 h. Bacterial proliferation was monitored in triplicate by measuring absorbance at 595 nm using a microplate reader. MIC was defined as the lowest concentration that completely prevents the visible growth of the indicator strain (Wayah and Philip, 2018; Cao et al., 2019). Negative controls were prepared using 200 µL BHI broth, while positive controls included 100 µL BHI broth with 100 µL of the 10^6 CFU mL^{-1} *L. monocytogenes* DSM 20600 suspension. Protein concentration of the peptides was measured by Coomassie Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA) (Cintas et al.,1998). All experiments were performed in triplicate.

2.5 Prediction of the bacteriocin gene cluster

To identify genes encoding bacteriocins, the genome sequences of the strains *B. cereus* MHI 86 (NCBI GenBank accession number LABK00000000; Böhm et al., 2015; Jessberger et al., 2015) and *B. thuringiensis* 5–29 (NCBI GenBank accession number SAMN60754827; kindly provided by Dr. Mareike Wenning, Bavarian Health and Food Safety Authority, Oberschleißheim, Germany) were used. AntiSMASH 8.0.4 (https://antismash.secondarymetabolites.org/#!/start) was used to identify secondary metabolite gene clusters in the genomes of *B. cereus* MHI 86 and *B. thuringiensis* 5–29. Potential bacteriocin genes were screened and identified using the BAGEL4 web server (http://bagel4.molgenrug.nl/; van Heel et al., 2018), and the predicted bacteriocin sequences were analysed for similarity using NCBI BlastP (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. DNA extraction and PCR

Samples for DNA extraction and transcription analysis were collected during growth kinetic experiments from cultures grown in 150 mL TSB at 37°C under continuous shaking at 130 rpm. Genomic DNA from the *Bacillus* strains was extracted using the DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany). Specific primers for the thuricin were designed using Primer3web (https://primer3.ut.ee/). The primers are listed in Table S1. The amplification process was carried out in an S1000 thermal cycler (Biometra GmbH, Göttingen, Germany) following the cycling conditions specified in Table S1.

2.7 RNA extraction, cDNA synthesis, and reverse transcription polymerase chain reaction

Following genomic DNA extraction and PCR-based confirmation using species-specific primers, total RNA was extracted from the confirmed strains using the RNeasy Plus Mini Kit Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) based on absorbance at 260 nm; duplicate measurements were performed for each RNA sample, and the mean value was used for subsequent analyses. Subsequently, cDNA synthesis was carried out using the Protoscript cDNA Synthesis Kit (New England Biolabs, (UK) Ltd., Hitchin, UK). Two µL of the resulting cDNA of the samples were amplified by the reverse transcription polymerase chain reaction (RT-PCR) (20 µL) using the primers and parameters shown in Table S1 and an S1000 thermal cycler (Biometra GmbH, Göttingen, Germany). Original sample DNA was used as positive control in each reaction. As a negative control, water was used in place of the cDNA template. One-tenth of the amplification mixture was then analyzed by gel electrophoresis on a 1.5% agarose gel. The 100-bp DNA ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to estimate the length of the PCR products.

2.8. Purification of bacteriocins

CFSs were obtained by cultivation, centrifugation, and filtration as stated in 2.1 and 2.2. After that, they were subjected to ammonium sulfate precipitation at 70% saturation and incubated at 4°C for 12 h. The precipitate, recovered by centrifugation (15,000 ×g, 20 min, 4°C), was resuspended in 10 mM PBS (pH 7.0) using the minimum necessary volume. The suspension was dialyzed using a 2 kDa cut-off dialysis membrane (Thermo Fisher Scientific Inc., Waltham, MA, USA) against the same buffer at 4°C for 24 h. Following dialysis, 2 mL suspension was applied to a Sephadex® G-10 matrix (Merck KGaA, Darmstadt, Germany) in a glass column (1 x 20 cm, Bio-Rad Laboratories GmbH, Feldkirchen, Germany) equilibrated with 10 mM PBS (pH 7.0) at a flow rate of 1 mL/min. Active fractions of 2 mL were collected and concentrated by serially passing through Amicon® ultra centrifugal filters with molecular weight cut-offs of 10 kDa and 3 kDa as recommended by the supplier (Merck KGaA, Darmstadt, Germany).

2.9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

To estimate the molecular weight of partially purified bacteriocins, SDS-PAGE analysis was performed on a gradient gel (16%, 10%, 4%) according to Schägger (2006); electrophoresis was carried out at a

constant voltage of 180 V for 1.5 h in 100 mM tris-tricine buffer (pH 8.25). A low range protein marker (3-200 kDa; Thermo Fisher Scientific Inc., Waltham, MA, USA) was used as molecular weight standard. Half of the gel was tested for antibacterial activity following the procedures described by Gaspar et al. (2018). The gel was fixed for 1 h in a solution containing 70% sterile water, 20% isopropanol, and 10% acetic acid to remove SDS from the gel and fix the bands. This was followed by rinsing for at least 3 h in sterilized water. Subsequently, the gel was aseptically transferred to a petri dish. LB agar containing the indicator strain *L. monocytogenes* DSM 20600 (~ 10^8 CFU/mL) was added to the Petri dish and incubated at 37°C for 12 hours to observe the inhibition zones. As a control, CFS from non-bacteriocin-producing *B. cereus* DSM 4222 strain was used. To identify the protein bands, the remaining part of the gel was stained with 0.02% Coomassie R-250 in 30% methanol and 10% acetic acid for 20 minutes and destained in 8% acetic acid until the bands were visible.

2.10. Ultra-performance liquid chromatography-electrospray ionization-mass spectrometry (UPLC-ESI-MS) analysis

Mass spectrometry (MS) was performed using a Premier Mass Spectrometer (Waters Inc., Milford, MA, USA) operating in electrospray ionization (ESI) positive mode (V-mode). The parameters were as follows: capillary voltage = 3.0 kV, sample cone voltage = 20 V, extraction cone voltage = 2.0 V, source temperature = 105°C, desolvation temperature = 250°C, and desolvation gas flow = 650 L/h nitrogen. The mass range was set to 70–3000 m/z, with a scan time of 0.5 s. Chromatographic separation was achieved using an Acquity UPLC system (Waters Inc.) equipped with a binary solvent manager, sample manager, column manager, and Acquity UPLC BEH300 C4 1.7 µm LC column (50 × 2.1 mm) (Waters Inc., Milford, MA, USA) at 35°C. The mobile phase consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid), with the following gradient: 5% B (0 min) → 80% B (4.5.0 min) → 80% B (6.5 min) → 5% B (6.6 min). The flow rate was set at 0.4 mL/min, the total run time was 8 min per injection, and the injection volume was 5 µL.

2.11. Effect of pH, temperature, and enzymes on bacteriocin activity

The stability of the bacteriocins was assessed at various pH levels, temperatures, and enzyme treatments according to Ansari et al. (2018). Thermal stability assays were conducted by exposing the CFSs to temperatures of 24°C (as control, similar to section 2.4), 65°C, 80°C, 100°C, and 121°C for 10 minutes. pH stability assays were performed by adjusting the CFSs to pH values ranging from 3.0 to 9.0 and incubating at room temperature (24°C) for 10 minutes. pH 7.0 served as control (similar to section 2.4). pH adjustments were achieved using 50 mM citrate buffer (pH 3–5), 50 mM phosphate buffer (pH 6–7), and 50 mM glycine-NaOH buffer (pH 8–9). Proteolytic sensitivity was evaluated by incubating the CFSs with enzymes including proteinase K, pronase E, trypsin, lipase, amylase (Merck KGaA, Darmstadt, Germany), and chymotrypsin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at 37°C for 1 h at a concentration of 1 mg/mL. Prior to analysis, enzyme activity was stopped by heating at 80°C for 10

minutes. Following each treatment, the samples were evaluated for the presence and size of inhibition zones using the agar well diffusion assay as described in section 2.4.

2.12. Enzyme immunoassays

Representative for enterotoxin production, the B component of the non-hemolytic enterotoxin of *B. cereus* was detected in sandwich enzyme immunoassays (EIAs) as described previously, using monoclonal antibodies 2B11 (5 µg/mL) and 1E11-HRP (1:4000) for detection (Dietrich et al., 2005; Moravek et al., 2006). Culture supernatants of highly toxic reference strain DSM 4222 gained after 6 h of growth in casein-glucose-yeast (CGY) medium (Jessberger et al., 2015) was used as positive control. Microtiter plates were analyzed in a Tecan Sunrise photometer using Magellan 50 software (Tecan Trading AG, Männedorf, Switzerland). Results are shown as titters, which are defined as the reciprocal of the highest dilutions, resulting in an absorbance value of ≥ 1.0.

2.13. Cytotoxicity assays

Cytotoxic activity of CFSs was assessed using WST-1 bioassays on CaCo-2 cells, following the methodology described by Jessberger et al. (2014). Briefly, CaCo-2 cells were sourced from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) and maintained in culture media as recommended by the supplier and outlined by Jessberger et al. (2014). CFSs were tested in triplicate, and absorbance measurements at 450 nm were performed using a Tecan microplate reader (Tecan Trading AG, Männedorf, Switzerland).

2.14. Statistical analysis

All experiments were repeated three times. One-way ANOVA, t-tests, Tukey post-hoc tests were performed for statistical analysis. Differences were considered statistically significant at the $p < 0.05$ (two-tailed) level with a 95% confidence interval. Growth curves, dose-response curves of WST-1 biological assays, and half-maximal inhibitory concentrations (IC_{50}) were generated and calculated using GraphPad Prism v10.0.0 (GraphPad Software Inc., Boston, MA, USA; www.graphpad.com).

3. Results

3.1. Selection of isolates due to antibacterial activity against Listeria spp.

Of the 135 tested *B. cereus* and *B. thuringiensis* isolates, 10 showed antibacterial activity against *L. monocytogenes, L. innocua, Ent. faecalis*, *Ent. hirae*, and *B. cereus* DSM 4222 in preliminary agar well diffusion assays (Table S2). None of the tested isolates were active against *C. jejuni* BfR C19285, *Cand. albicans* DSM 1386, *E. coli* DSM 1103, *Lact. casei* DSM 20011, *S.* Typhimurium DSM 1020, or *Staph. aureus* ATCC 29213. Based on maximum inhibition and high repeatability of the results, *B. cereus* MHI 86 and *B. thuringiensis* 5–29 were selected for further investigations ($p < 0.05$).

3.2. Growth and bacteriocin production kinetics

Bacterial growth phases and antibacterial activity of the CFSs at different time points were compared for the two selected strains MHI 86 and 5–29 over a 32-h period. In general, MHI 86 showed slightly higher growth than 5–29 in TSB medium, but this difference was not statistically significant ($p > 0.05$). Both strains entered the exponential growth phase between 4 and 6 h of incubation (Fig. 1A). After 8 h incubation, during the mid to late exponential growth phase, antibacterial activity of the CFS of both strains was detectable, with stronger activity of MHI 86, as indicated by size of inhibition zone diameters ($p < 0.05$). Antibacterial activity of strain 5–29 reached its maximum after 10 h, while activity of MHI 86 increased gradually and reached its peak during stationary growth after 24 h incubation (Fig. 1A). Furthermore, the two CFSs showed activity titers of 2083 AU/mL (MHI 86) and 1042 AU/mL (5–29) after 24 h incubation (Fig. 1B). The MICs of the partially purified thuricin S from MHI 86 and 5–29 against *L. monocytogenes* DSM 20600 were 5.73 µg/mL and 10.77 µg/mL, respectively, whereas ampicillin showed an MIC of 0.20 µg/mL.

3.3. Identification of thuricin S gene clusters

Genome mining of *B. cereus* MHI 86 and *B. thuringiensis* 5–29 using BAGEL4 and antiSMASH consistently identified a single thuricin-related RiPP locus in each genome. BAGEL4 further predicted thuricin-like core peptides within these loci (Supplementary Fig. S1A and B), whereas no additional bacteriocin biosynthetic gene clusters were detected. In agreement with the BAGEL4 predictions, antiSMASH detected a RiPP-like peptides within contig 39.1 in *B. cereus* MHI 86 and within contig 62.1 in *B. thuringiensis* 5–29, both showing similarity to thurincin H-like peptides. Genome mining with antiSMASH provided complementary support for the BAGEL4 predictions by identifying RiPP-like loci in the corresponding contigs. Therefore, only the BAGEL4 output was used for downstream annotation and comparative analyses. Multiple sequence alignment using BLAST, supplemented by manual validation via BLASTp, confirmed that the predicted mature core peptides from *B. cereus* MHI 86 and *B. thuringiensis* 5–29 exhibited amino acid sequence identity with known subclass IId bacteriocins, including thurincin H, thuricin 17, and thuricin S (Supplementary Fig. S1C). Further analyses revealed that both genomes harbor three tandem bacteriocin-encoding structural genes. Supplementary Fig. S2 shows the results of a PCR analysis using specific primers (compare Table S1). Three different amplicons were detected (577, 348, and 120 bp), confirming that the bacteriocin precursor gene clusters comprise three tandem repeats. This was verified by amplification of another 833 bp fragment applying primers spanning the entire region (Supplementary Fig. S2A). Sequence analyses identified nucleotide and amino acid sequences encoding tandem repeats 1, 2, and 3 of thuricin S (Supplementary Fig. S2B).

Areas of interest identified in the genomes of *B. cereus* MHI 86 and *B. thuringiensis* 5–29 shared consistent features, including ATP-binding cassette (ABC) transporters, proteases, and multiple ORFs. Both core peptide-containing contigs encoded the post-translational modification gene *bmbF*, while the contig from strain 5–29 additionally included *lanT*, a conserved lantibiotic transporter gene (Supplementary Fig. S1A and B). The ABC transporter genes and the *bmbF* gene sequence from both strains are identical in size (864 bp for ABC transporters and ~ 1400 bp for *bmbF*), as verified by BLAST alignment.

3.4. Transcription of the thuricin S gene cluster

The transcriptional activity of the bacteriocin-encoding genes, associated with the production of mature thuricin S, was analyzed. cDNA samples of *B. cereus* MHI 86 and *B. thuringiensis* 5–29 were collected over a 24-h growth period. Agarose gel electrophoresis (Fig. 2) revealed time-dependent transcriptional activity of the bacteriocin gene cluster. Prominent bands corresponding to the expected size of the gene products (120 bp) were observed at all sampling times after 8 h growth, indicating their consistent transcriptional presence. A single 833 bp transcript corresponding to the thuricin S gene was detected by transcriptional analysis (Supplementary Fig. S2C).

3.5. Molecular weight and mass spectra of thuricin S

For molecular weight analyses, partially purified thuricin S from *B. cereus* MHI 86 and *B. thuringiensis* 5–29 were used (see 2.8). Tricine SDS-PAGE analysis revealed distinct bands at approximately 3.1 kDa (Fig. 3A). To correlate bioactivity with molecular weight, half of the gel was subjected to agar well diffusion assays using *L. monocytogenes* DSM 20600 as indicator. A clear inhibition zone was observed at the region corresponding to the 3.1 kDa bands (Fig. 3B). ESI-MS analysis revealed two thuricins, which were predominantly found as double and triple charged ions. The main peptide was calculated with a monoisotopic mass of 3137.6 ± 0.2 (double charged ion $[M + 2H]^{2+}$ = 1569.79 and triple charged ion $[M + 3H]^{3+}$ = 1046.86), whereas the second minor peptide showed a monoisotopic mass of 3091.6 ± 0.2 (double charged ion $[M + 2H]^{2+}$ = 1547.8 and triple charged ion $[M + 3H]^{3+}$ = 1032.21). Thus, the monoisotopic masses of both antimicrobially active metabolites of *B. cereus* MHI 86 and *B. thuringiensis* 5–29 are consistent with thuricin S (Fig. 4).

3.6. Stability of thuricin S towards enzymes, pH, and temperature

The stability of the thuricin S containing CFSs was evaluated under various conditions, including enzyme treatments, heat exposure, and pH alterations (Table 1). Both thuricin S peptides demonstrated full antibacterial activity after exposure to neutral pH values (6.0–7.0). Activity was also well preserved after exposure to acidic pH levels (3.0–5.0), with a minimum activity of 94%. In contrast, a notable, more than 10% decline in antibacterial activity was observed after exposure to alkaline pH values (8.0–9.0), with the effect being more pronounced under more alkaline conditions. Regarding temperature, the peptides retained over 89% of their activity after exposure to temperatures of up to 80°C. Antibacterial activity was maintained at 87% following treatment at 100°C but declined to 63% and 57% after exposure to 121°C for *B. cereus* MHI 86 and *B. thuringiensis* 5–29, respectively. These results indicate that the isolated bacteriocins exhibited remarkable heat tolerance, though their stability diminished under extreme heat conditions. Finally, the thuricin S peptides remained stable after enzymatic treatment with trypsin, chymotrypsin, α-amylase, and lipase, with residual antibacterial activities ranging from 76% to 94%. In contrast, their activity was completely gone after proteinase K and pronase E treatment (Table 1).

Table 1

Effect of pH, temperature, and enzymes on the antibacterial activity of the thuricin S containing CFSs against *L. monocytogenes* DSM 20600. For this, inhibition zones (in mm) were determined in agar well diffusion assays in triplicate. The inhibition zones of each positive control (no treatment) were set to 100% and the inhibition zones determined after treatment were calculated according to these values.

Treatment	***B. cereus* MHI 86 *B. thuringiensis* 5–29**	
	Antibacterial Activity in %	
pH		
3.0	97	96
5.0	95	94
6.0	100	100
7.0 (non-treated control)	100	100
8.0	90	83
9.0	75	72
Temperature		
24°C (non-treated control)	100	100
65°C	97	91
80°C	95	89
100°C	87	87
121°C	63	57
Enzymes (1 mg/mL)		
Non-treated (control)	100	100
Proteinase K	0	0
Pronase E	0	0
Trypsin	87	81
Chymotrypsin	85	76
α-amylase	93	94
Lipase	93	93
Enzyme inactivation control (10 min 80°C)	95	90

3.7. Safety assessment of B. cereus MHI 86 and B. thuringiensis 5–29

The production of the non-hemolytic enterotoxin (Nhe) component B associated with the diarrheal form of disease caused by *B. cereus* was tested using sandwich enzyme immunoassay. Strains *B. cereus* MHI 86, *B. thuringiensis* 5–29 and even the highly cytotoxic reference strain *B. cereus* DSM 4222 grown for 24 h at 37°C in TSB (see above) exhibited minimal toxin production with reciprocal titers of 0, while the control CFS (DSM 4222 grown for 6 h in CGY medium) demonstrated substantial enterotoxin production with a reciprocal titer of 4000 ± 300 (Fig. 5A). The cytotoxic effect of the bacterial CFSs on CaCo-2 cells was evaluated using WST-1 bioassay. *B. cereus* MHI 86 and *B. thuringiensis* 5–29 showed no detectable cytotoxic activity (reciprocal titer = 0), and the cells remained intact as evidenced by microscopic examination (Fig. 5B). *B. cereus* DSM 4222 grown for 24 h in TSB medium exhibited minimal cytotoxicity with a reciprocal titer of approximately 50, with microscopy revealing partially intact cells. In contrast, DSM 4222 grown for 6 h in CGY medium demonstrated high cytotoxic activity as positive control. The negative control (no CFS applied) showed no cytotoxic effect, and the cells remained fully intact. All measurements were performed in triplicate, with cytotoxicity assessed through both WST-1 absorbance readings and microscopic observation of cellular integrity.

4. Discussion

In the present study, *B. cereus* MHI 86 and *B. thuringiensis* 5–29 were found to produce thuricin S with high antibacterial activity. This is not only the first time that *B. cereus* has been identified as a producer of thuricin S, but also the first time that two (closely related) species have been compared in terms of occurrence and transcription of thuricin S gene clusters, thuricin S production kinetics, molecular weight and mass spectra, as well as stability of thuricin S. Previous studies showed that *Bacillus* strains produce closely related class IId bacteriocins such as thuricin 17, thurincin H, and bacthuricin F4 (Gray et al., 2006a; Gray et al., 2006b; Kamoun et al., 2005; Chehimi et al., 2007; Lee et al., 2009b; Wang et al., 2014). Thuricin S, produced by *B. thuringiensis* subsp. *entomocidus* HD198, has also been described before, exhibiting similar properties as thuricin 17 and targeting Gram-positive pathogens such as *L. monocytogenes* and *B. cereus* (Chehimi et al., 2007). Accordingly, the antimicrobial spectrum of the CFSs of MHI 86 and 5–29 confirms the selective efficacy of thuricin S against Gram-positive bacteria, particularly *B. cereus* and *L. monocytogenes*. This is particularly important, as these species are prominent foodborne pathogens responsible for severe health hazards and economic losses in food production systems (Bogaerts et al., 2023; Hoffmann et al., 2024; Olanya et al., 2019). The absence of antimicrobial activity against *Lact. casei* and *Staph. aureus* is noteworthy, as previous studies have reported resistances of these bacteria to bacteriocins (Chapot-Chartier and Kulakauskas, 2014; Radaic et al., 2020). The lack of inhibitory activity against Gram-negative bacteria is consistent with observations for other bacteriocins such as subtilin (Wang et al., 2015). The lack of activity against *Cand. albicans* suggests that thuricin S does not possess antifungal properties.

Searching for putative antimicrobial gene clusters, three tandem genes encoding thuricin S were identified, similar to thuricin 17 and thurincin H (Lee et al., 2009a; Casados-Vázquez et al., 2018). The presence of the thuricin-specific "DWTXWSXLVXAAXSVELL" sequences should be noted, as this is a conserved feature required for the antimicrobial activities of thuricin S (Lee et al., 2009b; Abriouel et al., 2011). Notably, in the BAGEL analysis, a *lanT* ABC transporter gene (Marsh et al., 2010; O'Sullivan et al., 2011; Letzel et al., 2014), annotated as an alpha-hemolysin translocation ATP-binding protein and typically involved in the export of modified peptides, was detected in the 5–29 gene cluster. This gene also plays a key role in removing the leader peptide from the precursor to release the mature bacteriocin (Teber and Asakawa, 2024). However, it was not detected in the MHI 86 gene cluster, which may reflect variations in gene cluster organization or alternative processing mechanisms. Additionally, ABC transporters and proteases were identified, which are essential for the transport, activation, and regulation of bacteriocins. This underlines that accessory genes are often co-located with bacteriocin gene clusters and play functional roles in bacteriocin production and activity. For instance, *bmbF* was reported in *B. safensis* KCTC 12796BP, *B. cereus* CC-1, *B. spizizenii* SW83, and *B. subtilis* subsp. *subtilis* BS155 as well as *lanT* in *B. velezensis* 9912D and *B. weihaiensis* Alg07 (Teber and Asakawa, 2024).

Transcriptional analysis of the genes associated with thuricin S production in MHI 86 and 5–29 revealed insights into the regulation of thuricin S synthesis. Similar to thurincin H, where three tandem *thnA* genes (*thnA1, thnA2*, and *thnA3*) encode equivalent pre-peptides under a single promoter to ensure production (Lee et al., 2009b; Sit et al., 2011), our cDNA transcriptional profiling revealed time-dependent expression patterns for the thuricin S encoding genes, supporting the hypothesis that its synthesis is tightly regulated during the stationary growth phase. Additionally, Lee et al. (2009) proposed that the thurincin H structural genes likely arose through tandem gene duplication and are transcribed as a single transcriptional unit from one promoter, with conserved intergenic regions between repeats in both strains. Notably, this transcriptional organization observed in thuricin S bears a striking resemblance to that experimentally established for thurincin H, where tandem gene duplication coupled with single-promoter transcriptional control has been demonstrated, suggesting that this regulatory strategy may represent a conserved mechanism among structurally related bacteriocins. Given these structural and regulatory parallels, it is highly probable that the three tandem repeats of thuricin S in MHI 86 and 5–29 adopt a similarly unified transcriptional architecture, a hypothesis that warrants further investigation through dedicated promoter and transcriptional termination analyses.

Furthermore, we observed antimicrobial activity of MHI 86 and 5–29 in late exponential, peaking during stationary growth phase. Secondary metabolites, including bacteriocins, are often synthesized in response to environmental stress such as nutrient deprivation, and their production peaks when bacterial growth slows down. The observed phase-dependent production of thuricin S aligns with findings from other studies on bacteriocins produced by *Bacillus* species, including *B. subtilis* and *B. licheniformis* (Alam et al., 2011; Guo et al., 2012). The activity titers and MIC values obtained for thuricin S from MHI 86 and 5–29 indicate substantial antimicrobial potential, while their differences most likely reflect strain-specific production efficiency and peptide yield. İt also suggests that the antibacterial

activity of thuricin S produced from MHI 86 and 5–29 could be enhanced in future studies by optimising production parameters or applying additional purification steps.

While Tricine SDS-PAGE analysis and gene sequencing determine the molecular weight of both thuricin S peptides to be approx. 3.1 kDa, mass spectrometry analyses revealed two distinct peptides. The primary peptide exhibited a monoisotopic mass of 3137.6 ± 0.2 Da, derived from the double charged ion $[M + 2H]^{2+}$ at m/z 1569.79 and the triple charged ion $[M + 3H]^{3+}$ at m/z 1046.86. These values closely align with those reported for thuricin S in previous studies (Chehimi et al., 2007; Sit et al., 2011). A secondary minor peptide was also detected, with a monoisotopic mass of 3091.6 ± 0.2 Da (doubly charged ion $[M + 2H]^{2+}$ at m/z 1547.8 and triply charged ion $[M + 3H]^{3+}$ at m/z 1032.21), which may represent a variant or degradation product not emphasized in the references. Alternatively, the observed mass differences between the major and minor peptide forms may indicate post-translational modifications such as dehydration, oxidation, or partial proteolytic processing, which are common among bacteriocins (Cheng et al., 2024; Sugrue et al., 2024). The available structural and functional data differentiate thuricin S from thurincin H and other thuricin-like bacteriocins (Sit et al., 2011; Mozolewska et al., 2017; Ruiz-de Anda et al., 2025). Although the full primary sequence of thuricin S has not been reported in the original literature (Chehimi et al., 2007; Chehimi et al., 2012), its measured molecular mass of 3137.61 Da has been reported previously and was reproduced by our mass spectrometry analysis, whereas thurincin H is fully sequenced with a mass of 3139.51 Da (Lee et al., 2009b; Wang et al., 2014), a difference that while small is analytically resolvable. Importantly, the precise match between our MS value (3137.61 Da) and the value reported by Chehimi et al (2007), together with the peptide's N-terminal sequence and its antimicrobial spectrum, provides strong experimental support for assigning the bacteriocin we characterized to thuricin S rather than to thurincin H. Nevertheless, this underscores the critical need for comprehensive structural characterization in future studies to establish homologous relationships among recently reported yet incompletely characterized bacteriocins from other *Bacillus* species (Salazar-Marroquín et al., 2016; Mercado & Olmos, 2022; Doshi et al., 2024; Ruiz-de Anda et al., 2025).

Importantly, we found a broad tolerance of thuricin S from MHI 86 and 5–29 to various environmental conditions, enhancing their potential for industrial application as bio preservatives or antimicrobial agents. Full activity was found in acidic to neutral pH (3.0–7.0), similar to other bacteriocins such as nisin (Rollema et al., 1995). This makes thuricin S well-suited as an additive in acidic environments such as fermented foods, especially fermented meat products. Thermostability is another critical factor for the practical use of bacteriocins in food preservation, and thuricin S demonstrated a high tolerance to heat. Retaining over 89% of its activity at temperatures up to 80°C suggests that thuricin S could be effective under various food-processing conditions where temperatures often exceed 60–70°C. This thermal stability is particularly relevant in the event of post-pasteurization contamination of ready-to-eat meat products, such as cooked-cured sausages being sliced, as the direct addition of bacteriocins as a food additive could effectively contribute to food safety, especially when integrated into hurdle concepts (Woraprayote et al., 2016). The stability of thuricin S against enzymatic degradation by trypsin, chymotrypsin, α-amylase, and lipase suggests that it is resistant to common digestive enzymes, which is a trait for applications in food preservation or therapeutic settings. This finding is consistent with

previous reports on other bacteriocins such as nisin and lacticin 3147 (Darbandi et al., 2022; Ryan et al., 2021). The complete loss of activity following proteinase K and pronase E treatments further confirm the peptidic nature of thuricin S.

Finally, the low enterotoxin production and cytotoxic activity towards CaCo-2 cells of MHI 86 and 5–29 suggest a favorable safety profile for these strains, which is important for potential application in food preservation systems. It must be noted that the *nhe* operon is present in both strains, which encodes the protein components of Nhe associated with the diarrheal form of disease caused by *B. cereus* (Dietrich et al., 2021). Both strains therefore meet the genetic requirements, but do not appear to produce Nhe under the cultivation conditions applied in the present study. Nhe production is also the main characteristic for differentiation between high and low toxic *B. cereus* strains (Jessberger et al., 2019). Furthermore, even under conditions optimal for enterotoxin production (6 h growth in CGY medium at 32°C), these two strains produce only small amounts of Nhe (MHI 86: Jessberger et al., 2015; 5–29: data unpublished). This finding further confirms these strains safe for biotechnological and antimicrobial applications.

5. Conclusion

With *B. cereus* MHI 86 and *B. thuringiensis* 5-29, the present study identified two new, promising candidates producing thuricin S with strong antimicrobial properties towards major foodborne pathogens such as *B. cereus* and *L. monocytogenes,* but with minimal further toxic effects. These strains or purified thuricin S obtained from them can be used in food safety and preservation applications, extending shelf life and reducing the risks associated with the use of traditional food preservatives. This study also contributes to clarifying the genetic background of thuricin S production, which may in turn enable the optimization of both production and function of the bacteriocin. The observed heat, pH and enzyme stability is of special importance, which makes the identified bacteriocins particularly suitable for application in fermented foods or for counteracting post-pasteurization contamination.Ongoing studies are focusing on optimization of the bacteriocins' spectrum of activity, further elucidating their mode of action, and evaluating their practical implementation in diverse food matrices as well as their long-term stability under food processing conditions.

Declarations

CRediT authorship contribution statement

S. Gülsüm Batman: Methodology, Investigation, Formal analysis, Writing - Original Draft; **Sophie Kittler**: Conceptualization, Funding acquisition, Methodology, Supervision, Project administration, Writing - Review & Editing; **Madeleine Plötz**: Funding acquisition, Writing - Review & Editing; **Gerald Dräger**: Methodology, Investigation, Writing - Review & Editing; **Nadja Jessberger**: Conceptualization, Funding acquisition, Methodology, Supervision, Project administration, Writing - Original Draft.

Funding

This work was funded by the Fritz-Ahrberg foundation.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

The authors gratefully acknowledge the excellent technical assistance provided by Iris Oltrogge, Inna Pahl, and Olena Walter.

Supplementary Material

The Supplementary material for this article can be found online at:

References

1. Abriouel, H., Franz, C. M., Omar, N. B., Gálvez, A., 2011. Diversity and applications of *Bacillus* bacteriocins. FEMS Microbiology Reviews, 35(1), 201-232. https://doi.org/10.1111/j.1574-6976.2010.00244.x.
2. Aka-Gbezo, S., N'Guessan, F. K., Djeni, T. N. D., Djè, M. K., Bonfoh, B., 2014. Partial characterization of bacteriocins from two *Pediococcus acidilactici* strains isolated during traditional sorghum beer processing in Côte d'Ivoire. Advances in Microbiology, 4(16), 1250-1259. http://dx.doi.org/10.4236/aim.2014.416135.
3. Alam, S. I., Kamran, M., Sohail, M., Ahmad, A., Khan, S. A., 2011. Partial characterization of bacteriocin like inhibitory substance from *Bacillus subtilis* BS15, a local soil isolate. Pakistan Journal of Botany, 43(4), 2195-2199.
4. Almeida-Santos, A. C., Novais, C., Peixe, L., Freitas, A. R., 2021. *Enterococcus* spp. as a producer and target of bacteriocins: a double-edged sword in the antimicrobial resistance crisis context. Antibiotics, 10(10), 1215. https://doi.org/10.3390/antibiotics10101215.
5. Ansari, A., Zohra, R. R., Tarar, O. M., Qader, S. A. U., Aman, A., 2018. Screening, purification and characterization of thermostable, protease resistant bacteriocin active against methicillin resistant *Staphylococcus aureus* (MRSA). BMC Microbiology, 18, 1-10. https://doi.org/10.1186/s12866-018-1337-y.
6. Balouiri, M., Sadiki, M., Ibnsouda, S. K., 2016. Methods for in vitro evaluating antimicrobial activity: A review. Journal of pharmaceutical analysis, 6(2), 71-79. https://doi.org/10.1016/j.jpha.2015.11.005.
7. Basi-Chipalu, S., P. Sthapit, Dhital, S., 2022. A review on characterization, applications and structure-activity relationships of *Bacillus* species-produced bacteriocins. Drug Discoveries & Therapeutics, 16(2), 55-62. https://doi.org/10.5582/ddt.2021.01087.

8. Batdorj, B., Dalgalarrondo, M., Choiset, Y., Pedroche, J., Metro, F., Prevost, H., Chobert, J. M., Haertle, T., 2006. Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. Journal of Applied Microbiology, 101(4), 837-848. https://doi.org/10.1111/j.1365-2672.2006.02966.x.
9. Biggel, M., Jessberger, N. Kovac, J., Johler, S., 2022. Recent paradigm shifts in the perception of the role of *Bacillus thuringiensis* in foodborne disease. Food Microbiology, 105, 104025. https://doi.org/10.1016/j.fm.2022.104025.
10. Bogaerts, B., Fraiture, M.-A., Huwaert, A., Van Nieuwenhuysen, T., Jacobs, B., Van Hoorde, K., De Keersmaecker, S. C. J., Roosens, N. H. C., Vanneste, K., 2023. Retrospective surveillance of viable *Bacillus cereus* group contaminations in commercial food and feed vitamin B2 products sold on the Belgian market using whole-genome sequencing. Frontiers in Microbiology, 14, 1173594. https://doi.org/10.3389/fmicb.2023.1173594.
11. Böhm, M. E., Huptas, C., Krey, V. M., Scherer, S., 2015. Massive horizontal gene transfer, strictly vertical inheritance and ancient duplications differentially shape the evolution of *Bacillus cereus* enterotoxin operons *hbl, cytK* and *nhe*. BMC Evolutionary Biology, 15, 246. https://doi.org/10.1186/s12862-015-0529-4.
12. Cao, S., Du, R., Zhao, F., Xiao, H., Han, Y., Zhou, Z., 2019. The mode of action of bacteriocin CHQS, a high antibacterial activity bacteriocin produced by *Enterococcus faecalis* TG2. Food Control, 96, 470–478. https://doi.org/10.1016/j.foodcont.2018.09.028.
13. Casados-Vázquez, L. E., Bideshi, D. K., Barboza-Corona, J. E., 2018. Regulator ThnR and the ThnDE ABC transporter proteins confer autoimmunity to thurincin H in *Bacillus thuringiensis.* Antonie van Leeuwenhoek, 111(12), 2349–2360. https://doi.org/10.1007/s10482-018-1124-7.
14. Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard C., Mahillon, J., 2019. Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group. Frontiers in Microbiology, 10, 302. https://doi.org/10.3389/fmicb.2019.00302 .
15. Chapot-Chartier, M. P., Kulakauskas, S., 2014. Cell wall structure and function in lactic acid bacteria. Microbial Cell Factories, 13(1), 9. https://doi.org/10.1186/1475-2859-13-S1-S9.
16. Chehimi, S., Delalande, F., Sable, S., Hajlaoui, M. R., Van Dorsselaer, A., Limam, F., Pons, A. M., 2007. Purification and partial amino acid sequence of thuricin S, a new anti-*Listeria* bacteriocin from *Bacillus thuringiensis*. Canadian Journal of Microbiology, 53(2), 284-290. https://doi.org/10.1139/w06-116.
17. Chehimi, S., Limam, F., Lanneluc, I., Delalande, F., van Dorsselaer, A., & Sable, S. (2012). Identification of three novel B. thuringiensis strains that produce the Thuricin S bacteriocin. *Bt Res*, *3*(1). https://doi.org/10.5376/bt.2012.03.0002.
18. Chen, Y., Wang, J., Li, G., Yang, Y., Ding, W. (2021) Current advancements in sactipeptide natural products. Front Chem 9:290. https://doi.or g/10.3389/fchem.2021.595991.
19. Cheng, Z., He, B. B., Lei, K., Gao, Y., Shi, Y., Zhong, Z., Liu, H., Liu, R., Zhang, H., Wu, S., Zhang, W., Tang, X., Li, Y. X., 2024. Rule-based omics mining reveals antimicrobial macrocyclic peptides against drug-

resistant clinical isolates. Nature Communications, 15(1), 4901. https://doi.org/10.1038/s41467-024-49215-y.

20. Cintas, L. M., Casaus, P., Fernández, M. F., Hernández, P. E., 1998. Comparative antimicrobial activity of enterocin L50, pediocin PA-1, nisin A and lactocin S against spoilage and foodborne pathogenic bacteria. Food Microbiology, 15(3), 289-298. https://doi.org/10.1006/fmic.1997.0160.
21. Darbandi, A., Asadi, A., Mahdizade Ari, M., Ohadi, E., Talebi, M., Halaj Zadeh, M., Kakanj, M. 2022. Bacteriocins: properties and potential use as antimicrobials. Journal of Clinical Laboratory Analysis, 36(1), e24093. https://doi.org/10.1002/jcla.24093.
22. Dietrich, R., Moravek, M., Bürk, C., Granum, P. E., Märtlbauer, E., 2005. Production and characterization of antibodies against each of the three subunits of the *Bacillus cereus* nonhemolytic enterotoxin complex. Applied and Environmental Microbiology, 71(12), 8214-20. https://doi.org/10.1128/AEM.71.12.8214-8220.2005.
23. Dietrich, R., Jessberger, N., Ehling-Schulz, M., Märtlbauer, E., Granum, P. E., 2021. The food poisoning toxins of *Bacillus cereus*. Toxins (Basel), 13(2), 98. https://doi.org/10.3390/toxins13020098.
24. Doshi, M. N., Badr, K., Ejaz, M., Hassan, Z. U., & Jaoua, S. (2024). Investigation of novel bacteriocin producers of Bacillus thuringiensis and partial characterization of two new bacteriocins: Thuricin 466 and thuricin 4Q7. *Bioresource Technology Reports*, *25*, 101760. https://doi.org/10.1016/j.biteb.2024.101760.
25. Frees, D., Brøndsted, L., & Ingmer, H. (2013). Bacterial proteases and virulence. *Regulated proteolysis in microorganisms*, 161-192.
26. Fugaban, J. I. I., Vazquez Bucheli, J. E., Holzapfel, W. H., Todorov, S. D., 2021. Characterization of partially purified bacteriocins produced by *Enterococcus faecium* strains isolated from soybean paste active against *Listeria* spp. and vancomycin-resistant enterococci. Microorganisms, 9(5), 1085. https://doi.org/10.3390/microorganisms9051085.
27. Gaona-Mendoza, A.S., Bravo-Rivas, M.C., Barboza-Corona, J.E., Massange-Sánchez, J.A., Casados-Vázquez, L.E. (2022) Expression of thurincin H, ChiA74 and cry proteins at the sporulation phase in Bacillus thuringiensis HD1. J Appl Microbiol 132(4):3049–3057. https://doi.org/10.1111/JAM.15434.
28. Gaspar, C., Donders, G. G., Palmeira-de-Oliveira, R., Queiroz, J. A., Tomaz, C., Martinez-de-Oliveira, J., Palmeira-de-Oliveira, A., 2018. Bacteriocin production of the probiotic Lactobacillus acidophilus KS400. Amb Express, 8(1), 153. https://doi.org/10.1186/s13568-018-0679-z.
29. Gradisteanu Pircalabioru, G., Popa, L. I., Marutescu, L., Gheorghe, I., Popa, M., Czobor Barbu, I., Cristescu, R., Chifiriuc, M. C., 2021. Bacteriocins in the era of antibiotic resistance: rising to the challenge. Pharmaceutics, 13(2), 196. https://doi.org/10.3390/pharmaceutics13020196.
30. Gray, E. J., Di Falco, M., Souleimanov, A., Smith, D. L., 2006a. Proteomic analysis of the bacteriocin thuricin 17 produced by *Bacillus thuringiensis* NEB17. FEMS Microbiology Letters, 255(1), 27-32. https://doi.org/10.1111/j.1574-6968.2005.00054.x.

31. Gray, E. J., Lee, K. D., Souleimanov, A. M., Di Falco, M. R., Zhou, X., Ly, A., Charles, T. C., Driscoll, B. T., Smith, D. L., 2006b. A novel bacteriocin, thuricin 17, produced by plant growth promoting rhizobacteria strain *Bacillus thuringiensis* NEB17: isolation and classification. Journal of Applied Microbiology, 100(3), 545-554. https://doi.org/10.1111/j.1365-2672.2006.02822.x.
32. Guo, Y., Yu, Z., Xie, J., Zhang, R., 2012. Identification of a new *Bacillus licheniformis* strain producing a bacteriocin-like substance. Journal of Microbiology, 50, 452-458. https://doi.org/10.1007/s12275-012-2051-3.
33. Halami, P.M., 2019. Sublichenin, a new subtilin-like lantibiotics of probiotic bacterium *Bacillus licheniformis* MCC 2512T with antibacterial activity. Microbial Pathogenesis, 128, 139-146. https://doi.org/10.1016/j.micpath.2018.12.044.
34. Hoffmann, S., White, A. E., McQueen, R. B., Ahn, J. W., Gunn-Sandell, L. B., Scallan Walter, E. J., 2024. Economic Burden of Foodborne Illnesses Acquired in the United States. Foodborne Pathogens and Disease. https://doi.org/10.1089/fpd.2023.0157 .
35. Jessberger, N., Dietrich, R., Bock, S., Didier, A., Märtlbauer, E., 2014. *Bacillus cereus* enterotoxins act as major virulence factors and exhibit distinct cytotoxicity to different human cell lines. Toxicon, 77, 49–57. https://doi.org/10.3389/fmicb.2015.00560.
36. Jessberger, N., Krey, V. M., Rademacher, C., Böhm, M. E., Mohr, A. K., Ehling-Schulz, M., Scherer, S., Märtlbauer, E., 2015. From genome to toxicity: a combinatory approach highlights the complexity of enterotoxin production in *Bacillus cereus*. Frontiers in Microbiology, 6, 560. https://doi.org/10.3389/fmicb.2015.00560.
37. Jessberger, N., Kranzler, M., Da Riol, C., Schwenk, V., Buchacher, T., Dietrich, R., Ehling-Schulz, M., Märtlbauer, E., 2019. Assessing the toxic potential of enteropathogenic *Bacillus cereus*. Food Microbiology, 84, 103276. https://doi.org/10.1016/j.fm.2019.103276.
38. Jessberger, N., Dietrich, R., Granum, P. E., 2023. *Bacillus cereus*, *Bacillus anthracis* and Other Pathogenic *Bacillus* Species. Update of P. E. Granum, L Stenfors Arnesen, From, Bacteria: *Bacillus cereus* and Other Pathogenic *Bacillus* Species, Editor(s): Yasmine Motarjemi, Encyclopedia of Food Safety, Academic Press, 2014, 356 363, ISBN 9780123786135.
39. Kamoun, F., Mejdoub, H., Aouissaoui, H., Reinbolt, J., Hammami, A., Jaoua, S., 2005. Purification, amino acid sequence and characterization of Bacthuricin F4, a new bacteriocin produced by *Bacillus thuringiensis*. Journal of Applied Microbiology, 98(4), 881-888. https://doi.org/10.1111/j.1365-2672.2004.02513.x.
40. Lee, K. D., Gray, E. J., Mabood, F., Jung, W. J., Charles, T., Clark, S. R., Ly, A., Souleimanov, A., Zhou, X., Smith, D. L., 2009a. The class IId bacteriocin thuricin-17 increases plant growth. Planta, 229(4), 747-755. https://doi.org/10.1007/s00425-008-0870-6.
41. Lee, H., Churey, J. J., Worobo, R. W., 2009b. Biosynthesis and transcriptional analysis of thurincin H, a tandem repeated bacteriocin genetic locus, produced by *Bacillus thuringiensis* SF361. FEMS Microbiology Letters, 299, 205-213. https://doi.org/10.1111/j.1574-6968.2009.01749.x.

42. Letzel, A. C., Pidot, S. J., Hertweck, C., 2014. Genome mining for ribosomally synthesized and post-translationally modified peptides (RiPPs) in anaerobic bacteria. *BMC genomics*, *15*(1), 983. https://doi.org/10.1186/1471-2164-15-983.
43. Marsh, A. J., O'Sullivan, O., Ross, R. P., Cotter, P. D., Hill, C., 2010. In silico analysis highlights the frequency and diversity of type 1 lantibiotic gene clusters in genome sequenced bacteria. *BMC genomics*, *11*(1), 679. https://doi.org/10.1186/1471-2164-11-679.
44. Mayr-Harting, A., Hedges, A. J., Berkeley, R. C. W., 1972. Methods for studying bacteriocins. In: Methods in Microbiology, 7, 315-422. Academic Press.
45. Medema, M.H.; Blin, K.; Cimermancic, P.; De Jager, V.; Zakrzewski, P.; Fischbach, M.A.; Weber, T.; Takano, E.; Breitling, R. antiSMASH: Rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res. 2011, 39, W339–W346. https://doi.org/10.1093/nar/gkr466.
46. Mercado, V., Olmos, J., 2022. Bacteriocin Production by *Bacillus* Species: Isolation, Characterization, and Application. Probiotics and Antimicrobial Proteins, 14(6), 1151-1169. https://doi.org/10.1007/s12602-022-09966-w.
47. Molujin, A. M., Abbasiliasi, S., Nurdin, A., Lee, P. C., Gansau, J. A., Jawan, R., 2022. Bacteriocins as potential therapeutic approaches in the treatment of various cancers: A review of *in vitro* studies. Cancers, 14(19), 4758. https://doi.org/10.3390/cancers14194758.
48. Morandini, L., Caulier, S., Bragard, C., Mahillon, J., 2024. *Bacillus cereus sensu lato* antimicrobial arsenal: An overview. Microbiological Research, 283, 127697. https://doi.org/10.1016/j.micres.2024.127697.
49. Moravek, M., Dietrich, R., Bürk, C., Broussolle, V., Guinebretiére, M. H., Granum, P. E., Nguyen-The, C., Märtlbauer, E., 2006. Determination of the toxic potential of *Bacillus cereus* isolates by quantitative enterotoxin analyses. FEMS Microbiology Letters, 257(2), 293-298. https://doi.org/10.1111/j.1574-6968.2006.00185.x.
50. Mozolewska, M.A., Sieradzan, A.K., Niadzvedstki, A., Czaplewski, C., Liwo, A., Krupa, P. (2017) Role of the sulfur to α-carbon thioether bridges in thurincin H. J Biomol Struct Dyn 35(13):2868–2879. h ttps://doi.org/10.1080/07391102.2016.1234414.
51. Murinda, S. E., Rashid, K. A., & Roberts, R. F. (2003). In vitro assessment of the cytotoxicity of nisin, pediocin, and selected colicins on simian virus 40–transfected human colon and Vero monkey kidney cells with trypan blue staining viability assays. *Journal of food protection*, *66*(5), 847-853. https://doi.org/10.4315/0362-028X-66.5.847.
52. Nazari, M., Smith, D. L., 2020. A PGPR-Produced Bacteriocin for Sustainable Agriculture: A Review of Thuricin 17 Characteristics and Applications. Frontiers in Plant Science, 11: 916. https://doi.org/10.3389/fpls.2020.00916.
53. Olanya, O. M., Hoshide, A. K., Ijabadeniyi, O. A., Ukuku, D. O., Mukhopadhyay, S., Niemira, B. A., Ayeni, O., 2019. Cost estimation of listeriosis (*Listeria monocytogenes*) occurrence in South Africa in 2017

and its food safety implications. Food Control, 102, 231-239. https://doi.org/10.1016/j.foodcont.2019.02.007.

54. O'Sullivan, O., Begley, M., Ross, R. P., Cotter, P. D., Hill, C., 2011. Further Identification of novel lantibiotic operons using LanM-based genome mining. Probiotics and Antimicrobial Proteins, 3(1), 27-40. https://doi.org/10.1007/s12602-011-9062-y.
55. Palma, L., Muñoz, D., Berry, C., Murillo, J., Caballero, P., 2014. *Bacillus thuringiensis* Toxins: An Overview of Their Biocidal Activity. Toxins, 6(12), 3296-3325. https://doi.org/10.3390/toxins6123296.
56. Parada Fabián, J. C., Álvarez Contreras, A. K., Natividad Bonifacio, I., Hernández Robles, M. F., Vázquez Quiñones, C. R., Quiñones Ramírez, E. I., & Vázquez Salinas, C. (2025). Toward safer and sustainable food preservation: a comprehensive review of bacteriocins in the food industry. *Bioscience Reports*, *45*(04), 277-302. https://doi.org/10.1042/BSR20241594.
57. Puan, S. L., Erriah, P., Baharudin, M. M. A., Yahaya, N. M., Kamil, W., Ali, M. S. M., Ahmad, S. A., Oslan, S. N., Lim, S., Sabri, S., 2023. Antimicrobial peptides from *Bacillus* spp. and strategies to enhance their yield. Applied Microbiology and Biotechnology, 107(18), 5569-5593. https://doi.org/10.1007/s00253-023-12651-9.
58. Radaic, A., de Jesus, M. B., Kapila, Y. L., 2020. Bacterial anti-microbial peptides and nano-sized drug delivery systems: The state of the art toward improved bacteriocins. Journal of Controlled Release, 321, 100-118. https://doi.org/10.1016/j.jconrel.2020.02.001.
59. Rollema, H. S., Kuipers, O. P., Both, P., de Vos, W. M., Siezen, R. J., 1995. Improvement of solubility and stability of the antimicrobial peptide nisin by protein engineering. Applied and Environmental Microbiology, 61(8), 2873–2878. https://doi.org/10.1128/aem.61.8.2873-2878.1995.
60. Ruíz-De-Anda, D., Mendoza-Acosta, M. F., & Casados-Vázquez, L. E. (2025). Exploring thurincin H as a biotechnologically relevant sactipeptide after fifteen years of research. *Archives of Microbiology*, *207*(8), 171.
61. Ryan, A., Patel, P., O'Connor, P. M., Ross, R. P., Hill, C., Hudson, S. P., 2021. Pharmaceutical design of a delivery system for the bacteriocin lacticin 3147. Drug Delivery and Translational Research, 11, 1735-1751. https://doi.org/10.1007/s13346-021-00984-9.
62. Salazar-Marroquín, E. L., Galán-Wong, L. J., Moreno-Medina, V. R., Reyes-López, M. Á., Pereyra-Alférez, B., 2016. Bacteriocins synthesized by *Bacillus thuringiensis*: generalities and potential applications. Reviews and Research in Medical Microbiology, 27(3). https://doi.org/10.1097/mrm.0000000000000076.
63. Schägger, H., 2006. Tricine-SDS-PAGE. Nature Protocols, 1(1), 16-22. https://doi.org/10.1038/nprot.2006.4.
64. Scholz, R., Vater, J., Budiharjo, A., Wang, Z., He, Y., Dietel, K.,Schwecke, T., Herfort, S.,Lasch, P., Borriss, R., 2014. Amylocyclicin, a novel circular bacteriocin produced by *Bacillus amyloliquefaciens* FZB42. Journal of Bacteriology, 196(10), 1842-1852. https://doi.org/10.1128/jb.01474-14.

65. Sharma, G., Dang, S., Gupta, S., Gabrani, R., 2018. Antibacterial Activity, Cytotoxicity, and the Mechanism of Action of Bacteriocin from *Bacillus subtilis* GAS101. Medical Principles and Practice, 27(2), 186-192. https://doi.org/10.1159/000487306.
66. Simons, A., Alhanout, K., Duval, R., 2020. Bacteriocins, Antimicrobial Peptides from Bacterial Origin: Overview of Their Biology and Their Impact against Multidrug-Resistant Bacteria. Microorganisms, 8(5), 639. https://doi.org/10.3390/microorganisms8050639.
67. Sit, C. S., van Belkum, M. J., McKay, R. T., Worobo, R. W., Vederas, J. C., 2011. The 3D solution structure of thurincin H, a bacteriocin with four sulfur to α-carbon crosslinks. Angewandte Chemie International Edition, 50(37), 8718–8721. https://doi.org/10.1002/anie.201102527.
68. Sugrue, I., Ross, R. P., Hill, C., 2024. Bacteriocin diversity, function, discovery and application as antimicrobials. Nature Reviews Microbiology, 22(9), 556-571. https://doi.org/10.1038/s41579-024-01045-x.
69. Teber, R., Asakawa, S. 2024. *In Silico* Screening of Bacteriocin Gene Clusters within a Set of Marine Bacillota Genomes. International Journal of Molecular Sciences, 25(5), 2566. https://doi.org/10.3390/ijms25052566.
70. Todorov, S. D., Nyati, H., Meincken, M., Dicks, L. M. T., 2007. Partial characterization of bacteriocin AMA-K, produced by *Lactobacillus plantarum* AMA-K isolated from naturally fermented milk from Zimbabwe. Food control, 18(6), 656-664. https://doi.org/10.1016/j.foodcont.2006.03.003.
71. Todorov, S. D., Popov, I., Weeks, R., Chikindas, M. L., 2022. Use of bacteriocins and bacteriocinogenic beneficial organisms in food products: Benefits, challenges, concerns. Foods, 11(19), 3145. https://doi.org/10.3390/foods11193145.
72. van Heel, A. J., de Jong, A., Song, C., Viel, J. H., Kok, J., Kuipers, O. P., 2018. BAGEL4: A user-friendly web server to thoroughly mine RiPPs and bacteriocins. Nucleic Acids Research, 46(W1), W278–W281. https://doi.org/10.1093/nar/gky383.
73. Wang, G., Manns, D. C., Guron, G. K., Churey, J. J., Worobo, R. W., 2014. Large-scale purification, characterization, and spore outgrowth inhibitory effect of thurincin H, a bacteriocin produced by *Bacillus thuringiensis* SF361. Probiotics and Antimicrobial Proteins, 6, 105-113. https://doi.org/10.1007/s12602-014-9159-1.
74. Wang, T., Liang, Y., Wu, M., Chen, Z., Lin, J., Yang, L., 2015. Natural products from *Bacillus subtilis* with antimicrobial properties. Chinese Journal of Chemical Engineering, 23(4), 744-754. https://doi.org/10.1016/j.cjche.2014.05.020.
75. Woraprayote, W., Malila, Y., Sorapukdee, S., Swetwiwathana, A., Benjakul, S., & Visessanguan, W. (2016). Bacteriocins from lactic acid bacteria and their applications in meat and meat products. *Meat Science*, *120*, 118-132. https://doi.org/10.1016/j.meatsci.2016.04.004.

Figures

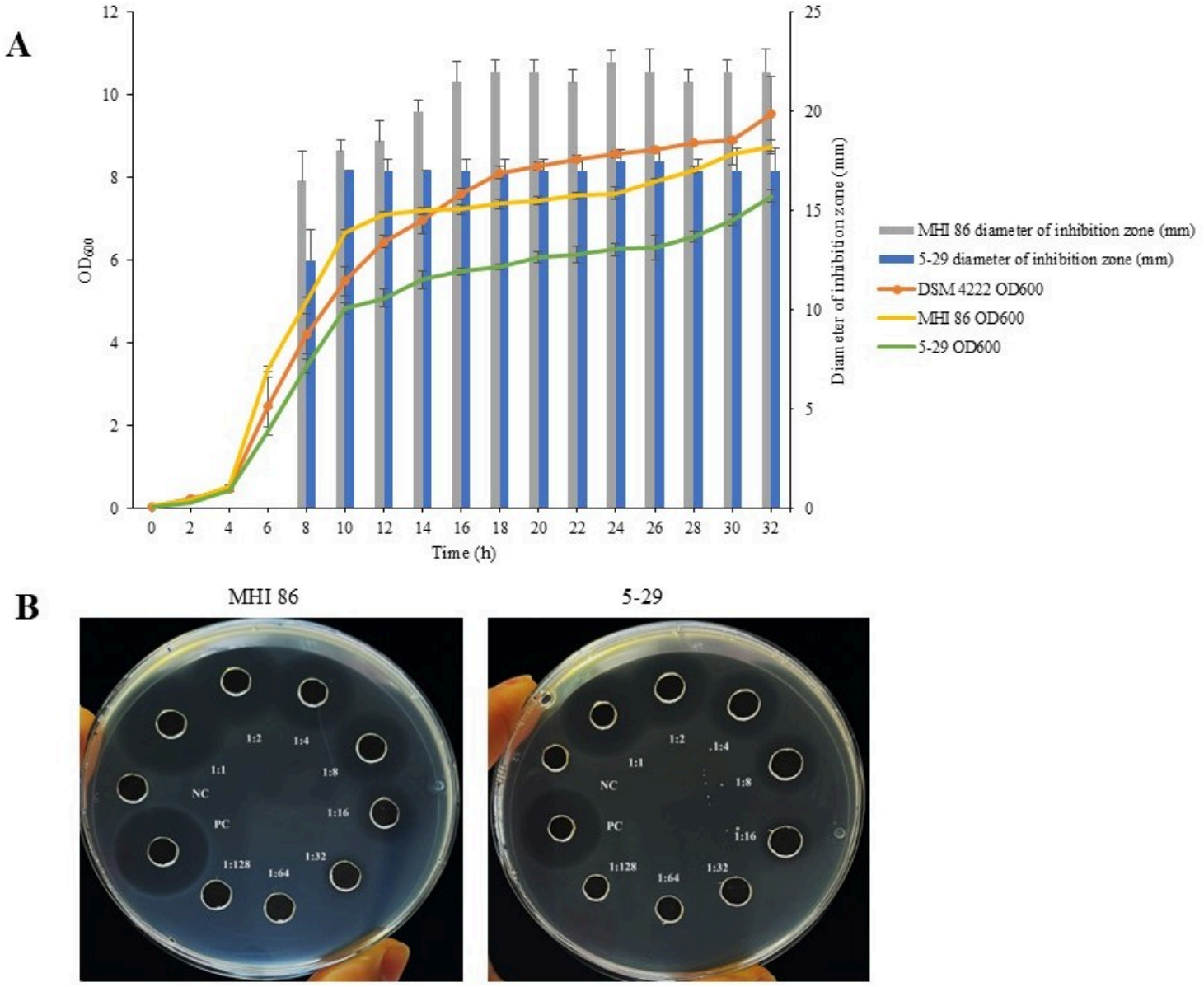


Figure 1

Antibacterial activity of *B. cereus* MHI 86 and *B. thuringiensis* 5-29 against *L. monocytogenes* DSM 20600. (A) Growth curves of *B. cereus* MHI 86 and *B. thuringiensis* 5-29, alongside the antibacterial activity of their cell-free supernatants (CFSs) against *L. monocytogenes* DSM 20600, depicted as inhibition zone diameters. *B. cereus* DSM 4222 (no antibacterial activity) was added as control. In this experiment, *Bacillus*strains were grown in 150 mL TSB for 32 h at 37 °C under shaking. At the indicated time points, 2 mL samples were taken, filtered, and added to the agar well diffusion assay. The experiment was conducted in triplicate, with error bars indicating the standard deviation of the mean. (B) Application of 1:2 serial dilutions of the CFSs after 24 h of growth to the agar well diffusion assay against *L. monocytogenes* DSM 20600. Bacteriocin activity (AU/mL) was calculated based on the highest dilution showing a visible inhibition zone (compare section 2.4). Left: *B. cereus* MHI 86; Right: *B. thuringiensis* 5-29. Positive control (PC): Ampicillin, and negative control (NC): *B. cereus* DSM 4222 CFS.

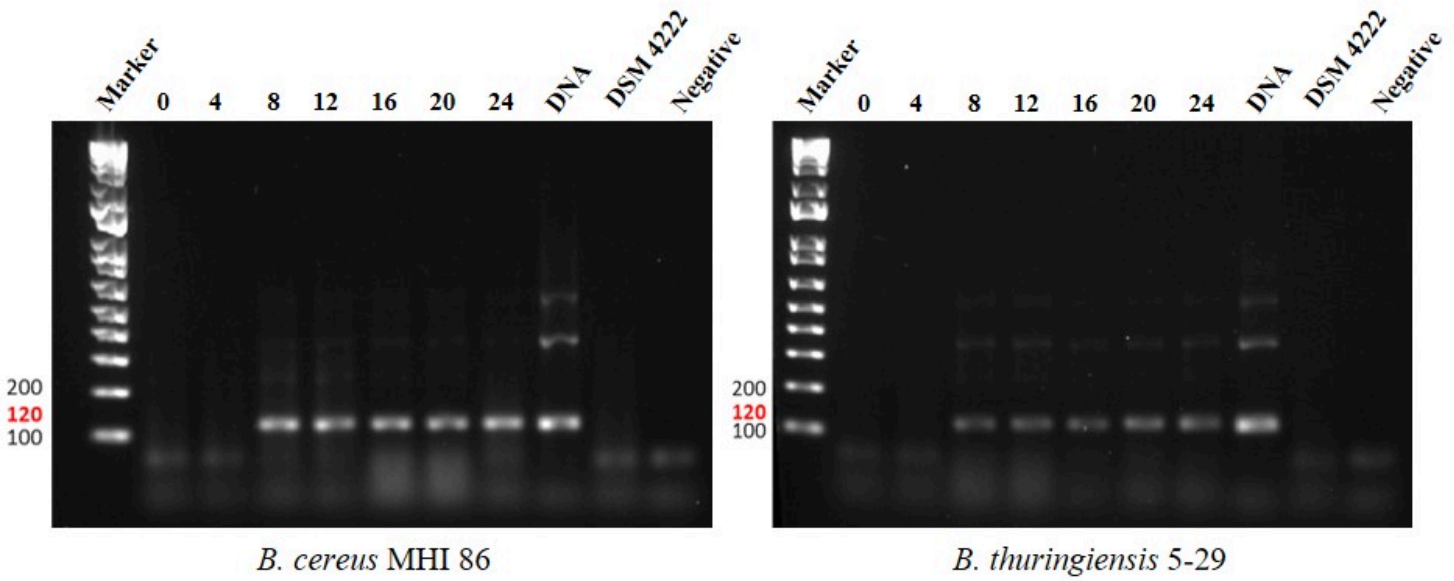


Figure 2

Time-dependent transcriptional analysis of the thuricin S encoding genes by reverse transcription polymerase chain reaction (RT-PCR). At the indicated time points, 2 mL samples were taken, total ribonucleic acid (RNA) was prepared, transcribed into cDNA, and used as template for polymerase chain reaction (PCR) with thuricin S specific primers (compare Supplementary Fig. S1and Table S1). Left: *B. cereus* MHI 86. Right: *B. thuringiensis* 5-29. DNA: genomic DNA as positive control. DSM 4222: genomic DNA of the *B. cereus* strain DSM 4222 as negative control. Negative: non-template control.

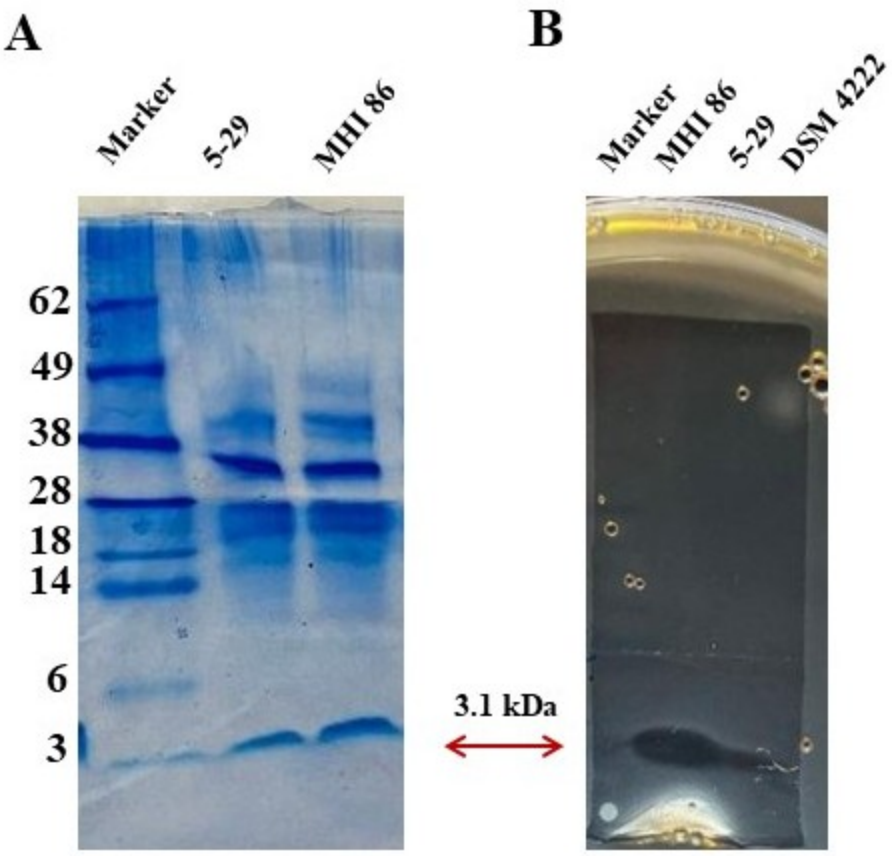


Figure 3

Determination of molecular weight and activity of thuricin S. (A) SDS-PAGE profile of thuricin S produced by *B. cereus* MHI 86 and *B. thuringiensis* 5-29. Marker: low molecular weight, 3-200 kDa. (B) Antibacterial activity of the bands of interest against *L. monocytogenes* DSM 20600. Negative control (NC): *B. cereus* DSM 4222 CFS.

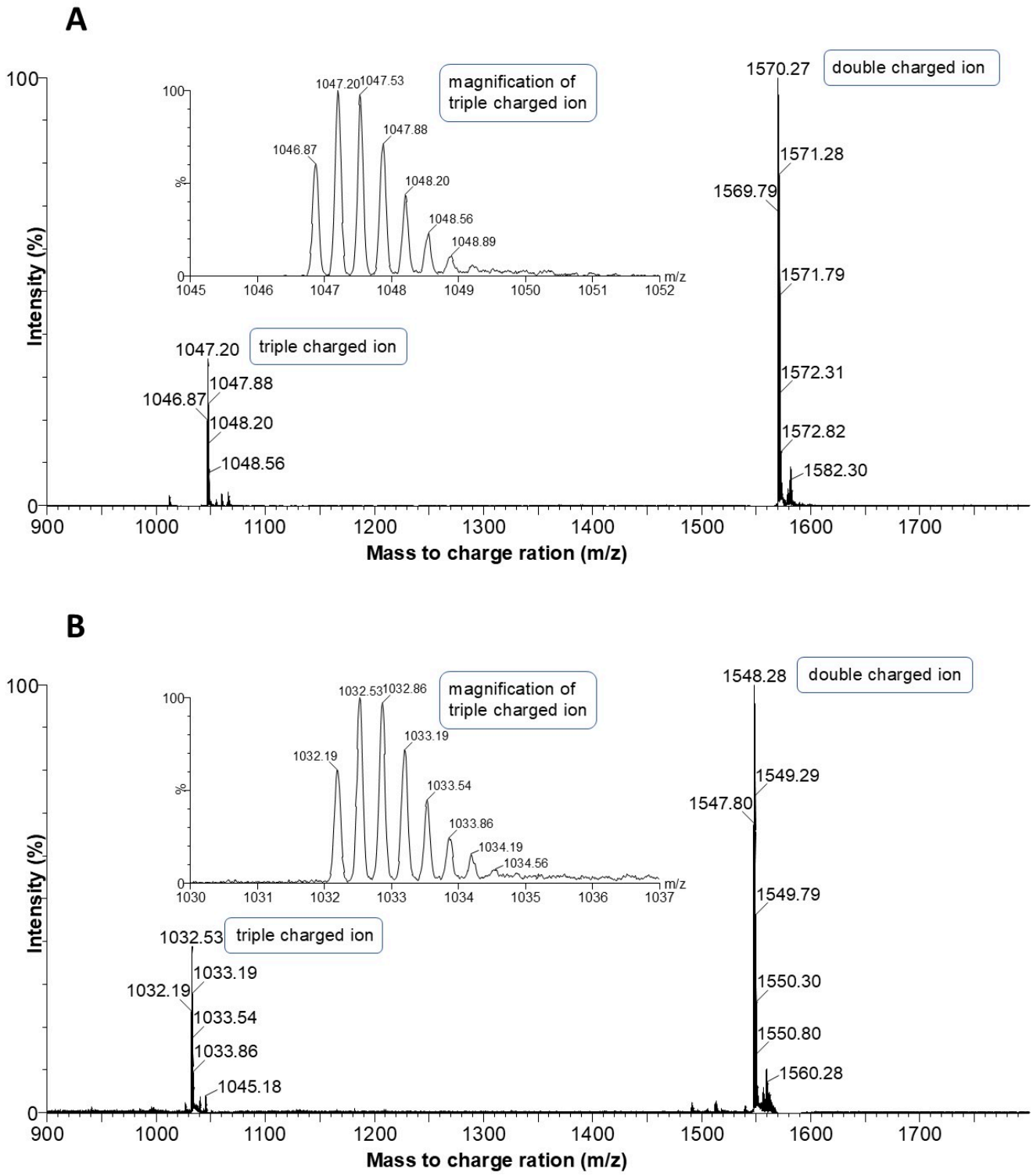


Figure 4

Mass spectrum analysis of both partially purified bacteriocins from (A) *B. cereus* MHI 86 and (B) *B. thuringiensis* 5-29. We calculated the monoisotopic mass of the main peptide to be 3,137.6 (±0.2), with double- and triple-charged ions of 1,569.79 and 1,046.86, respectively.

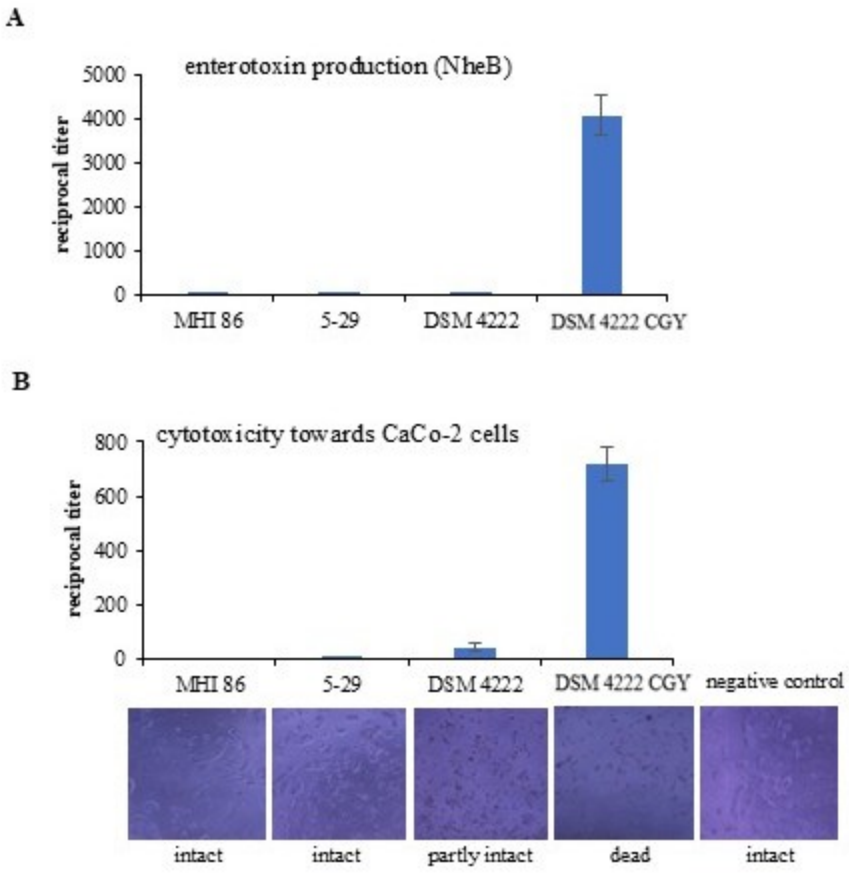


Figure 5

Assessment of the pathogenic potential of CFSs of strains MHI 86 and 5-29. CFSs of strain DSM 4222 were added as controls; first, after 24-h growth in tryptic soy broth (TSB) (as used for bacteriocin production, refer to Fig. 2A) and second, after 6-h growth in casein-glucose-yeast (CGY) medium. The latter is known to induce maximum enterotoxin production and cytotoxic activity of this highly toxic reference strain also known as F837/76 (Jessberger et al., 2015; Dietrich et al., 2021). (A) The B component of the non-hemolytic entertoxin (NheB) was determined via sandwich enzyme immunoassay (EIA); (B) Cytotoxicity towards CaCo-2 cell was determined via WST-1 bioassay. The images show examples of cells to which the CFSs were added in a 1:20 dilution.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterialPAPBatman.docx
- GraphicalAbstract.png