Simultaneous Production of Multiple Antimicrobial Compounds by *Bacillus velezensis* ML122-2 Isolated From Assam Tea Leaf [*Camellia sinensis* var. *assamica* (J.W.Mast.) Kitam.]

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*Bacillus velezensis* ML122-2 is an antimicrobial-producing strain isolated from the leaf of Assam tea or Miang [*Camellia sinensis* var. *assamica* (J.W.Mast.) Kitam.]. The cell-free supernatant (CFS) of strain ML122-2 exhibits a broad-spectrum antimicrobial activity against various Gram-positive and Gram-negative bacteria as well as the mold *Penicillium expansum*. The genome of *B. velezensis* ML122-2 was sequenced and in silico analysis identified three potential bacteriocin-associated gene clusters, that is, those involved in the production of mersacidin, amylocyclicin, and LCI. Furthermore, six gene clusters exhibiting homology (75–100% DNA sequence identity) to those associated with the secondary metabolites bacilysin, bacillibactin, surfactin, macrolactin H, bacillaene, and plipastatin were identified. Individual antimicrobial activities produced by *B. velezensis* ML122-2 were purified and characterized by Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry analysis, revealing three antimicrobial peptides with molecular masses corresponding to surfactin, plipastatin, and amylocyclicin. Transcriptional analysis of specific genes associated with mersacidin (*mrsA*), amylocyclicin (*acnA*), plipastatin (*ppsA*), and surfactin (*srfAA*) production by *B. velezensis* ML122-2 were purified and characterized by Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry analysis, revealing three antimicrobial peptides with molecular masses corresponding to surfactin, plipastatin, and amylocyclicin. Transcriptional analysis of specific genes associated with mersacidin (*mrsA*), amylocyclicin (*acnA*), plipastatin (*ppsA*), and surfactin (*srfAA*) production by *B. velezensis* ML122-2 showed that the first was not transcribed under the conditions tested, while the latter three were consistent with the presence of the associated peptides as determined by mass spectrometry analysis. These findings demonstrate that *B. velezensis* ML122-2 has the genetic capacity to produce a wide range of antimicrobial activities that may support a specific community structure and highlight the biotechnological properties of Assam tea.

Keywords: amylocyclicin, bacteriocin, biocontrol, gene cluster, Miang, plipastatin, RT-qPCR, surfactin
INTRODUCTION

Members of the *Bacillus* genus, which represent Gram-positive and endospore-forming bacteria, are widespread in a variety of environments including air, soil, aquatic ecosystems, foods, skin, and the gastrointestinal tract of animals (Abriouel et al., 2011). Some species of *Bacillus* are believed to play a key role in biological control through the production of antimicrobial compounds (e.g., bacteriocins, non-ribosomal polypeptides, and polyketides) and in plant growth promotion, such as *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Bacillus tequilensis* (Chen et al., 2007; Gao et al., 2017; Li et al., 2018). The antimicrobial metabolites produced by *Bacillus* spp. are used in clinical settings to achieve inhibition of pathogens, such as *Bacillus cereus*, *Clostridium difficile*, *Listeria monocytogenes*, and methicillin-resistant *Staphylococcus aureus* (MRSA; Sabaté and Audisio, 2013; Lv et al., 2020; Rungsirivanich and Thongwai, 2020). Additionally, certain species of *Bacillus* have been reported to elicit probiotic potential, in particular *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus saniensis*, and *Bacillus subtilis* (Du et al., 2018; Jeżewska-Frąckowiak et al., 2019; Rungsirivanich et al., 2020).

Bacteriocins are ribosomally synthesized antimicrobial peptides which exhibit antimicrobial activity mostly against closely related bacterial species (Klaenhammer, 1993). Bacteriocins have been classified into three major classes: Class I bacteriocins are small peptides which undergo post-translational modifications, while Classes II and III are small (0.77–10 kDa) and large (>10 kDa) unmodified linear antimicrobial proteins, respectively (Abriouel et al., 2011; Cotter et al., 2013; Alvarez-Sieiro et al., 2016). In addition to bacteriocins, several species of *Bacillus* have been described to produce non-ribosomally synthesized peptides (NRPs) and polyketides (PKs) with antimicrobial properties (Patel et al., 1995; Pathak and Keharia, 2013). NRPs and PKs are synthesized by large multi-modular synthetases, non-ribosomal peptide synthetases (NRPSs), polyketide synthetases (PKSs), or hybrid NRPS/PKS enzymes. NRPSs typically consist of one or more modules, each responsible for the enzymatic incorporation of a specific amino acid in a growing peptide. An individual NRPS module typically consists of three core domains, that is, domains responsible for adenylation, thiolation, and condensation. Similarly, a given PKS enzyme comprises acyl transferase, acyl carrier, and ketosynthase domains (Mootz et al., 2002; Wang et al., 2014; Aleti et al., 2015). Prediction of gene clusters responsible for the biosynthesis of antimicrobial compounds using genome-mining tools has been applied for the identification and subsequent characterization of genes associated with antimicrobial compound production (Medema et al., 2011; Sekurova et al., 2019). BAGEL is a powerful prediction tool aimed at the identification of bacteriocin-associated genes (De Jong et al., 2006). AntiSMASH is a genome database for gene cluster analysis responsible for the synthesis of secondary metabolite compounds, such as NRPs, PKs, and other antimicrobials (Medema et al., 2011; Weber et al., 2015).

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is an analytical technique used for evaluating chemical components which are ionized into charged molecules. It has been applied for identification and analysis of biological molecules, especially proteins and peptides (Singhal et al., 2015). MALDI-TOF MS has also been used to identify and analyze antimicrobial peptides, such as amphotericin (Scholz et al., 2014), iturin, fengycin, surfactin (Yang et al., 2015; Théatre et al., 2021), and mersacidin (Viel et al., 2021).

Antimicrobial peptides (AMPs) have received substantial attention as an effective treatment of bacterial infections and as an alternative to antibiotics ( Cotter et al., 2005), in many cases supported by their low toxicity to human cells (Yang et al., 2014). Furthermore, specific AMPs have not only been used in the food industry as preservatives but also in agricultural applications as antimicrobial compounds (Dischinger et al., 2014). Several studies have identified antimicrobial-producing *Bacillus* strains associated with soils and plants and are therefore believed to contribute to the biocontrol of plant pathogens (Shafi et al., 2017; Andrić et al., 2020). The mode of action of bacteriocins may be through interaction with specific membrane receptors causing bacterial membrane disruption and associated electrolyte leakage from bacterial cells, ultimately leading to cell death (Tymoszewska et al., 2017; Perez et al., 2018). In contrast, antibiotics typically act as enzyme inhibitors in DNA replication, protein, and fatty acid synthesis, or cell wall biosynthesis (Zhang et al., 2018; O’Rourke et al., 2020). Previous studies by Rungsirivanich and Thongwai (2020) and Rungsirivanich et al. (2020) revealed the antimicrobial activity of *B. velezensis* ML122-2 isolated Assam tea [*Camellia sinensis* var. *assamica* (J.W.Mast.) Kitam.] leaf surface against *S. aureus*, including MRSA. Moreover, this strain was also shown to exhibit tannin tolerance and probiotic properties. In the current study, we describe the identification, purification, and characterization of antimicrobial compounds produced by *B. velezensis* ML122-2 revealing co-production of several distinct antimicrobial compounds. Genome and transcriptional analysis of *B. velezensis* ML122-2 revealed expression of the corresponding gene clusters for these antimicrobial activities.

MATERIALS AND METHODS

Bacterial Strain and Growth Condition

*B. velezensis* ML122-2 (previously named *Bacillus siamensis* ML122-2; GenBank accession no. MH796212) was isolated from an Assam tea leaf [*Camellia sinensis* var. *assamica* (J.W.Mast.) Kitam.] harvested in the Phrae province, Thailand. Strain ML122-2 was grown in tryptic soy broth (TSB, Merck™, Germany) at 37°C with shaking at 150 rpm for 24 h, as previously described by Rungsirivanich and Thongwai (2020).

Antimicrobial Activity Assay

Antibacterial activity was assayed using an agar well diffusion method according to the modified protocol of Sewify et al. (2017). The indicator bacteria (listed in Table 1) were grown in brain heart infusion (BHI, Oxoid™, Basingstoke, England), de Man, Rogosa, and Sharpe (MRS, Oxoid™, Basingstoke, England), or M17 (Oxoid™, Basingstoke, England) containing...
### Draft Genome Sequencing and Sequence Analysis

Chromosomal DNA of strain ML122-2 was extracted using a NucleoBond® kit (Macherey-Nagel, Germany). Genome sequencing of *B. velezensis* ML122-2 was performed using the Pacific Bioscience (PacBio) SMRT RSII sequencing platform (PacBio, Menlo Park, CA, United States). The obtained raw reads were assembled with the Hierarchical Genome Assembly Process (HGAP) pipeline using the protocol RS_Assembly2 implemented in SMRT Smart Analysis portal v2.3 (Altschul et al., 1990). Genome sequencing was also performed using an Illumina MiSeq platform by the commercial sequencing service provider Probiogenomics (University of Parma, Italy) using the chromosomal DNA of strain ML122-2, which was extracted using a PureLink™ Genomic DNA extraction kit according to the manufacturer's instructions (Invitrogen™, CA, United States). Genomic libraries were constructed using the TruSeq DNA PCR-Free LT Kit (Illumina®) and 2.5 μg of genomic DNA, which was fragmented with a Bioruptor NGS ultrasonicator (Diagenode, United States) followed by size evaluation using Tape Station 2,200 (Agilent Technologies, Santa Clara, CA, United States). Library samples were loaded into a Flow Cell V3 600 cycles (Illumina®). Fastq files of the paired-end reads (2 × 250 bp) were used as input for genome assemblies through the MEGAnnotator pipeline in default mode (Lugli et al., 2016). Open reading frames prediction was performed by Prodigal v2.6.3 (Strepis et al., 2020). Protein-encoding genes were automatically annotated using a BlastP v2.2.26 (cut-off value of E 0.0001) sequence alignments against the non-redundant protein (nr) database curated by NCBI. The bacteriocin/antimicrobial gene clusters were predicted with BAGEL4 software. Meanwhile, gene clusters involved in the biosynthesis of secondary metabolites, such as those involved in the production of NRPs, and PKs, were predicted by antiSMASH software. The genome sequence was deposited in GenBank under accession number JAGTWM000000000.

### Purification and Identification of Antimicrobial Compounds in Cell Fractions

Strain ML122-2 was cultivated in 800 ml clarified TSB, which had been passed through a column containing Amberlite XAD-2 resin beads (Sigma-Aldrich™, St. Louis, MO, United States) to remove hydrophobic peptides, and incubated at 150 rpm, 37°C for 48 h prior to centrifugation at 8,000×g at 4°C for 20 min. The resulting cell pellet was removed, and the cell-free supernatant (CFS, ~800 ml) was passed through an Econo column containing 30 g Amberlite® XAD16N beads (Phenomenex, Cheshire, UK) prewashed with Milli Q water.

### TABLE 1 | Antimicrobial activity of cell-free supernatant (CFS) produced by *B. velezensis* ML122-2 against indicator microorganisms.

| Microorganism                        | Zone of Inhibition (mm) |
|--------------------------------------|-------------------------|
| *Bacillus cereus* TISTR 687<sup>a</sup> | 10.1 ± 0.2              |
| *Bacillus subtilis* NCDO 1769<sup>b</sup> | 0                      |
| *Bacillus subtilis* NCDO 10073<sup>c</sup> | 0                      |
| *Enterobacter aerogenes* NGMIB 10102<sup>d</sup> | 9.0 ± 0.0              |
| *Escherichia coli* DH5α<sup>e</sup> | 0                      |
| *Fusarium penangensis* ML061-4<sup>f</sup> | 11.3 ± 0.3             |
| *Lactococcus lactis* H4<sup>g</sup> | 13.9 ± 0.9             |
| *Leuconostoc paramesenteroides* | 12.0 ± 0.0              |
| *NCDO 1012* | 16.5 ± 0.0              |
| *Penicillium expansum* TISTR 687<sup>a</sup> | 14.5 ± 0.8             |
| *Penicillium expansum* SA-C12<sup>a</sup> | 15.3 ± 0.8             |
| *Penicillium expansum* Rap 51<sup>c</sup> | 0                      |
| *Penicillium expansum* Rap 43<sup>d</sup> | 13.1 ± 0.4             |
| *Penicillium expansum* Rap 51<sup>d</sup> | 14.9 ± 0.2             |
| *Penicillium expansum* ATCC 347<sup>g</sup> | 14.8 ± 0.3             |
| *Listeria innocua* UCC3<sup>c</sup> | 11.1 ± 0.2             |
| *Methicillin-resistant* Staphylococcus aureus DMST 20826<sup>a</sup> | 10.4 ± 0.4             |
| *Penicillium digitatum* DSM 2731<sup>c</sup> | 0                      |
| *Penicillium expansum* DSM 1282<sup>c</sup> | 29.8 ± 0.8             |
| *Pseudomonas aeruginosa* PA 01<sup>c</sup> | 10.1 ± 0.2             |
| *Staphylococcus aureus* ATCC 25923<sup>c</sup> | 10.4 ± 0.4             |
| *Staphylococcus aureus* NCDO 947<sup>c</sup> | 9.6 ± 0.6              |
| *Streptococcus dysgalactiae* grp B<sup>h</sup> | 10.3 ± 0.3             |
| *Weissella cibaria* R16<sup>i</sup> | 11.9 ± 0.2             |
| *Weissella confusa* IS<sup>i</sup> | 12.0 ± 0.4             |

Data are expressed as mean ± standard deviation (n=3).<sup>a</sup>obtained from Thailand Institute of Scientific and Technological Research. <sup>b</sup>obtained from National Collection of Dairy Organisms, Scotland. <sup>c</sup>obtained from National Collection of Industrial, Food and Marine Bacteria, UK. <sup>d</sup>obtained from University College Cork culture collection. <sup>e</sup>obtained from Chiang Mai University culture collection. <sup>f</sup>obtained from American Type Culture Collection. <sup>g</sup>obtained from Department of Medical Sciences Thailand. <sup>h</sup>obtained from German Collection of Microorganisms and Cell Cultures.
Following this, the beads were washed with 250 ml 40% ethanol (Fisher Scientific, UK), and bound peptides were eluted from the column with 250 ml 70% (v/v) isopropanol-containing 0.1% (v/v) trifluoroacetic acid (IPA). In parallel, cells from the corresponding cell pellet were mixed with 250 ml IPA and stirred at room temperature for 3–4 h. Subsequently, the mixture was centrifuged at 8,000 × g at 4°C for 20 min. Both IPA eluent and IPA supernatant obtained from CFS and cell pellets, respectively (20 ml each), were applied to a 1 g Strata-E C18 SPE column (Phenomenex, Cheshire, UK) which was pre-equilibrated with 40% methanol and water. Each column was subsequently washed with 20 ml of 40% ethanol and then eluted using 20 ml IPA. The C18 SPE IPA eluents were assessed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Axima TOF2 MALDI-TOF mass spectrometer, Shimadzu Biotech, Manchester, UK) and the molecular mass of bacteriocins determined in positive ion linear mode according to the protocol described by Hill et al. (2020).

**RP-HPLC Purification of Antimicrobial Peptides**

Antimicrobial peptides (except surfactin, see below) were purified from CFS and cell pellets using C18 SPE and a reversed phase HPLC (RP-HPLC). The C18 SPE IPA eluents obtained as described here (Figure 1A) were applied to a semi prep Proteo Jupiter C12 (250 × 10 mm, 4 μ, 90 Å) followed by running a 40 to 85% isopropanol 0.1% trifluoroacetic acid (TFA) gradient. Eluent B was 99.9% isopropanol-containing 0.1% TFA at a flow rate of 2.5 ml/min. Peptide-containing fractions were detected by measuring the absorbance at 214 nm. Fractions that exhibited antimicrobial activity were collected and pooled, subjected to rotary evaporation, and then lyophilized. Each purified antimicrobial peptide was resuspended in 600 μl 50% isopropanol (Figure 1A). Antibacterial activity of fractions was assessed in duplicate using 6 mm diameter wells, and 25 μl of a given fraction/well, and employing Bacillus cereus TISTR 687, B. subtilis NCDO 10073, Escherichia coli DH5α, Listeria innocua UCC3, Leuconostoc paramesenteroides NCDO 869, methicillin-resistant S. aureus (MRSA) DMST 20625, or S. aureus ATCC 25923 as indicator strains. Purified antimicrobial peptides from active fractions were then subjected to MALDI-TOF MS analysis.

**Partial Purification of Surfactin From Cell-Free Supernatant**

Partial purification of surfactin was achieved by organic solvent extraction according to the protocol described by Lei et al. (2020) with modifications as follows. Strain ML122-2 was cultured in TSB and incubated at 37°C, on a rotating platform at 150 rpm for 48 h before centrifugation at 5,000 × g at 4°C for 15 min. The supernatant was subsequently filtered through a 0.20 μm nylon membrane filter. 25 ml ethyl acetate (Sigma-Aldrich™, St. Louis, MO, United States) was mixed with 25 ml filtered cell-free supernatant using a vortex mixer for 10 min prior to centrifugation at 5,000 × g at 4°C for 60 min. Subsequently, the top phase (organic phase), approximately 25 ml, was transferred into a glass bottle. Solvent evaporation was performed using Genevac™ miVac centrifugal concentrator (Genevac Limited, Suffolk, UK) at room temperature for 80 min. The evaporated solvent extract was resuspended in 0.01 M PBS (5 ml; Figure 1B). Antibacterial activity was investigated using the agar well diffusion method described above. B. cereus TISTR 687, B. subtilis NCDO 10073, E. coli DH5α, L. innocua UCC3,
Leu. paramesenteroides NCDO 869, MRSA DMST 20625, and S. aureus ATCC 25923 were used as the indicator strains. The antimicrobial-containing crude extract was then subjected to MALDI-TOF MS analysis.

RT-qPCR Analysis
Transcriptional activity of genes associated with gene clusters predicted to be responsible for mersacidin, amyloliquecin, lipstatin, and surfactin production was investigated using RT-qPCR analysis, whereby the mrsA, anca, ppsA, and srfAA genes served as target genes, respectively. The housekeeping gene rpsE was used as reference for this analysis. Primers were designed using Primer3Plus and listed in Table 2. B. velezensis ML122-2 was cultivated in TSB and incubated overnight at 37°C on an orbital platform shaker (150 rpm) prior to centrifugation at 5000 x g at 4°C for 10 min. The resulting cell pellet was washed twice with 0.85% (w/v) NaCl and adjusted to an OD₆₀₀ of 0.1. A 1% (w/w) of resuspended culture was inoculated into TSB and then incubated at 37°C at 150 rpm for 48 h. Cells were harvested at 24 and 48 h of incubation by centrifugation at 2000 x g for 5 min. RNA extraction and cDNA synthesis were carried out using High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) and SuperScript™ III Reverse Transcriptase (Invitrogen™, CA, United States), respectively. RT-qPCR analysis of the genes or interest and reference gene were performed using a SYBR Green I Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) on the LightCycler® 480 II System (Roche Diagnostics GmbH, Mannheim, Germany) employing the following PCR conditions: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10s, 50°C for 15s, and 72°C for 15s. The relative expression level was calculated using the comparative 2⁻ΔΔCT method (Livak and Schmittgen, 2001).

RESULTS

B. velezensis ML122-2 Exhibits a Broad Antimicrobial Range
In a previous study, B. velezensis (formerly Bacillus siamensis) ML122-2 had been demonstrated to exert antimicrobial activity against certain S. aureus strains (Rungsirivanich et al., 2020). Conversely, the strain did not elicit any observable antimicrobial activity against Bacillus cereus TISTR 687 or E. coli O157:H7 DMST 12743. To establish the inhibitory spectrum of this strain, the antimicrobial activity of the strain was evaluated against a panel of 23 bacterial strains and two mold species. The CFS of B. velezensis ML122-2 was investigated using an agar well diffusion method. B. velezensis ML122-2 CFS was demonstrated to inhibit 19 out of 23 assessed bacterial strains, and one out of the two molds tested in agar well diffusion assays with inhibitory/clearing zones ranging between 9.0 and 16.5 mm. The CFS of B. velezensis ML122-2 was shown to elicit the most potent antimicrobial activity against Leu.

The Genome of B. velezensis ML122-2 Harbors Multiple Gene Clusters Associated With Antimicrobial Compound Biosynthesis
B. velezensis ML122-2 exhibits antimicrobial activity against S. aureus ATCC 25923 and MRSA DMST 20625 (Rungsirivanich et al., 2020), as well as various other microorganisms (see results above). This broad range of antimicrobial activity against a panel of microbes prompted an investigation into the nature of the antimicrobial compound(s) produced by this strain based on genome sequence analysis. To identify the antimicrobial compounds that may be produced by this strain, the genome of B. velezensis ML122-2 was sequenced using a combination of Illumina and PacBio sequencing technologies. The chromosome of B. velezensis ML122-2 was assembled into a single contig using a hybrid assembly approach employing the obtained PacBio and Illumina sequence data. This chromosomal contig consists of 4,083,790 base pairs with a 46.61% GC content, and 3,922 predicted open reading frames (ORFs). Congruently, the whole genome of B. velezensis ML122-2 exhibits 98.3% (94% query coverage) and 86.6% (54% query coverage) sequence identity with B. velezensis FZB42 (GenBank accession no. CP000560) and B. subtilis subsp. subtilis str. 168 (GenBank accession no. AL009126), respectively.

B. velezensis ML122-2 was previously assigned to the B. siamensis species based on 16S rRNA gene sequencing (Rungsirivanich et al., 2020). However, it has been suggested that rpoB represents a more robust marker (than the 16S rRNA gene) to determine the phylogeny of bacilli that belong to the so-called “operational group Bacillus amyloliquefacienfs,” the latter constituting the closely related species B. amyloliquefaciens, B. velezensis, and B. siamensis (Fan et al., 2017). BlastN analysis of the rpoB gene of strain ML122-2 revealed 100% sequence identity with that of Bacillus velezensis strains and with reduced sequence identity to rpoB of B. siamensis (<98.8%) and B. amyloliquefaciens (<99.8%). This finding confirms that strain ML122-2 belongs to the B. velezensis species rather than B. siamensis. To validate this, the average nucleotide identity (ANI) of ML122-2 was analyzed in comparison with those of strains of the B. amyloliquefaciens, B. siamensis, and B. velezensis species. ML122-2 exhibits ANI values of 97.86, 97.85, and 94.65% with B. amyloliquefaciens FBZ42, and B. siamensis SCSIO 05746, respectively. The ML122-2 genome was shown to lack identifiable CRISPR-Cas systems, while it is predicted to contain three prophage-associated regions (13.6, 31.8, and 28.7 kb in length, respectively). Two of these appear to represent incomplete prophage regions, while one is predicted to be intact and located within positions 1,213,485-1,245,310 on the genome. This prophage region contains genes predicted to encode DNA replication enzymes, capsid and tail structural components,

https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi
and lysis functions. BlastN analysis of this putative prophage region highlights that it is highly conserved among the sequenced genomes of *B. velezensis* strains.

Further *in silico* analysis was performed using BAGEL4 and antiSMASH to identify genes involved in the production of antimicrobial or bioactive compounds. A total of four putative bacteriocin or bacteriocin-like gene clusters were predicted by BAGEL4 software including those encoding the biosynthetic and immunity genes for mersacidin, amylocyclicin, ComX, and LCI (Table 3). The predicted ML122-2 mersacidin gene cluster was shown to comprise of mrsK2, mrsR2, mrsF, mrsG, mrsE, mrsA, mrsR1, mrsD, mrsM, and mrsT and is similar to that of *Bacillus* sp. HIL-Y85/54728 (Genbank accession no. AJ250862; 98%) which was previously described by Altena et al. (2000; Figure 2A). Therefore, it appears that a complete mersacidin gene cluster is present in the *B. velezensis* ML122-2 genome. The ML122-2 genome also contains a gene cluster with high identity (98%) to the amylocyclicin cluster of *B. velezensis* FZB42 (Scholz et al., 2014; Figure 2B). The comX gene cluster of *B. velezensis* ML122-2 elicits 35% identity with that of *B. velezensis* FZB42 which encodes the competence pheromone ComX peptide, while the lci gene encodes a putative antimicrobial peptide, and exhibits 89% identity with the corresponding lci gene of *B. velezensis* FZB42 (Supplementary Figure 1). Gene clusters with nucleotide sequence similarity values below 30% were deemed insignificant.

In addition to ribosomally synthesized antimicrobial peptides, *Bacillus* spp. have been reported to produce non-ribosomally synthesized antimicrobial compounds. Sequence analysis using antiSMASH identified nine gene clusters predicted to be involved in the production of secondary metabolites including NRPs and PKs, of which six were shown to exhibit 75–100% nucleotide identity to known NRP/PK clusters from strains of *Bacillus* spp. (Table 3). Of these latter six clusters, five are predicted to encode NRPs (bacilysin, bacilliabactin, surfactin, macrolactin H, and plipastatin), while the remaining one is associated with the biosynthesis of a PK (bacillaene). Genes associated with macroactin biosynthesis are typically identified on the genomes of *B. velezensis* strains, while they have not been observed among the genomes of *B. siamensis* or *B. amyloliquefaciens* strains (Fan et al., 2017). This finding supports the reassignment of this strain as a *B. velezensis* strain. The bacilysin- and bacilliabactin-associated clusters display 100% sequence identity with equivalent clusters in *B. velezensis* FZB42 which includes seven (*bacABCDEFG*) and five (*dhbACEBF*) subunit genes (Figures 3A,B), respectively. The bacilliabactin biosynthesis cluster exhibits 75% nucleotide identity with its counterpart in *B. subtilis* subsp. *subtilis* str. 168, while the surfactin gene cluster (*srfAA, srfAB, srfAC, and srfAD*) exhibits 98 and 79% nucleotide identity with *B. velezensis* FZB42 and *B. subtilis* JH642, respectively. The genome of *B. velezensis* ML122-2 was shown to lack the *ycxBCD* genes located downstream of the *sfp* gene (Figure 3C). Furthermore, the macroactin H biosynthesis gene cluster, *mnnABCDEFH*, exhibits 100% sequence identity to those of *B. velezensis* FZB42, whereas the plipastatin biosynthesis gene cluster, *ppsABCD*, displays 97% identity with that of *B. velezensis* FZB42 (Figures 3D,E). The bacillaene-associated gene

### Table 2: Primers for RT-qPCR used in this study.

| Gen product          | Gene target | Primer (5′ → 3′) | NCBI reference sequence number | Locus tag number | Reference               |
|----------------------|-------------|-----------------|--------------------------------|-----------------|------------------------|
| Mersacidin           | mrsA        | mrsA-F: CATTTGTTAGGCGAACAGATT mrsA-R: GCCACCAGGCAATGTAAAAG | WP_224272223     | KC480_00765 | This study             |
| Amylocyclicin        | ancA        | ancA-F: GTTGCTGTTGCAACGATT ancA-R: TTTTTGCTGTTGCAACGATT | WP_003151973     | KC480_04455 | This study             |
| Plipastatin synthetase subunit I | ppsA | ppsA-F: TGACACAGAGAAGCCGAATG ppsA-R: TACAGCTCGCCGAATTCTTT | WP_014418073     | KC480_16150 | This study             |
| Surfactin synthetase subunit I | srfAA | srfAA-F: TGACACAGAGAAGCCGAATG srfAA-R: CCAAGATCGCTAGGCGTAAG | WP_057766256     | KC480_07345 | This study             |
| 30S ribosomal protein SS | rpsE | rpsE-F: GCGTCGTATTGACCCAAGC rpsE-R: TACCAGTACCGAATCCTACG | WP_003328273     | KC480_19470 | Jordan et al., 2006   |

Further *in silico* analysis was performed using BAGEL4 and antiSMASH to identify genes involved in the production of antimicrobial or bioactive compounds. A total of four putative bacteriocin or bacteriocin-like gene clusters were predicted by BAGEL4 software including those encoding the biosynthetic and immunity genes for mersacidin, amylocyclicin, ComX, and LCI (Table 3). The predicted ML122-2 mersacidin gene cluster was shown to comprise of mrsK2, mrsR2, mrsF, mrsG, mrsE, mrsA, mrsR1, mrsD, mrsM, and mrsT and is similar to that of *Bacillus* sp. HIL-Y85/54728 (Genbank accession no. AJ250862; 98%) which was previously described by Altena et al. (2000; Figure 2A). Therefore, it appears that a complete mersacidin gene cluster is present in the *B. velezensis* ML122-2 genome. The ML122-2 genome also contains a gene cluster with high identity (98%) to the amylocyclicin cluster of *B. velezensis* FZB42 (Scholz et al., 2014; Figure 2B). The comX gene cluster of *B. velezensis* ML122-2 elicits 35% identity with that of *B. velezensis* FZB42 which encodes the competence pheromone ComX peptide, while the lci gene encodes a putative antimicrobial peptide, and exhibits 89% identity with the corresponding lci gene of *B. velezensis* FZB42 (Supplementary Figure 1). Gene clusters with nucleotide sequence similarity values below 30% were deemed insignificant.

In addition to ribosomally synthesized antimicrobial peptides, *Bacillus* spp. have been reported to produce non-ribosomally synthesized antimicrobial compounds. Sequence analysis using antiSMASH identified nine gene clusters predicted to be involved in the production of secondary metabolites including NRPs and PKs, of which six were shown to exhibit 75–100% nucleotide identity to known NRP/PK clusters from strains of *Bacillus* spp. (Table 3). Of these latter six clusters, five are predicted to encode NRPs (bacilysin, bacilliabactin, surfactin, macrolactin H, and plipastatin), while the remaining one is associated with the biosynthesis of a PK (bacillaene). Genes associated with macroactin biosynthesis are typically identified on the genomes of *B. velezensis* strains, while they have not been observed among the genomes of *B. siamensis* or *B. amyloliquefaciens* strains (Fan et al., 2017). This finding supports the reassignment of this strain as a *B. velezensis* strain. The bacilysin- and bacilliabactin-associated clusters display 100% sequence identity with equivalent clusters in *B. velezensis* FZB42 which includes seven (*bacABCDEFG*) and five (*dhbACEBF*) subunit genes (Figures 3A,B), respectively. The bacilliabactin biosynthesis cluster exhibits 75% nucleotide identity with its counterpart in *B. subtilis* subsp. *subtilis* str. 168, while the surfactin gene cluster (*srfAA, srfAB, srfAC, and srfAD*) exhibits 98 and 79% nucleotide identity with *B. velezensis* FZB42 and *B. subtilis* JH642, respectively. The genome of *B. velezensis* ML122-2 was shown to lack the *ycxBCD* genes located downstream of the *sfp* gene (Figure 3C). Furthermore, the macroactin H biosynthesis gene cluster, *mnnABCDEFH*, exhibits 100% sequence identity to those of *B. velezensis* FZB42, whereas the plipastatin biosynthesis gene cluster, *ppsABCD*, displays 97% identity with that of *B. velezensis* FZB42 (Figures 3D,E). The bacillaene-associated gene
Identifying gene clusters involved in the ribosomally synthesized bacteriocins and secondary metabolite synthesis by B. velezensis ML122-2 using BAGEL4 (clusters 1, 3, 5, and 8) and antiSMASH (remainder of presented clusters).

| Cluster | Genome location | Type | Bacteriocins or Secondary metabolites | Nucleotide identity (%) | Expected molecular mass (Da) | Reference |
|---------|-----------------|------|--------------------------------------|-------------------------|-----------------------------|-----------|
| 1       | 141,570         | Lantipeptide class II | Mersacidin | 98 | 1,826 | Herzner et al., 2011 |
| 2       | 300,796         | Other⁴ | Bacilysin | 100 | 270 | Walker and Abraham, 1970 |
| 3       | 867,484         | Class I small RfPs | Amylocyclicin | 98 | 6,381 | Scholz et al., 2014 |
| 4       | 872,945         | NRPS | Bacillabant | 100 | 882 | Cheon et al., 2019 |
| 5       | 917,801         | ComX pheromone | ComX | 35 | – | – |
| 6       | 1,185,571       | RfP-PKs | Unknown | 100 | – | – |
| 7       | 1,401,273       | NRPS | Surfactin | 98 | 1,036 | Gao et al., 2017 |
| 8       | 1,461,209       | Bacteriocin class II | LCI | 89 | 5,468 | Zhu et al., 2001 |
| 9       | 1,539,768       | TransAT-PKS⁴ | Rhizoccin A | 22 | 351 | Rapp et al., 1988 |
| 10      | 2,183,974       | TransAT-PKS | Difficidin | 46 | 544 | Wilson et al., 1987 |
| 11      | 2,283,261       | PKS-like⁵ | Butirosin A/butirosin B | 7 | 555 | Dion et al., 1972 |
| 12      | 2,409,385       | Terpene⁴ | Unknown | – | – | – |
| 13      | 2,725,345       | TransAT-PKS | Macrolactin H | 100 | 376 | Nagao et al., 2001 |
| 14      | 3,036,690       | TransAT-PKS | Bacilaena | 97 | 580 | Patel et al., 1995 |
| 15      | 3,201,579       | TransAT-PKS | Pilipastatin | 97 | 1,464 | Li et al., 2012 |
| 16      | 3,300,156       | TransAT-PKS | Pilipastatin | 97 | 1,464 | Li et al., 2012 |
| 17      | 3,399,466       | Terpene⁴ | Unknown | – | – | – |
| 18      | 3,491,466       | TRPKS | Unknown | – | – | – |
| 19      | 3,606,746       | TransAT-PKS-like⁴ | Difficidin | 53 | 544 | Wilson et al., 1987 |
| 20      | 4,016,677       | TransAT-PKS-like | Difficidin | 26 | 544 | Wilson et al., 1987 |

Secondary metabolite cluster class abbreviations according to antiSMASH:
- cluster containing a secondary metabolite-related protein that does not fit into any other category.
- other unspecified ribosomally synthesized and post-translationally modified peptide product (RfP) cluster.
- trans-AT PKS.
- trans-AT-PKS fragment, with trans-AT domain not found.
- type III PKS.

Antimicrobial Purification and Mass Spectrometry Analysis

Based on genome analysis, B. velezensis ML122-2 has the genetic capacity to produce a considerable number of distinct antimicrobial compounds. Accordingly, in order to assess which of the predicted antimicrobial compounds are responsible for the observed antimicrobial activity of B. velezensis ML122-2, we characterized the antimicrobial peptides produced in cell pellets and CFS extracts and analyzed the active fractions by MALDI-TOF MS (see "Materials and Methods"; Figure 1). The MALDI-TOF mass spectrum displayed major ion peaks [M + H]⁺ at m/z values of 1,059.25, 1,464.33, and 6,381.58, which correspond to the deduced molecular masses of surfactin (1,036 kDa; Pathak et al., 2014), pilipastatin (1,464 kDa; Dimić et al., 2017), and amylocyclicin (6,381 kDa; Scholz et al., 2014), respectively (Table 3; Figure 4A).

Further purification by RP-HPLC allowed separation of antimicrobial activities in two active fractions, one of which corresponded to ion peaks with m/z values of 1,449.9, 1,463.9, 1,471.9, 1,487.9, 1,485.9, and 1,501.9 (Figure 4B), and one which corresponded to the peaks at m/z 6,381.4 and 3,190.3 (Figure 4C). Since surfactin could not purified by RP-HPLC, possibly due to its inherent hydrophobic nature, (partial) purification of this compound was achieved by solvent extraction with ethyl acetate. Ethyl acetate possesses a lower polarity than isopropanol, which was used in the RP-HPLC purification and may explain its (near) absence in the original purification. Moreover, a previous study revealed that surfactin extraction by ethyl acetate is associated with high purity and yield of the compound (Chen and Juang, 2008). The MALDI-TOF mass spectra of unpurified extract obtained from solvent extraction represented the peaks at m/z 1,032.38, 1,046.25, 1,060.24, and 1,103.99 (Figure 4D). Different molecular weights for purified pilipastatin and purified surfactin have previously been described regarding the production of surfactin and fengycins/plipastatin with fatty acid side chains of 15 to 17 carbon atoms in a Bacillus strain (Koumoutsi et al., 2004), resulting in incremental molecular mass increases of 14 Da for purified pilipastatin and surfactin. A previous study by Pathak et al. (2014) reported the mass spectrum [M + H]⁺ of surfactin from Bacillus strain at m/z 994.7, 1,008.7, 1,022.7, 1,036.7, 1,064.7, 1,078.7, and 1,092.7 consistent with unsaturated C₁₂-C₁₇ β-hydroxy fatty acids. Similarly, [M + H]⁺ ions at m/z 1,433.8, 1,447.8, 1,461.8, 1,475.8, and 1,489.9 were assigned to pilipastatin isomers that correspond to unsaturated C₁₄-C₁₆ β-hydroxy fatty acids (Gao et al., 2017). Here, we successfully purified surfactin via solvent extraction. Furthermore, based on peak height, it appeared that cells represented a better source of amylocyclicin and lipopeptides than CFS (Supplementary Figure 2).
Genome analysis identified intact gene clusters associated with the biosynthesis of additional ribosomally (mersacidin) and non-ribosomally (macrolactin, bacillaene, bacilysin, and bacillibactin) synthesized compounds. However, the molecular masses associated with mersacidin, bacilysin, bacillibactin, macrolactin H, and bacillaene (Table 3) were not detected through MALDI-TOF MS in either the crude or purified extracts suggesting that these compounds are not produced under the applied laboratory conditions.

The antibacterial activity of individually purified amylocyclicin, plipastatin, and surfactin against B. cereus TISTR 687, B. subtilis NCDO 10073, E. coli DH5α, L. innocua UCC3, Leu. paramesenteroides NCDO 869, MRSA DMST 20625, and S. aureus ATCC 25923 as determined by the agar well diffusion method is presented in Table 4 and Supplementary Figure 3. The purified amylocyclicin was shown to inhibit growth of all test indicator strains with the inhibitory value ranging between 7.8 and 24.0 mm, while purified plipastatin represented antimicrobial activity against B. cereus TISTR 687, Leu. paramesenteroides NCDO 869, MRSA DMST 20625, and S. aureus ATCC 25923, with an associated zone of inhibition ranging from 7.5 to 7.8 mm, and with no inhibition observed for B. subtilis NCDO 10073 and L. innocua UCC3. The purified surfactin obtained via solvent extraction was demonstrated to elicit antimicrobial activity against L. innocua UCC 3 and Leu. paramesenteroides NCDO 869, producing a zone of inhibition of 11.8 and 11.7 mm, respectively, whereas no inhibition was observed when B. cereus TISTR 687, B. subtilis NCDO 10073, E. coli DH5α, and MRSA DMST 20625 and S. aureus ATCC 25923 were used as indicator bacteria (Table 4; Supplementary Figure 3).

Transcriptional Activity of Genes Associated With the Gene Clusters

To validate the mass spectrometry-based identification of the (partially) purified compounds, transcriptional analysis of genes...
**FIGURE 3** The gene clusters associated with the non-ribosomally synthesized peptides, bacilsin (A), bacillibactin (B), surfactin (C), macro lactin H (D), and pipastatin (E), and polyketide, bacillaene (F), of *B. velezensis* ML122-2 (red) compared to equivalent clusters in reference *Bacillus* strains. The predicted functions and sequence similarity are color-coded according to the legend.
associated with amylocyclicin, surfactin, and plipastatin biosynthesis was undertaken. RT-qPCR analysis was performed for variation analysis of specific genes associated with amylocyclicin (acnA), plipastatin (ppsA), and surfactin (srfAA) production at different time points (24 and 48 h) of *B. velezensis* ML122-2 cultivation. The *rpsE* gene which encodes the 30S-associated ribosomal protein S5 was used as a reference. Furthermore, since mersacidin was not detected (among others)
in the analysis, it was selected as a representative negative control for the transcriptional analysis. At 24-h cultivation, ancA, ppsA, and srfAA genes were upregulated 1.57-, 2.80-, and 1.16-fold, while after 48-h incubation, the transcription levels were upregulated 1.23-, 1.75-, and 2.53-fold, respectively. The relative expression level of gene mrsA at 48-h incubation (0.18-fold) was not significant when compared with 24-h incubation (0.15-fold; Figure 5). The low transcription levels measured for the mrsA gene suggest lack of expression of this gene cluster, being consistent with a failure to detect mersacidin.

### DISCUSSION

A previous study by Rungsirivanich and Thongwai (2020) revealed antibacterial activity of B. velezensis ML122-2 against S. aureus ATCC 25923 and MRSA DMST 20625 which may play a role in microbiological control strategy in Assam tea plantation areas as well as Assam tea fermentation processes. Consequently, the aim of the current study was to identify, purify, and characterize antimicrobial compounds produced by B. velezensis ML122-2 isolated from an Assam tea leaf. Antimicrobial activity assays revealed that various indicator bacteria are inhibited by the CFS of B. velezensis ML122-2. Analysis of the genome sequence of B. velezensis ML122-2 using BAGEL4 identified two distinct bacteriocin gene clusters including those associated with the production of mersacidin and amylocyclicin genes, while six gene clusters potentially involved in the synthesis of secondary metabolites consisting of bacilysin, bacillibactin, surfactin, macrolactin H, bacillaene, and plipastatin identified using antiSMASH. Although the genome of B. velezensis ML122-2 appeared to contain the complete mersacidin gene cluster, mersacidin did not appear to be synthesized by B. velezensis ML122-2 under laboratory conditions which is consistent with the study by Herzner et al. (2011). The failure to detect mersacidin may be due to low expression levels of mrsA and associated genes. Possibly, the expression of the mersacidin-associated genes may be induced under stress conditions or specific media and deserves further investigation. It has previously been shown that inactivation of mrsR1 causes loss of mersacidin production in synthetic medium by inhibiting MrsA synthesis (Guder et al., 2002), while knockouts of mrsR2K2 genes explicitly have been shown to prevent induction of mersacidin transcription (Schmitz et al., 2006).

Several species of Bacillus can produce secondary metabolites with antimicrobial activity against plant pathogenic bacteria and fungi (Lv et al., 2020), and plant growth-promoting activity with plant hormone production ability, such as indole-3-acetic acid (IAA) and 2,3-butanediol (Chen et al., 2007). Various studies have reported the production of multiple antimicrobial compounds by Bacillus strains. A previous study by Han et al. (2018) revealed a broad-spectrum of antimicrobial activity produced by B. amyloliquefaciens WY047 as a result of the simultaneous production of six antimicrobial substances. Gao et al. (2017) presented an engineered B. subtilis pB2-L with the ability to co-produce surfactin and plipastatin. In the current study, B. velezensis ML122-2 was observed to co-produce amylocyclicin, plipastatin, and surfactin. Several reports highlight the role of amylocyclicin (Chen et al., 2009), plipastatin/fengycin, and surfactin (Ongena et al., 2007) in the biological control of plant pathogens. The study by Scholz et al. (2014) revealed that amylocyclicin produced by B. velezensis FZB42 exhibits high antibacterial activity against Gram-positive bacteria (e.g., B. subtilis, B. cereus, Micrococcus luteus, and Paenibacillus granivorans). Surfactin exhibits antimicrobial and emulsification activities and inhibits biofilm formation (Chen et al., 2015). Plipastatin, also known as fengycin, has been reported to demonstrate antibacterial (e.g., L. monocytogenes, S. aureus, and Salmonella Typhimurium) and antifungal (e.g., Fusarium oxysporum and Pythium ultimum) activities causing cellular membrane distortion and cell membrane pore formation and

### TABLE 4 | Antibacterial activity of CFS, purified amylocyclicin and purified plipastatin and purified surfactin against indicator strains using an agar well diffusion assay.

| Indicator strain                  | Zone of inhibition |
|-----------------------------------|--------------------|
|                                  | CFS                | Purified amylocyclicin | Purified plipastatin | Purified surfactin |
| B. cereus TISTR 687              | 10.1 ± 0.2         | 7.8 ± 0.2             | 7.8 ± 0.6           | 0                 |
| B. subtilis NCDO 1007/3          | 0                  | 10.5 ± 0.4            | 0                   | 0                 |
| L. innocua UCC3                  | 11.1 ± 0.2         | 19.3 ± 0.2            | 0                   | 11.8 ± 0.2        |
| Leu. paramesenteroides NCDO 869  | 16.5 ± 0.0         | 24.0 ± 0.4            | 7.8 ± 0.2           | 11.7 ± 0.5        |
| MRSA DMST 20625                  | 10.4 ± 0.4         | 10.8 ± 0.6            | 7.5 ± 0.4           | 0                 |
| S. aureus ATCC 25923             | 10.4 ± 0.4         | 11.8 ± 0.2            | 7.5 ± 0.4           | 0                 |

Data are expressed as mean ± standard deviation (n = 3).
ultimately death of cells (Gao et al., 2017; Jeong et al., 2018; Lin et al., 2020). The ability to produce multiple antimicrobial compounds has been described to increase the potential for biological control (Han et al., 2018). Our findings support the notion that B. velezensis ML122-2 found on Assam tea leaf plays a role in microbiological control in Assam tea or Miang cultivation via the production of antimicrobial peptides (Rungsirivanich et al., 2019). Consequently, B. velezensis that can be found both on Assam tea leaves (Rungsirivanich et al., 2020) and in fermented Assam tea products (Unban et al., 2020) may exert a powerful biocontrol function in environments, preventing food spoilage through the production of antimicrobial compounds, such as amylolysicin, plipastatin, and surfactin.

CONCLUSION

B. velezensis ML122-2 exhibits strong and broad-spectrum antimicrobial activity. Three antimicrobial peptides produced by B. velezensis ML122-2, that is, amylolysicin, plipastatin, and surfactin, were purified from CFS and cell pellets, and their masses confirmed by MALDI-TOF mass spectrometry, this being consistent with transcriptional activity of specific marker genes for the corresponding gene clusters. Each purified peptide was shown to be antimicrobial, with amylolysicin, in particular, eliciting substantial antimicrobial activity. These findings show that B. velezensis has the potential to play an important role in microbial biocontrol in Assam tea cultivation and Assam tea fermentation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, JAGTWM0000000000, MH796212, CP000560, AL009126, AJ250862, and X70356.

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AUTHOR CONTRIBUTIONS

PR, EP, JM, NT, and DS designed the experiments. PR, EP, PO’C, and DF analyzed the data. PR, JM, and DS investigated the data. NT and DS acquired the funding. PR and NT prepared the original draft. PR, PO’C, JM, NT, and DS reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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