Purification, characterization, mode of action and enhanced production of Salivaricin mmaye1, a novel bacteriocin from Lactobacillus salivarius SPW1 of human gut origin

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ABSTRACT

Background: Emergence of antibiotic resistance among pathogenic and food spoilage bacteria such as *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus pyogenes*, *Streptococcus sanguinis*, *Streptococcus mutans*, *Bacillus cereus,* and *Listeria monocytogenes* triggered the search for alternative antimicrobials. An investigation aimed at purifying, characterizing, elucidating the mode of action, and enhancing the production of salivaricin from *Lactobacillus salivarius* of human gut origin was conducted.
Results: Salivaricin mmaye1 is a novel bacteriocin purified from *L. salivarius* isolated from human feces. It is potent at micromolar concentrations and has a molecular weight of 1221.074 Da as determined by MALDI-TOF mass spectrometry. It has a broad spectrum of antibacterial activity. Salivaricin mmaye1 showed high thermal and chemical stability and moderate pH stability. The proteinaceous nature of salivaricin mmaye1 was revealed by the complete loss of activity after treatment with pepsin, trypsin, α-chymotrypsin, protease, and proteinase. Salivaricin mmaye1 is cell wall associated, and adsorption–desorption of the bacteriocin from the cell wall of the producer by pH modification proved successful. It exhibited a bactericidal mode of action mediated by pore formation. Its biosynthesis is regulated by a quorum sensing mechanism. Enhanced production of salivaricin mmaye1 was achieved in a newly developed growth medium.
Conclusions: A novel, cell wall adhering, highly potent bacteriocin with a broad spectrum of inhibitory activity, membrane-permeabilizing ability, and enhanced production in a newly constituted medium has been isolated. It has a quorum sensing regulatory system and possesses interesting physicochemical characteristics favoring its future use in food biopreservation. These findings pave the way for future evaluation of its medical and food applications.

1. Introduction

There are increasing reports on antibiotic resistance among pathogens [1,2]. Furthermore, some pathogens have developed multidrug resistance to commercially available antibiotics [3,4,5,6]. Furthermore, consumers' preference for food products containing less chemical preservatives continues to increase [7,8,9]. The use of natural products as antibiotics and food preservatives is receiving increasing attention because they do not significantly alter the microbiota of the human gut unlike synthetic drugs and chemical preservatives [8,10,11].

One of the classes of natural products that have been receiving increasing attention recently is bacteriocins. Bacteriocins are low-molecular-weight peptides that exhibit either a narrow or broad spectrum of inhibitory activities against other microbiota [8]. Bacteriocins are one of the inherent defense systems of bacteria required for effective niche competition [12]. They differ from most antibiotics in that they are ribosomally synthesized and are generally harmless to humans and the environment. Bacteriocins are gaining more interest owing to their novel modes of action and inhibitory activities against several pathogenic multiresistant bacteria. Recent studies have shown that they possess a huge potential in the treatment of multiple human and animal infections, and their potential use as biopreservatives and probiotics are becoming major areas of scientific interest [13]. The fact that bacteriocins are quick acting and potent even at nanomolar concentrations reduces chances of resistance development [13].

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Although bacteriocins are produced by different bacteria, those from lactic acid bacteria (LAB) are generally recognized as safe [14]. Furthermore, the bacteriocins are heat stable and inactivated by gut proteases; their biosynthetic gene cluster are plasmid encoded, thereby facilitating the use of genetic engineering approaches in improving production [15,16]. Heterologous expression of genes encoding bacteriocins in genetically amenable and more robust hosts such as *Escherichia coli* is one of the approaches that can be used to enhance bacteriocin production [17]. Enhanced production of pediocin PA-1 and bactofencin A was achieved by expressing their structural and transporter genes in *E. coli* [17]. The yield and antimicrobial activity of enterocin A when heterologously produced in *Kluyveromyces lactis* and *Pichia pastoris* were higher than those in its native producer (*Enterococcus faecium*) [18].

Lactobacillus salivarius is one of the promising LAB strains with potential probiotic applications. It is mostly found in human, avian, and porcine gastrointestinal tracts, the majority of which are bacteriocinogenic [10,15]. It is suggested that bacteriocin production is an essential attribute of the probiotic *L. salivarius* [10]. To date, *L. salivarius*-derived bacteriocins from mammalian sources are as follows: the two-peptide bacteriocin ABP-118, made up of ABP-118α and ABP-118bβ (4096.69 and 4333 Da, respectively) [19,20]; the two-peptide bacteriocin salivaricin P, made up of Sln1 and Sln2 (4096 and 4283 Da, respectively) [20]; the two-peptide bacteriocin salivaricin T, constituted of SalTa and SalTb (5656.25 and 5270.51 Da, respectively); the one-peptide bacteriocin salivaricin L (molecular weight not determined) [10]; the two-peptide bacteriocin salivaricin CRL 1328, made up of Salα and Salβ (4096.14 and 4333.12 Da, respectively) [21]; bacteriocin LS1 (10 kDa) [22]; bacteriocin-like substance (5 kDa) [22]; bacteriocin LS2 (4115.1 Da) [23]; two-peptide bacteriocin-like inhibitory substance (5655.58 Da and 5269.02 Da) [23]; and bactofencin A (2782 Da) [24]. The bacteriocins isolated from the avian intestine are as follows: bacteriocin FK22 (4331.70 Da) [19], OR-7 bacteriocin (5123 Da) [25], bacteriocin L-1077 (3454 Da) [26], and bacteriocin SMXD51 (5383.2 Da) [27].

Despite the prospects of the bacteriocinogenic *L. salivarius* as a future probiotic, only limited research efforts have been put to unravel its bacteriocins. The mechanism by which it regulates bacteriocin production has not been elucidated. Moreover, modes of action of its bacteriocins remain largely unknown. Furthermore, optimization and scale-up studies are quite limited. In this study, a novel bacteriocin (salivaricin mmaye1) produced by *L. salivarius* SPW1 of human fecal origin was purified and characterized. Its mode of action was investigated. Enhancement of salivaricin mmaye1 production through modification of growth media was studied.

2. Materials and methods

2.1. Bacterial strains and culture media

All streptococci (except *Streptococcus mutans*), enterococci, *Bacillus cereus*, *Micrococcus luteus,* and *Lactococcus lactis* were obtained from American Type Culture Collection (ATCC). *Listeria monocytogenes* NCTC 10890 was obtained from National Collection of Type Culture (NCTC). *Staphylococcus aureus* RF122, *S. mutans* GEJ11, *Pseudomonas aeruginosa* PA7, *Corynebacterium* spp. GH17, *Escherichia coli* UT181, *Lactobacillus plantarum* K25, *Lactobacillus agilis* Yanat1, and *Lactobacillus pentosus* CS2 were taken from the culture collection of Microbial Biotechnology Laboratory, Division of Microbiology, Institute of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. *L. salivarius* SPW1, *L. plantarum* K25, *L. agilis* Yanat1, and *L. pentosus* CS2 were maintained on De Man, Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany). All streptococci were maintained on Todd-Hewitt agar (Difco, Le Pont de Claix, France). *M. luteus* ATCC 10240, *B. cereus* ATCC 14579, *S. aureus* RF122, *P. aeruginosa* PA7, *Corynebacterium* spp. GH17, and *E. coli* UT181 were maintained on Mueller-Hinton agar (Merck, Darmstadt, Germany). *E. faecium* ATCC BAA-2127 and *E. faecium* ATCC 349 were maintained on Tryptic soy agar (Merck, Darmstadt, Germany), whereas other enterococcal strains and *L. monocytogenes* NCTC 10890 were maintained on Brain Heart Infusion agar (Merck, Darmstadt, Germany). *L. lactis* ATCC 11454 was maintained on M17 agar (Merck, Darmstadt, Germany) supplemented with 5% glucose (Merck, Darmstadt, Germany).

2.2. Isolation, identification, and screening of LAB for bacteriocin production

MRS broth (Merck, Darmstadt, Germany) was inoculated with human feces from a healthy adult human subject and incubated aerobically for 24 h at 37°C. The culture was serially diluted in peptone water (Merck, Darmstadt, Germany), and LAB was isolated by growing on the MRS agar plate (Merck, Darmstadt, Germany) at 37°C. MRS broth was inoculated with single colonies from a 24 h old MRS agar LAB culture and incubated aerobically at 37°C for 24 h. Screening of LAB for bacteriocin production was carried out using cell-free supernatant (CFS) by a well diffusion assay. MRS agar used for well diffusion assay was supplemented with 0.1% $CaCO_3$ (Friedemann Schmidt Chemical, Germany). *S. aureus* RF122, *M. luteus* ATCC 10240, *Streptococcus pyogenes* ATCC 12344, *Streptococcus sanguinis* ATCC 10556, *S. mutans* GEJ11, *B. cereus* ATCC 14579, and *L. monocytogenes* NCTC 10890 were used as indicators because they are the bacterial targets of interest.

Molecular identification of LAB was conducted by amplifying 16S rRNA gene by PCR using the universal primers 27F (5′-AGAGTTTGATC (A/C)TGGCTCAG-3′) and 1492R (5′-ACGG(C/T) TACCTTGTTACGACTT-3′). The 16S rRNA gene was sequenced, and a similarity search was performed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Purification, determination of molecular weight, and molar extinction coefficient of bacteriocin

L. salivarius SPW1 was grown in the MRS medium for 18 h in a bioreactor (Sartorius Stedim, Germany) at 37°C, with an impeller agitation speed of 150 rpm; the medium was then centrifuged (10,000 rpm for 20 min at 4°C) to collect the supernatant, which was subsequently filtered using a 0.22 μm filter to obtain a CFS. This was subjected to hydrophobic interaction chromatography in which acetonitrile (Merck, Darmstadt, Germany) gradient (20%, 40%, 60%, and 80% v/v) was used for elution of the peptides adsorbed onto the surfaces of amberlite XAD-16 particles (Sigma-Aldrich, St. Louis, USA) packed in a glass column. Fractions were evaporated, and activity was determined by a well diffusion assay. The active fraction was subjected to reverse-phase high-pressure liquid chromatography (RP-HPLC). The mobile phase consisted of two solvents: solvent A (95% Milli-Q water [Millipore, USA] and 5% acetonitrile [Merck, Germany]) and solvent B (100% acetonitrile). Elution was performed using a biphasic gradient of 20–80% (v/v) acetonitrile at a flow rate of 1 ml/min for 90 min. Fractions were collected and evaporated using a centrifugal vacuum evaporator (Thermo Fisher Scientific, Vantaa, Finland), and activity was tested. The molecular weight of the bacteriocin was determined by subjecting the active HPLC fraction to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. To ascertain the molar extinction coefficient, twofold dilutions of the bacteriocin were prepared, and the bacteriocin concentration was expressed in molar units. Absorbance at 280 nm was measured, and a standard curve was generated and used for determining the molar extinction coefficient.

2.4. Inhibitory spectrum and MIC

MRS broth was inoculated with a 24 h old culture of *L. salivarius* SPW1 at a concentration of 5% (v/v) and incubated at 37°C for 20 h. The CFS was subjected to precipitation using 80% ammonium sulfate, after which it was centrifuged (10,000 rpm, 20 min, 4°C) to obtain the precipitate. The precipitate was dissolved in minimum Milli-Q water, and the semi-purified bacteriocin was used to determine the antibacterial spectrum by a well diffusion assay.

Minimum inhibitory concentrations (MICs) of key bacterial targets of interest, namely, *S. mutans*, *S. sanguinis*, *M. luteus*, and *S. aureus* were determined by broth microdilution assay as described by Mota-Meira [28] with little modifications. Twofold dilutions of pure salivaricin mmaye1 were prepared in adequate media, and 10 μl of each was added to a 96-well microtiter plate. Overnight culture of the indicator bacteria was diluted to 1×10^8 CFU/ml, and 150 μl of this solution was added to each pure bacteriocin preparation. Wells containing indicators without bacteriocin were used as a positive control, whereas wells containing only the media were used as blank (negative control). The plate was incubated at 37°C, and optical density (OD) at 600 nm was determined with a multiskan spectrophotometer for a period of 24 h. MIC was defined as the bacteriocin preparation that caused growth reduction by more than 80% compared with the positive control.

2.5. Bacteriocin stability test

Bacteriocin stability test was carried out as described by Goh and Philip [29]. Bacteriocin was exposed to different temperatures: 40°C, 60°C, and 80°C for 40 min as well as 100°C and 121°C for 15 min. Samples were cooled to room temperature before testing for inhibitory activity. The stability of bacteriocin to enzymes (Sigma-Aldrich, St. Louis, USA), namely, proteinase K, lysozyme, pepsin, lyticase, catalase, trypsin, α-chymotrypsin, protease, proteinase, and hyaluronidase, was tested by adding different enzyme preparations, thereby making a final enzyme concentration of 1 mg/ml and then incubating this solution for 1 h at 37°C; then, the inhibitory activity was tested. Bacteriocin was adjusted to various pH values (2, 3, 5, 8, and 10) and then incubated for 2 h at room temperature, following which the inhibitory activity was again tested. The stability of the bacteriocin upon exposure to different chemicals, namely, 1% (v/v) Tween 80, 1% (v/v) Tween 20, 1% (w/v) sodium dodecyl sulfate (Fisher Scientific, New Jersey, USA), and 1% (v/v) Triton X-100 was investigated by adding these chemicals to the bacteriocin and then incubating for 2 h at room temperature followed by inhibitory activity test.

2.6. Bacteriocin–cell wall association assay

MRS broth was inoculated with 5% (v/v) of a 24 h old *L. salivarius* SPW1 culture and incubated at 37°C for 18 h after which it was centrifuged at 10,000 rpm for 20 min at 4°C to collect the cell pellet and the supernatant. Activity of CFS was tested by a well diffusion assay. The cell pellet was resuspended in 95% (v/v) methanol (Merck, Darmstadt, Germany) at pH 2 and stirred overnight at 4°C on a magnetic stirrer (Benchmark, Edison, NJ, USA). The cell suspension was centrifuged (10,000 rpm for 30 min at 4°C), and the supernatant was filtered using a 0.22 μm Millipore filter after which the residue methanol was evaporated on a water bath (Memmert, Schwalbach, Germany) at 40°C. The cell extract was reconstituted in ultrapure water and inhibitory activity tested by a well diffusion assay.

2.7. Mechanism of regulation of salivaricin mmaye1 production

Bacteriocin induction test was performed as described by Barbour et al. [30] with slight modifications. Colonies from an overnight MRS agar culture of *L. salivarius* SPW1 was added to 10 ml of MRS broth and grown for 18 h at 37°C. The culture was centrifuged (2000 rpm for 10 min) to collect the cell pellet after which it was resuspended in 0.85% saline solution and washed three times to remove most of the bacteriocin from the surface of the cell wall. Eppendorf tubes (1.5 ml) containing 900 μl of MRS broth were each inoculated with 100 μl of the cell suspension. Fifty microliters (50 μl) of four different bacteriocin preparations, namely, CFS, ammonium sulfate precipitate, Amberlite XAD-16 fraction, and pure salivaricin mmaye1 each showing inhibitory activity at 6 AU/ml, were added to the culture separately and marked as "induced," whereas culture tubes without bacteriocin preparation were marked as "control." All culture tubes were incubated at 37°C for 18 h after which 50 μl of each bacteriocin preparation was added to the corresponding control culture tubes. Well diffusion assay was then performed. Induction of bacteriocin production occurred when an "induced tube" showed activity while the corresponding "control" tube did not.

2.8. Mode of action

2.8.1. Time-killing assay

S. mutans GEJ11, *S. sanguinis* ATCC 10556, and *S. aureus* RF122 were grown for 10 h, and the medium was centrifuged (2000 rpm for 5 min) to collect cell pellet. Each cell pellet was resuspended in ice-cold 5 mM sodium phosphate buffer (pH 7.2) and washed twice. The cell suspension was mixed at a ratio of 1:1 with salivaricin mmaye1 (5 X MIC) and incubated at 37°C. The control consisted of bacterial suspension without the addition of salivaricin mmaye1. Log_{10} CFU/ml was measured at an interval of 20 min for a period of 220 min.

2.8.2. Pore-formation assay

M. luteus was grown in M17 supplemented with 5% glucose (GM17) until $OD_{600nm} = 0.45$ after which 5 μM SYTOX green stain (Invitrogen, USA) was added. Ninety microliters of stained bacteria was added to a MicroAmp Fast Optical 96-well reaction plate (Applied Biosystems, Life Technologies, USA). After a stable line base was attained, 10 μl of bacteriocin (5 X MIC) was added to the stained bacteria. Sodium phosphate buffer (5 mM) and nisin (SIGMA-Aldrich, USA) were added to the stained bacteria in different wells to serve as negative and positive controls, respectively. Fluorescence resulting from the binding of SYTOX green to leaked intracellular DNA was monitored using Real-Time PCR (Applied Biosystems, USA).

2.9. Enhancement of salivaricin mmaye1 production

2.9.1. Development of new media

A new medium named as SGSL was produced. The components of this medium are as follows: 1% tryptone (Difco, Le Pont de Claix, France), 1% peptone, (Difco, Le Pont de Claix, France), 1% yeast extract (Difco, Le Pont de Claix, France), 5% glucose (Merck, Darmstadt, Germany), 0.05% ascorbic acid (R & M Chemicals, Essex, UK), 0.2% sodium citrate (Peking Chemical Works, Peking, China), 0.005% manganese (II) sulfate (BDH Chemicals Ltd, Poole, England), 0.025% magnesium sulfate (Halewood Chemicals Ltd, Middlesex, England), 0.02% sodium chloride (John Kollin Corporation, USA), and 0.1% Tween 80 (Sigma-Aldrich, Missouri, USA). Another medium named as TPYGMA was also formulated. The composition is as follows: 1% tryptone, 1% peptone, 1% yeast extract, 1.5% glucose, 1% 2-morpholinoethanesulfonic acid, MESA (2-morpholinoethanesulfonic acid) (Merck, Darmstadt, Germany), and 0.05% ascorbic acid. Furthermore, TPYGCaT80 medium was also formulated, whose components are as follows: 1% tryptone, 1% peptone, 1% yeast extract, 1.5% glucose, 0.1% $CaCO_3$, and 0.1% Tween 80.

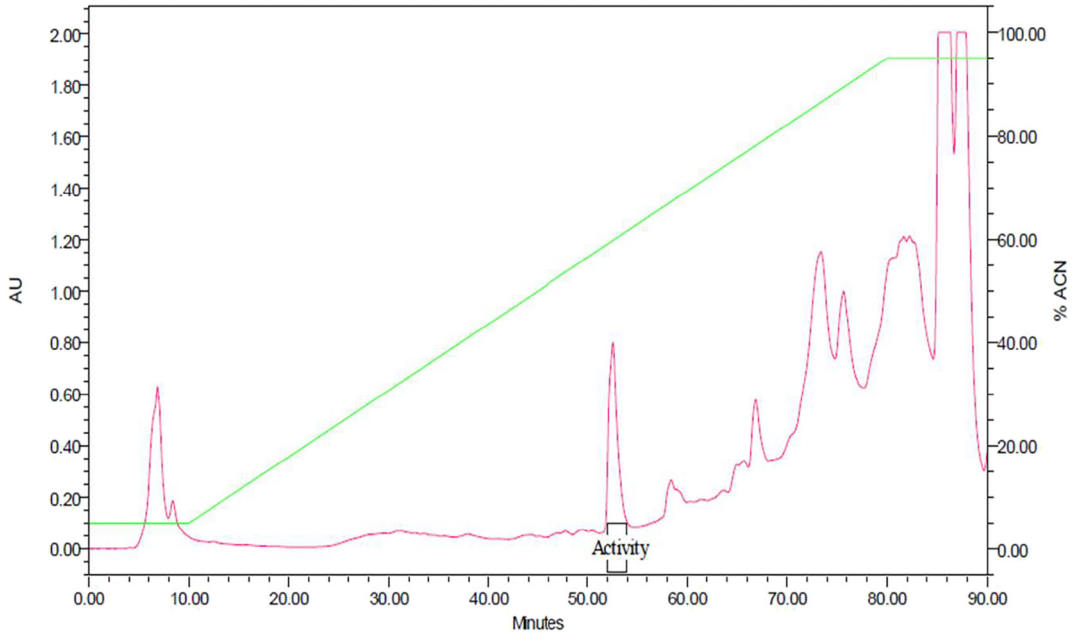

Fig. 1. Reverse-phase HPLC chromatogram of active XAD-16 fraction from *Lactobacillus salivarius* SPW1. Vertical lines indicate retention time. Active fraction from hydrophobic interaction chromatography was subjected to RP-HPLC.

2.9.2. Effect of media on salivaricin mmaye1 production

Different media (MRS, SGSL, TPYGMA, TPYGCaT80, Brain Heart Infusion broth, tryptic soy broth, Todd-Hewitt broth, M17 broth, M17 supplemented with 5% glucose, M17 supplemented with 5% sucrose, and M17 supplemented with 5% galactose were inoculated with 5% (v/v) of a 24 h old *L. salivarius* SPW1 culture and incubated at 37°C for 20 h. Two 12 h subcultures were performed, and the third culture was centrifuged (5000 rpm for 10 min) to collect the pellet, which was resuspended in 0.85% saline solution and washed twice using the same solution. The OD (600 nm) was adjusted to 0.1, and each medium was inoculated with 5% (v/v) of the corresponding cell suspension and incubated at 37°C for 18 h. CFS from each medium was used in the well diffusion assay using *M. luteus* as the indicator strain.

3. Results

3.1. Isolation, identification, and screening of LAB for bacteriocin production

Four LAB strains were successfully isolated from the feces of a healthy human subject. On the basis of 16S rRNA gene sequence homology searches, three strains were identified as *L. salivarius* and were designated as SPW1, SPW2, and SPW3, whereas one strain was *L. agilis* subsequently designated as Yanat1. Of all four strains, CFS from *L. salivarius* SPW1 exhibited the strongest and broadest inhibitory activity toward all indicators tested.

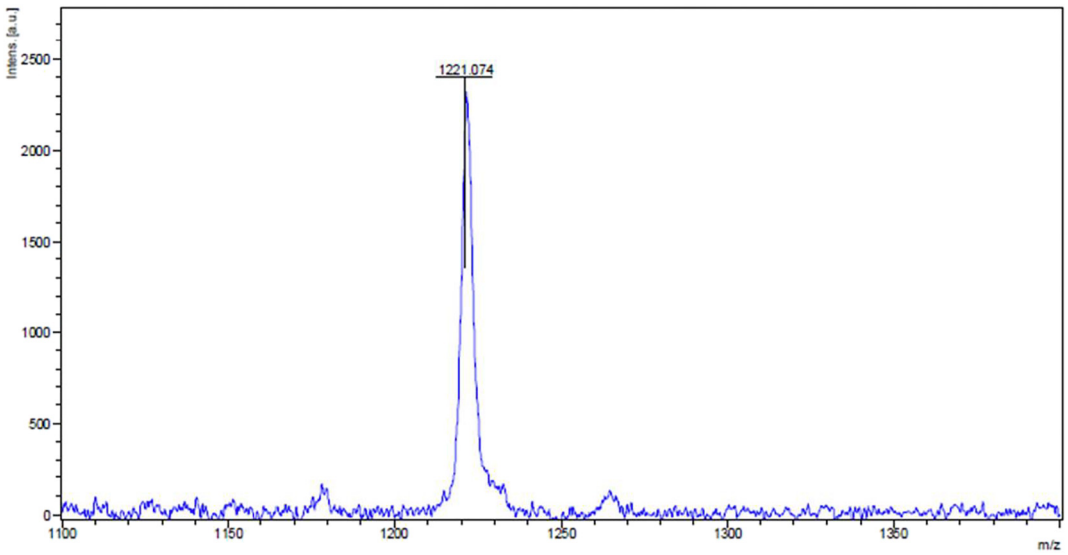

Fig. 2. MALDI-TOF mass spectrum of purified salivaricin mmaye1. Purified salivaricin mmaye1 was subjected to MALDI-TOF mass spectrometry.

Table 1
Inhibitory spectrum of salivaricin mmaye1.

Indicator	Zone of inhibition (mm)[a]	MIC (μM)
Streptococcus mutans GEJ11	++++	6.88
Streptococcus pyogenes ATCC 12344	++++	ND
Streptococcus equisimilis ATCC 12388	++++	ND
Streptococcus sanguinis ATCC 10556	++++	3.44
Streptococcus constellatus ATCC 27823	++	ND
Streptococcus pneumonia ATCC 6301	-	ND
Lactococcus lactis ATCC 11454	++	ND
Listeria monocytogenes NCTC 10890	++	ND
Staphylococcus aureus RF122	++++	6.88
MRSA	++	ND
Bacillus cereus ATCC 14579	-	ND
Corynebacterium spp. GH17	+++	ND
Pseudomonas aeruginosa PA7	++	ND
Escherichia coli UT181	++	ND
Micrococcus luteus ATCC 10240	++++	3.44
Lactobacillus plantarum K25	-	ND
Lactobacillus pentosus CS2	-	ND
Lactobacillus agilis Yanat1	+++	ND
Enterococcus faecium ATCC BAA-2318	+++	ND
Enterococcus faecium ATCC BAA-2127	+++	ND
Enterococcus faecium ATCC 27270	+++	ND
Enterococcus faecium ATCC 27273	++++	ND
Enterococcus faecium ATCC 19434	+++	ND
Enterococcus faecium ATCC 6569	+++	ND
Enterococcus faecium ATCC 349	+++	ND
Enterococcus faecium ATCC 25307	++++	ND

[a] ++++ Inhibition zone >20 mm, +++ Inhibition zone 15–20 mm, ++ Inhibition zone <15 mm, - No inhibition, ND Not determined.

3.2. Purification, determination of molecular weight, and molar extinction coefficient of bacteriocin

Bacteriocin was successfully purified to homogeneity by using a combination of hydrophobic interaction chromatography and RP-HPLC. Bacteriocin was collected at a retention time of 52–54 min (Fig. 1). The molecular weight of the bacteriocin as determined by MALDI-TOF mass spectrometry was 1221.074 Da (Fig. 2). The molar extinction coefficient of bacteriocin at 280 nm was 218.68 $M^{-1} \cdot cm^{-1}$.

3.3. Inhibitory spectrum and MIC

Upon investigating the inhibitory spectrum of this bacteriocin, results revealed that it inhibited the growth of bacterial targets found in 10 genera, namely, *Streptococcus*, *Lactococcus*, *Listeria*, *Staphylococcus*, *Corynebacterium*, *Pseudomonas*, *Escherichia*, *Micrococci*, *Lactobacillus,* and *Enterococci* (Table 1). It is important to note that salivaricin mmaye1 inhibited the growth of methicillin-resistant *S. aureus* (MRSA). However, it was not inhibitory toward *Streptococcus pneumoniae* ATCC 6301, *B. cereus* ATCC 14579, *L. plantarum* K25, and *L. pentosus* CS2.

MIC values obtained for *M. luteus* ATCC 10240 and *S. sanguinis* ATCC 10556 were lower (3.44 μM) than those obtained for *S. mutans* GEJ11 and *S. aureus* RF122 (6.88 μM) as shown in Table 1. These low values show the high potency of salivaricin mmaye1 against these targets.

3.4. Bacteriocin stability test

Residual activity was detected at various temperatures and after chemical and enzymatic treatments as shown in Table 2. Activity was retained at all temperatures investigated, the lowest being at 121°C. It also retained almost 100% activity after exposure to various chemicals. However, upon treatment with proteinase K, there was 50% loss of activity. Treatment with pepsin, trypsin, α-chymotrypsin, protease, and proteinase resulted in complete loss of activity. There was no loss of activity upon enzymatic treatment with lysozyme, lyticase, catalase, and hyaluronidase, thereby confirming its proteinaceous nature.

Table 2
Stability tests for bacteriocin (heat, enzyme, chemical, and pH).

Test	Zone of inhibition (mm)[a]
Heat	
40	++++
60	++++
80	++++
100	+++
121	+++
Enzyme	
Proteinase K	++
Lysozyme	++++
Pepsin	-
Lyticase	++++
Catalase	++++
Trypsin	-
α-Chymotrypsin	-
Protease	-
Proteinase	-
Hyaluronidase	++++
Chemical	
Triton X-100	++++
Sodium dodecyl sulfate	++++
Tween 80	++++
pH	
2	++++
3	++++
5	++++
8	++
10	++

[a] ++++ Inhibition zone >20 mm, +++ Inhibition zone 15-20 mm, ++ Inhibition zone <15 mm, - No inhibition.

Salivaricin mmaye1 was active in the pH range of 2–10, but the highest activity occurred in the acidic pH range (pH 2-5).

3.5. Bacteriocin–cell wall association assay

The total activity of salivaricin mmaye1 was 1.92×10^6 AU, out of which 66.67% of the total activity was detected on the cell wall, whereas 33.3% was found in the CFS (Table 3). An experiment was conducted to investigate whether bacteriocin can be adsorbed and desorbed from the cell wall of the producer by pH modification of the broth culture. Increase in pH of broth culture to 5.8 before centrifugation resulted in adsorption of bacteriocin onto the cell wall of the producer, which was evident by the absence of activity in the CFS but the presence of activity in the cell extract.

3.6. Mechanism of regulation of bacteriocin biosynthesis

This assay was carried out to investigate the regulatory mechanism for bacteriocin production by *L. salivarius* SPW1. By adding an inducible concentration of different preparations of the bacteriocin (CFS, ammonium sulfate precipitate, Amberlite XAD-16 fraction, and pure bacteriocin), salivaricin mmaye1 production by the bac^- phenotype of *L. salivarius* SPW1 was restored.

Table 3
Bacteriocin recovered from cell-free supernatant and cell extract of *Lactobacillus salivarius* SPW1.

Source of bacteriocin	Activity (AU)	Activity (%)
Cell-free supernatant	6.4×10^5	33.33
Cell extract	1.28×10^6	66.67
Total	1.92×10^6	100.00

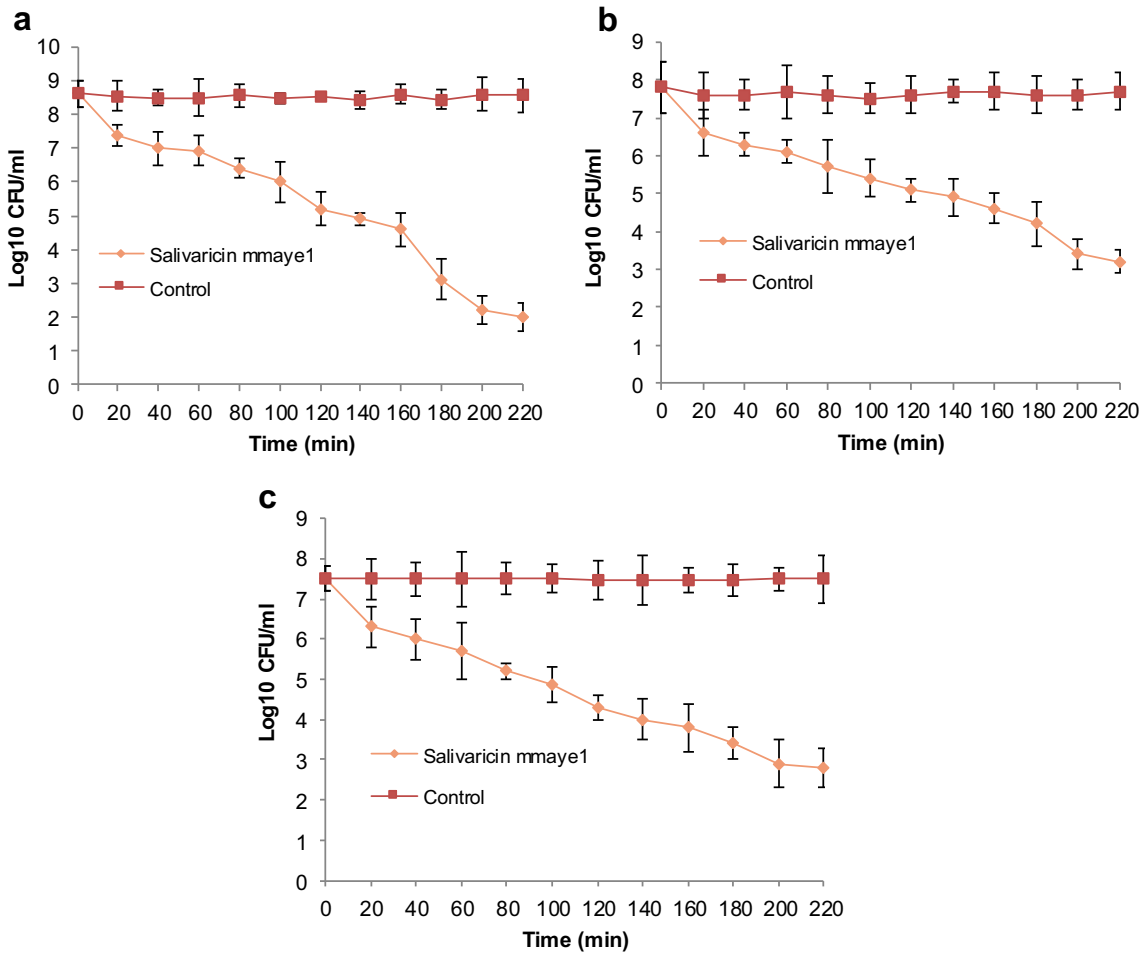

Fig. 3. Time-killing assay for salivaricin mmaye1 (a) *Streptococcus sanguinis* ATCC 10556 (b) *Streptococcus mutans* GEJ11, (c) *Staphylococcus aureus* RF122. Pure salivaricin mmaye1 was used to ascertain its mode and kinetics of action against selected bacterial targets.

3.7. Mode of action

3.7.1. Time-killing assay

Time-killing assay was carried out to investigate its mode of action and how quick it exerts its inhibitory effects. Results revealed that salivaricin mmaye1 exerts a bactericidal mode of action against its targets because there was continuous decline in $\log_{10}$ CFU/ml (Fig. 3) throughout the study period. After 220 min, $\log_{10}$ viable cell count had significantly decreased from 8.6 to 2.0 (76.74% reduction), 7.8 to 3.2 (58.97% reduction), and 7.5 to 2.8 (62.72% reduction) for *S. sanguinis*, *S. mutans*, and *S. aureus*, respectively. This reflects its quick-acting bactericidal nature.

3.7.2. Membrane permeabilization assay

This experiment was conducted to know whether salivaricin mmaye1 acts against its targets through pore formation or not. There was a steady increase in fluorescence intensity with time for both salivaricin mmaye1-treated and nisin-treated *M. luteus*

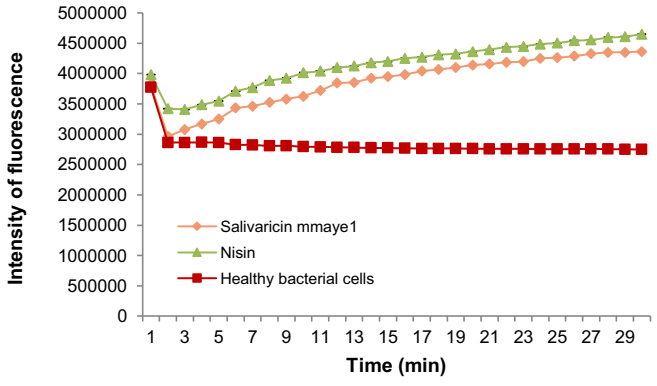

Fig. 4. Permeabilization of the cell membrane of *Micrococcus luteus* by salivaricin mmaye1. Pure salivaricin mmaye1 was added to *M. luteus* stained with SYTOX Green dye and fluorescence due to leakage of intracellular DNA was monitored by real-time PCR.

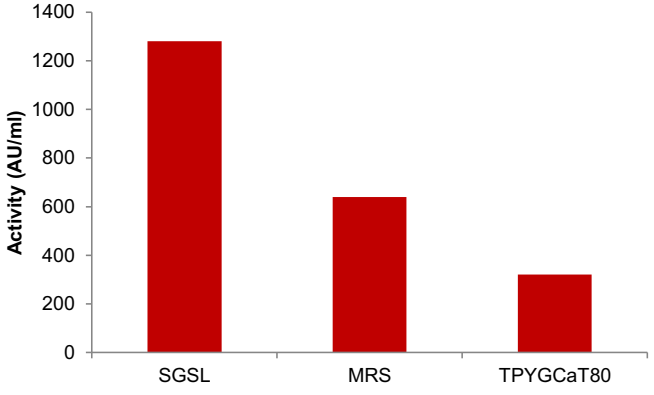

Fig. 5. Effect of media on bacteriocin production. Bacteriocin production was investigated in 11 media. Only three media (SGSL, MRS, and TPYGCaT80) supported bacteriocin production. *Micrococcus luteus* was used as the indicator bacterial strain.

albeit the fluorescence intensity of the latter was more than the former. Fluorescence intensity for the untreated bacterial cells remained unchanged (Fig. 4). Increase in fluorescence indicates pore formation.

3.8. Enhancement of salivaricin mmaye1 production

Three new media (SGSL, TPYGMA, and TPYGCaT80) were successfully synthesized. The compositions of these media were drawn from the constituents of commercially available media.

Bacteriocin production was observed in only three media, namely, SGSL, MRS, and TPYGCaT80 of the 11 media investigated (Fig. 5). The highest salivaricin titer was recorded in SGSL, whereas the least was obtained in TPYGCaT80.

4. Discussion

The presence of bacteriocinogenic *L. salivarius* SPW1 in the feces of healthy adult human subjects is important in the maintenance of the general well-being of a human subject. Moreover, *L. salivarius* SPW1 is nonhemolytic as well as catalase and oxidase negative, thereby indicating its health safety. *L. salivarius* is a major component of the human gut microflora and has not been reported to have any toxicity [31]. On the contrary, it has probiotic potential and improves the well-being of humans and animals [15]. It has been suggested that its bacteriocins can be used as biopreservatives [10]. However, the potential of this bacteriocin producer has been largely untapped. To date, only 14 *L. salivarius*-derived bacteriocins have been purified to homogeneity [10,19,20,21,23,24,25,26,27]. None of these bacteriocins have molecular weights equal to those of the bacteriocin described in this study. It is therefore considered as novel and subsequently named as salivaricin mmaye1. The late retention time of salivaricin mmaye1 (corresponding to high acetonitrile:water ratio) suggests that it contains a high amount of hydrophobic amino acid residues. Elucidation of the molar extinction coefficient would help in the quantitative estimation of salivaricin mmaye1, thus facilitating its future industrial application. Furthermore, it gives an insight into the biophysical property of salivaricin mmaye1.

Upon investigating the inhibitory spectrum of salivaricin mmaye1, it was discovered that it has a relatively broad spectrum of activity in that it inhibited selected bacterial strains within 10 genera. This attribute has also been observed in some selected bacteriocins [32,33,34]. The fact that it could not inhibit certain bacteria is thought to be an advantage because it is less likely to alter the gut microflora compared to a very broad-spectrum antimicrobial agent such as tetracycline [35,36]. Moreover, its broad spectrum of activity posits it as a future biopreservative considering the complex microflora of food [37]. *In vivo* study would be required to investigate its bioprotective potential. Interestingly, salivaricin mmaye1 was inhibitory toward two gram-negative bacteria (*P. aeruginosa* and *E. coli*). Although the mechanism of action against these gram-negative bacterial targets is unknown, we think that it possess a rare ability to penetrate their outer membrane. Inhibitory activity toward gram-negative bacteria has been reported for bacteriocin SMXD51(*Campylobacter jejuni*) produced by *L. salivarius* SMXD51 [38], plantaricin MG produced by *L. plantarum* KLDS1.0391 [39], bacteriocin GGN7 produced by *E. faecium* GGN7 [40], and bacteriocin BM1157 produced by *Lactobacillus crustorum* MN047 [41].

Although inhibition of *S. mutans* has also been observed for the bacteriocin LS1 produced by *L. salivarius* BGH01 (a human oral LAB strain) [22], unlike the aforementioned bacteriocin, salivaricin mmaye1 was also inhibitory toward *S. pyogenes*. This the first report of a *L. salivarius*-derived bacteriocin with inhibitory activity toward *S. pyogenes*, *Streptococcus equisimilis*, *S. sanguinis*, *Streptococcus constellatus*, *M. luteus, Corynebacterium* sp., and *L. lactis*. No single *L. salivarius*-derived bacteriocin has been reported to display inhibitory activity toward all the aforementioned pathogens except salivaricin mmaye1. This finding highlights the potential application of salivaricin mmaye1 in the treatment of oral infections and other diseases associated with the above-mentioned pathogens. *In vivo* potency against these pathogens would be required to substantiate its therapeutic potential.

S. mutans is the major cause of dental caries in humans [42]. *S. sanguinis* is the cause of infective endocarditis [43]. *M. luteus* is associated with peritonitis [44] and native valve endocarditis [45]. *S. aureus* is the cause of infections in human soft tissues and skin [46,47]. Moreover, the aforementioned pathogens are also major causes of food spoilage [48,49]. As such, MIC values for these selected bacterial targets were determined. Salivaricin mmaye1 was highly potent against *M. luteus* ATCC 10240, *S. sanguinis* ATCC 10556, *S. mutans* GEJ11, and *S. aureus* RF122. Although the potency of salivaricin mmaye1 against these targets is less than that of industrially produced nisin [50], its antibacterial spectrum is wider, thus suggesting wider future medical and food applications of salivaricin mmaye1 [37]. The observed difference in antibacterial activity could be a result of the very high purity level of commercially available nisin. We think that the antibacterial activity of salivaricin mmaye1 could be as high as or even better than nisin if the purity level of industrially produced nisin is attained. This may be achieved by employing chromatographic methods such as cation-exchange chromatography and immunoaffinity chromatography in a future study. However, in addition to the fact that salivaricin mmaye1 has a broader antibacterial spectrum than nisin, it also has a rare inhibitory activity toward pathogenic gram-negative bacteria, an attribute not reported for nisin. Moreover, studies suggest rare occurrence of resistance to nisin [51], but such report has not been available for *L. salivarius*-derived bacteriocins.

Time-killing assay showed that salivaricin mmaye1 inhibits its targets through a bactericidal mode of action. After 220 min, $\log_{10}$ viable cell count had significantly declined. This show that salivaricin mmaye1 acts quickly against its targets. This mode of action has been reported for several bacteriocins [50]. Its quick bactericidal mode of action makes it well positioned for future use in the treatment of infections caused by these targets [1]. Salivaricin mmaye1 acted on *M. luteus* through pore formation, thus leading to the loss of intracellular DNA as indicated by the increase in fluorescence. Moreover, a rapid increase in fluorescence intensity was observed, which signifies its quick onset of action. This is considered as an advantage because resistance to a quick-acting antimicrobial agent is less likely to occur compared with a slow-acting one [1,37]. Furthermore, its pore-forming ability was close to that of nisin. Although salivaricin CRL 1328 (isolated from *L. salivarius* CRL 1328) has been reported to cause dissipation of proton motive force of its target membrane, there was no evidence of pore formation [21]. We present the first report on membrane permeabilization as a mechanism of action of *L. salivarius*-derived bacteriocins.

Salivaricin mmaye1 was stable to various temperatures, chemicals, and enzymes. Activity was detected even after heating at 121°C for 15 min, thereby reflecting its thermal stability. Reduction in activity upon treatment with proteinase K and the complete loss of activity after treatment with pepsin, trypsin, α-chymotrypsin, protease, and proteinase is a reflection of its proteinaceous nature. To add credence to this fact, high activity was detected after enzyme treatment with lysozyme, lyticase, catalase, and hyaluronidase. Similar results were reported for plantaricin K25 [52] and bacteriocin ST8KF [53]. Salivaricin mmaye1 exhibited a high residual activity in the acidic pH range of 2–5. Reduced activity was observed in alkaline pH (8-10). These physical characteristics favor its future use in biopreservation of foods [37]. Although toxicity potential of salivaricin mmaye1 was not evaluated in this study, bacteriocins from *L. salivarius* have a GRAS status; hence, salivaricin mmaye1 can be considered safe for

consumption [15,54]. Future use in the treatment of human and animal infections would require improvements in its stability toward digestive proteases of the gastrointestinal tract, which may be achieved through bioengineering and encapsulation in nanoparticles [1,3,55].

Salivaricin mmaye1 is a cell wall-associated bacteriocin owing to the fact that 66.67% of the total activity was detected on the cell wall, whereas 33.3% of total activity was found in the CFS. This characteristic has never been reported for any *L. salivarius*-derived bacteriocin. We think that this attribute enhances its survival and competitiveness within the complex microbial communities of the human gastrointestinal tract. Adsorption–desorption of bacteriocin by pH modification of culture supernatant was achieved. This finding can be investigated in its purification by the adsorption–desorption approach, which has been documented for some bacteriocins [56].

CFS, ammonium sulfate precipitate, Amberlite XAD-16 fraction, and pure salivaricin mmaye1 all induced salivaricin mmaye1 production by the bac$^-$ phenotype of *L. salivarius* SPW1. This suggests that salivaricin mmaye1 production is regulated through a three-component quorum sensing mechanism involving a histidine protein kinase (HPK), a response regulator (RR), and an inducing peptide (IP). The IP in this case is salivaricin mmaye1. Quorum sensing regulatory mechanism, requiring the action of an IP, have been reported for ABP-118 produced by *L. salivarius* subsp. *salivarius* UCC118 [19]. However, the regulatory system for salivaricin mmaye1 production is slightly different in that the IP is the bacteriocin itself (autoinducible). This regulatory mechanism has been seen in *Streptococcus thermophilus* [57] and *Streptococcus salivarius* [57].

Salivaricin mmaye1 production occurred in only three (SGSL, MRS, and TPYGCaT80) of the 11 media tested. The reason for the lack of production of detectable levels of bacteriocin in other media is due to poor growth and absence of Tween 80. Production of the bacteriocin ST13BR by *L. plantarum* ST13BR was increased by 50% in the presence of Tween 80 [58]. SGSL and MRS had higher bacteriocin titer than TPYGCaT80 because they contain mineral salts. Manganese (II) sulfate greatly enhanced bacteriocin production by *Lactobacillus acidophilus* [59]. The highest bacteriocin production was observed in SGSL. This is attributed to the presence of tryptone, ascorbic acid, and sodium chloride in this medium. This observation suggests that tryptone (a component of SGSL) contains certain essential amino acids that are absent in meat extract (a component of MRS). Tryptone, ascorbic acid, and sodium chloride have been shown to influence bacteriocin production in *L. pentosus* ST151BR [60] and *L. salivarius* CRL 1328 [61]. Although both SGSL and MRS broths contain 10 components each, the cost of producing salivaricin mmaye1 in SGSL is cheaper than that in commercially available MRS broth. Therefore, it will be more economical to use SGSL for industrial production of the bacteriocin. It is pertinent to mention that the six-component medium (TPYGCaT80) being the cheapest of the three media may be a more cost-effective medium for salivaricin mmaye1 production, albeit further studies required to enhance yield.

5. Conclusions

Human gastrointestinal tract contains *L. salivarius* SPW1, which produces a novel bacteriocin named as salivaricin mmaye1. It is highly potent and possesses a broad spectrum of antibacterial activity. It is proteinaceous, displays high thermal and chemical stability, and exhibits moderate pH stability. Its production is regulated by a quorum sensing mechanism. Salivaricin mmaye1 is cell wall associated and possesses a bactericidal mode of action mediated by pore formation. Enhanced production of salivaricin mmaye1 occurred in a newly developed medium (SGSL). Having established the fact that salivaricin mmaye1 possesses interesting physicochemical properties favoring its use in food preservation, an *in vivo* evaluation of its biopreservative potential was not investigated. Future studies would focus on evaluating its economics of industrial applications.

Conflict of interest

The authors declare that they have no competing interest.

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