Pentocin MQ1: a Novel, Broad-spectrum, Pore-forming bacteriocin from Lactobacillus pentosus CS2 with Quorum Sensing Regulatory Mechanism and Biopreservative Potential

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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

Author contribution statement

Koshy Philip designed and supervised execution of the experiments and wrote the manuscript. Koshy Philip also edited the manuscript.

Samson Wayah designed the experiments, performed it and wrote the manuscript.

Keywords

bacteriocin, cell-wall associated bacteriocin, Quorum Sensing, Lactobacillus pentosus, bactericidal, pore formation, Broad-spectrum bacteriocin, biopreservation

Abstract

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Pentocin MQ1: a Novel, Broad-spectrum, Pore-forming bacteriocin from *Lactobacillus pentosus* CS2 with Quorum Sensing Regulatory Mechanism and Biopreservative Potential

Running title: Pentocin MQ1, a novel bacteriocin

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Abstract

Micrococcus luteus, *Listeria monocytogenes* and *Bacillus cereus* are major food-borne pathogenic and spoilage bacteria. Emergence of antibiotic resistance and consumer demand for foods containing less chemical preservatives triggered a search for natural antimicrobials. Pentocin MQ1 is a novel bacteriocin isolated from *Lactobacillus pentosus* CS2 of coconut shake origin. The purification strategy involved adsorption-desorption of bacteriocin followed by RP-HPLC. It has a molecular weight of 2110.672 Da as determined by MALDI-TOF mass spectrometry and a molar extinction value of 298.82 M^{-1} cm^{-1}. Pentocin MQ1 is not plasmid-borne and its biosynthesis is regulated by a quorum sensing mechanism. It ~~is~~ has a broad spectrum of antibacterial activity, exhibited high chemical, thermal and pH stability but sensitive to proteolytic enzymes. It is potent against *Micrococcus luteus*, *Bacillus cereus* and *Listeria monocytogenes* at micromolar concentrations. It is quick-acting and exhibited a bactericidal mode of action against its targets. Target killing was mediated by pore formation. Pentocin MQ1 is a cell wall-associated bacteriocin. Application of pentocin MQ1 improved the microbiological quality and shelf life of fresh banana. These findings place pentocin MQ1 as a potential biopreservative for further evaluation in food and medical applications.

INTRODUCTION

Consumer's requisition for food products containing less chemical preservatives (Barbosa *et al*., 2017) and emergence of antibiotic resistance among pathogenic and food spoilage bacteria prompted the search for novel antimicrobials (Berendonk *et al*., 2015; Jia *et al*., 2017). Bacteriocins are an attractive class of natural antimicrobials with potential for future use as synergist or replacement of antibiotics (Behrens *et al*., 2017; Collins *et al*., 2017) and currently used chemical preservatives (Kaškonienė *et al*., 2017; Wiernasz *et al*., 2017) because of their ability to inhibit some drug-resistant pathogens (Mathur *et al*., 2017).These interesting antimicrobial peptides are of bacterial origin and are

ribosomally synthesized (Langa *et al*., 2017). They have narrow or broad spectrum of inhibitory activity (Hanchi *et al*., 2017).

Bacteriocins are a highly diverse group of antimicrobial peptides with variations in molecular weight, inhibitory spectrum, mode of action, mechanism of biosynthesis and externalization, and self-protection mechanism (Salazar *et al*., 2017). They are part of the inherent defence system of bacteria and play other roles such as niche colonization, direct killing of competing strains and signaling (cross-talk and quorum sensing) within bacterial communities (Dobson *et al*., 2012; Inglis *et al*., 2013; Yang *et al*., 2014). They are commonly classified into two groups namely class I (undergo post-translational modification) and class II (unmodified). In less popular classification schemes class III (high molecular weight and heat-sensitive bacteriocins) (Alvarez-Sieiro *et al*., 2016) and class IV (bacteriocins with carbohydrate or lipid moieties) (Kaškonienė *et al*., 2017) were introduced. Bacteriocins are produced by lactic acid bacteria (LAB) and non-lactic acid bacteria (Mechoud *et al*., 2017). LAB bacteriocins are given more attention because they are generally recognized as safe (GRAS) facilitating their *use in situ* and *ex situ* in preservation of food (Bali *et al*., 2016; Castro *et al*., 2017; Hu *et al*., 2017). Moreover, they are inactivated by gut proteases, heat-stable, active at various pH, potent even at nanomolar concentration and their biosynthetic gene cluster is often plasmid-borne, facilitating the use of genetic engineering approaches in improving production (Cotter *et al*., 2013; Lakshminarayanan *et al*., 2013; Messaoudi *et al*., 2013; Woraprayote *et al*., 2016).

Biopreservation involves the use of microorganisms or their products and other natural bio-products to enhance safety and extend shelf life of food either by killing or reduction of the load of food spoilage microorganisms (Johnson *et al*., 2017; Saraoui *et al*., 2017). The concept of biopreservation of food has recently intensified due to growing consumer ~~growing~~ inclination towards foods containing biopreservatives or less synthetic chemical preservatives, fear of side effects of currently used chemical preservatives, demand for fresh-tasting and less processed food (Barbosa *et al*., 2017; Kashani[1] *et al*., 2012; Woraprayote *et al*., 2016). Fruits and vegetables are one of the major reservoirs of minerals, vitamins and fibre and are consumed worldwide. Fresh fruits such as banana have short shelf life due to their high moisture content (Joardder *et al*., 2014). Preservation of banana is a huge task especially if required fresh. Moreover, eating them fresh exposes consumers to food-borne pathogens (Berger *et al*., 2010; Tian *et al*., 2012).

Bacteriocin was first discovered in early 1925 when antagonistic activity was observed among strains of *Escherichia coli* (Ghazaryan *et al*., 2014). The discovery of colicin as the first bacteriocin was closely followed by that of nisin (1928), the first LAB bacteriocin (Shin *et al*., 2016). Despite the long history of LAB bacteriocins only nisin and pediocin PA-1/AcH have gained approval for preservation of selected foods (Barbosa *et al*., 2017; Saraniya and Jeevaratnam, 2014). The potential of bacteriocins in the biopreservation of fresh fruits or minimally processed fruits has been highly underexploited. Combined application of nisin-EDTA and chlorine was effective at reducing the surface microbial load of whole melon (Ukuku and Fett, 2002). Application of nisin, hydrogen peroxide, citric acid and sodium lactate effectively reduced the transfer of pathogens from the surface of melons to freshly cut pieces (Ukuku *et al*., 2005). Load of pathogens on the surfaces of minimally processed mangoes was controlled by packaging in nisin films (Barbosa *et al*., 2013). Enterocin AS-48 was effective at controlling contamination of raw fruits by *Listeria monocytogenes* (Molinos *et al*., 2008). Enterocin 416K1 inhibited the growth of *Listeria monocytogenes* on apples and grapes (Anacarso *et al*., 2011). The potential of preserving minimally processed papaya by applying alginate coatings containing pediocin has been demonstrated (Narsaiah *et al*., 2015). Biopreservation of fresh banana using bacteriocin has not been investigated.

Although *Lactobacillus pentosus* has been isolated from various sources, bacteriocinogenic strains are rare (Liu *et al*., 2008). Bacteriocinogenic strains of *Lactobacillus pentosus* with probiotic potential has been reported (Aarti *et al*., 2016). Bacteriocins of *Lactobacillus pentosus* origin have not been adequately studied. Pentocins have been poorly characterized and their regulatory mechanisms have not been sufficiently investigated. Their modes of action are unknown. Moreover biopreservation of fresh banana using *Lactobacillus pentosus*-derived bacteriocins (commonly called pentocins) has not been studied. In this study a novel bacteriocin (pentocin MQ1) from *Lactobacillus pentosus* CS2 of coconut shake origin was purified to homogeneity and characterized. Its regulatory mechanism and mode of action was investigated. Finally, its ability to preserve fresh bananas was studied.

MATERIALS AND METHODS

Bacterial strains and culture media

Streptococcus pyogenes, *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, *Streptococcus mutans* GEJ11, *Pseudomonas aeruginosa* PA7, *Corynebacterium* spp. GH17, *Escherichia coli* UT181, *Lactobacillus plantarum* K25 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. *Lactobacillus plantarum* K25 and *Lactobacillus pentosus* CS2 were maintained on MRS agar (Merck, Darmstadt, Germany). *Streptococcus pyogenes* ATCC 12344 and *Streptococcus mutans* GEJ11 were maintained on Todd-Hewitt agar (Difco, Le Pont de Claix, France). *Micrococcus luteus* ATCC 10240, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* RF122, *Pseudomonas aeruginosa* PA7, *Corynebacterium* spp. GH17 and *Escherichia coli* UT181 were maintained on Mueller-Hinton agar (Merck, Darmstadt, Germany). *Enterococcus faecium* ATCC BAA-2127 and *Enterococcus faecium* ATCC 349 were maintained on Tryptic Soy Agar (Merck, Darmstadt, Germany) while other enterococcal strains and *Listeria monocytogenes* NCTC 10890 were maintained on Brain-heart infusion agar (Merck, Darmstadt, Germany). *Lactococcus lactis* ATCC 11454 was maintained on M17 agar (Merck, Darmstadt, Germany) supplemented with 5 % glucose (Merck, Darmstadt, Germany).

Isolation and screening of LAB for bacteriocin production

Indigenously sourced coconut shake was inoculated into freshly prepared De man Rogose and Sharpe (MRS) broth and incubated at 37 ^{0}C for 24 hours. The culture was serially diluted in peptone water and lactic acid bacteria (LAB) was isolated by growing on MRS agar plate (Merck Germany) at 37 ^{0}C. MRS broth was inoculated with single colonies from a 24 hour old MRS agar LAB culture and incubated aerobically at 37 ^{0}C for 24 hours. Screening of LAB for bacteriocin production was carried out using well diffusion assay in which cell-free supernatant (CFS) was tested for inhibitory activity against *Micrococcus luteus* ATCC 10240, *Listeria monocytogenes* NCTC 10890, *Bacillus cereus* ATCC 14579 and *Staphylococcus aureus* RF122. MRS agar used for well diffusion assay was supplemented with 0.1 % $CaCO_3$ (Friedemann Schmidt Chemical, Germany).

Molecular identification of LAB was conducted by amplifying 16S rRNA gene via 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 similarity search was performed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Purification, determination of molecular weight and molar extinction coefficient

Bacteriocin was purified using adsorption-desorption approach followed by reversed-phase high performance liquid chromatography (RP-HPLC). A 24-hour old culture of *Lactobacillus pentosus* CS2 was subcultured in freshly prepared MRS broth in a bioreactor (Sartorius Stedim, Germany). The Bioreactor was set up (agitation at 150 rpm, temperature at 37 ^{0}C) and run for 20 hours after which the culture was collected and the pH was adjusted to 5.8 and allowed for 1 hour. The culture was centrifuged (9000 x g, for 20 minutes at 4 ^{0}C) and the cell pellet re-suspended in 95 % methanol (Merck, Darmstadt, Germany) with pH value adjusted to 2. The cell suspension was stirred overnight at 4 ^{0}C and subsequently centrifuged (9000 x g for 30 minutes at 4 ^{0}C) to obtain the supernatant which was filtered using 0.22 µm sterilized cellulose membrane (Millipore). The clear supernatant was evaporated to dryness at 40 ^{0}C using a water bath and the crude bacteriocin was reconstituted in ultrapure water. Inhibitory activity was tested using well diffusion assay and crude bacteriocin was subjected to RP-HPLC containing SemiPrep RP-18e 100-10 mm column. The mobile phase consisted of two solvents: A (95 % Mili-Q water Millipore, USA) and 5 % acetonitrile (Merck, Germany) and B (100 % acetonitrile). Elution was done using a biphasic gradient of 20-80 % acetonitrile at a flow rate of 1 ml/minute over 65 minutes. Fractions were collected and evaporated using a vacuum evaporator. Antibacterial activity of HPLC fractions were tested. Molecular weight of the bacteriocin was determined by subjecting the active HPLC fraction to MALDI-TOF mass spectrometry. To ascertain the molar extinction coefficient, 2-fold dilutions of the bacteriocin were prepared and bacteriocin concentration was expressed in molar units. Absorbance at 280 nm was measured and a standard curve was generated from which the molar extinction coefficient was determined.

Antibacterial spectrum

This experiment was done to ascertain the inhibitory spectrum of pentocin MQ1. Bacteriocin producer was grown in MRS broth for 20 hours and CFS was used in well diffusion assay to test antibacterial activity against selected targets. All agar plates were supplemented with 0.1 % $CaCO_3$ (Friedemann Schmidt Chemical, Germany) to neutralize acidity.

Bacteriocin-cell wall association assay

An investigation was done to assess the association of pentocin MQ1 with the cell wall of its producer. Overnight broth culture of bacteriocin producer was centrifuged (9000 x g for 20 minutes) at 4 ^{0}C. Antibacterial activity of CFS was tested using well diffusion assay. The cell pellet was re-suspended in 95 % methanol (Merck, Darmstadt, Germany) adjusted to pH = 2 and stirred overnight at 4 ^{0}C on a magnetic stirrer. The cell suspension was centrifuged (9000 x g for 30 minutes at 4 ^{0}C) and the supernatant was filtered using a Millipore filter (0.22 µm) after which methanol was evaporated on a water bath at 40 ^{0}C. The cell extract was reconstituted in ultrapure water and antibacterial activity was tested.

Bacteriocin stability test

In order to ascertain the stability of pentocin MQ1, bacteriocin preparation was exposed to different temperatures: 40 ^{0}C, 60 ^{0}C and 80 ^{0}C for 40 minutes; 100 ^{0}C and 121 ^{0}C for 15 minutes. Samples were cooled to room temperature before testing antibacterial activity. Stability of bacteriocin to different 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 to a final enzyme concentration of 1 mg/ml and incubating for 1 hour at 37 ^{0}C after which inhibitory activity was tested. Bacteriocin was adjusted to various pH (2, 3, 5, 8 and 10)

and incubated for 2 hours at room temperature. Antibacterial activity was tested. Stability of bacteriocin upon exposure to different chemicals viz: 1 % (v/v) Tween 80, 1 % (v/v) Tween 20, 1 % (w/v) sodium dodecyl sulfate (SDS) (Fisher scientific, New Jersey, USA) and 1 % (v/v) triton X-100 was investigated by adding these chemicals to the bacteriocin and incubating for 2 hours at room temperature after which antibacterial activity was tested.

Plasmid isolation

To investigate if plasmids harbor the bacteriocin structural gene or not, plasmid isolation was carried out. This was done using easy pure® plasmid miniprep kit (TransGen Biotech, Beijing) according to manufacturer's instruction.

Regulatory mechanism

This experiment was done to understand the regulatory system of pentocin MQ1 production. Ten milliliters (10 ml) of fresh MRS broth was inoculated with colonies from an overnight culture of *L. pentosus* CS2 and incubated at 37 ^{0}C for 20 hours. Cell pellet was collected by centrifugation at 2000 rpm for 5 minutes. It was re-suspended in saline solution (0.85 %) and washed three times after which 100 µl was used to inoculate 900 µl of fresh MRS broth in 2 ml Eppendorf tube and 50 µl of 0.21 µM pentocin MQ1, ammonium sulphate precipitate, and amberlite XAD-16 fraction (each having an activity of 8 AU/ml) was added. These tubes were marked as "induced" while tubes that do not contain the bacteriocin were marked as "control". All tubes were incubated at 37 ^{0}C for 20 hours after which 50 µl of pentocin MQ1was added to the control tube. All tubes were tested for antibacterial activity. Induction of pentocin MQ1 production was said to occur if an induced tube produced inhibition zone while the control did not.

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined by employing the broth microdilution assay as described by Mota-Meira *et al*. (2000) with little modifications. Two-fold dilutions of bacteriocin were prepared in adequate media and 10 µl for pentocin MQ1 was added to 96-well microtiter plate. Overnight culture of indicator bacteria was diluted (1 X 10^8 CFU/ml) and added to 150 µl of each bacteriocin preparation. Wells containing indicator without pentocin MQ1 were used as positive control while wells containing only the media were used as blank. Incubation was done at 37 ^{0}C and optical density at 600 nm was monitored with a multiskan GO microplate reader (Multiskan GO, Thermo Scientific) over a period of 24 hours. MIC was defined as the bacteriocin preparation which caused growth reduction by more than 90 % compared with the positive control.

Mode of action

Time-killing

This assay was done to investigate the mode and speed of action of pentocin MQ1. Indicators were grown for 10 hours and centrifuged (2000 rpm for 5 minutes) to collect cell pellet. Each cell pellet was re-suspended 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 the bacteriocin preparation (5 X MIC) and incubated at 37 ^{0}C. Control consisted of bacterial suspension without the addition of bacteriocin. Growth was monitored over a period of 120 minutes.

Membrane permeabilization

Pore formation assay was done to understand the mechanism of action of pentocinMQ1. *Micrococcus luteus* was grown in Mueller Hinton broth until OD_{600nm} = 0.45 after which 5 µM SYTOX green dye (Invitrogen, USA) was added. Ninety microliters (90 µl) of stained bacteria was added to MicroAmp Fast Optical 96-well reaction plate (Applied Biosystems, Life Technologies, USA). After a stable line base was attained, 10 µl of pentocin MQ1 (5 X MIC) was added to the stained bacteria. Sodium phosphate buffer (5 mM) and nisin (Sigma-Aldrich, USA) were added to stained bacteria in different wells to serve as negative and positive controls respectively. Fluorescence as a result of binding of SYTOX green to leaked intracellular DNA was monitored using Real-Time PCR (Applied Biosystems, USA).

Biopreservation of banana

To investigate the biopreservative potential of pentocin MQ1 bacteriocin preparation (66.4 µM) was topically applied to mature, fresh banana samples. Some pentocin MQ1-treated banana samples were kept at ambient condition while others were refrigerated. Control samples consisted of non-pentocin MQ1-treated banana samples kept at ambient condition and others refrigerated. Samples were monitored for morphological changes. At the onset of deterioration of control samples, sterile cotton swabs were used to collect surface microflora of both control and pentocin MQ1-treated banana samples and bacterial count (CFU/ml) was measured. The experiment was allowed to proceed until the onset of deterioration of bacteriocin-treated banana samples. Shelf-life was measured. Experiments were done in triplicates.

RESULTS

Isolation and screening of LAB for bacteriocin production

Sixteen (16) different strains of lactic acid bacterial were isolated and identified based on 99 % sequence homology. *Lactobacillus pentosus* CS2 exhibited the strongest antibacterial activity and broadest antibacterial spectrum.

Purification, determination of molecular weight and molar extinction coefficient

Purification of bacteriocin by a combination adsorption-desorption method and RP-HPLC proved successful. Bacteriocin was obtained at a retention time of 31-33 minutes **(Figure 1).** MALDI-TOF mass spectrometry revealed that the molecular weight is 2110.672 Da **(Figure 2).** A molar extinction coefficient of 298.82 M^{-1} cm^{-1} was obtained.

Antibacterial spectrum

Pentocin MQ1 displayed strong inhibitory activity towards *Listeria monocytogenes* NCTC 10890, *Micrococcus luteus* ATCC 10240 and *Bacillus cereus* ATCC 14579. It was also inhibitory albeit to a less extent towards *Streptococcus pyogenes* ATCC 12344, *Staphylococcus aureus* RF122, *Pseudomonas aeruginosa* PA7, *Enterococcus faecium* ATCC 19434, *Enterococcus faecium* ATCC 27270, *Enterococcus faecium* ATCC 27273, *Enterococcus faecium* ATCC BAA-2318, *Enterococcus faecium* ATCC BAA-2127, *Enterococcus faecium* ATCC 6569, *Enterococcus faecium* ATCC 25307 and *Enterococcus faecium* ATCC 349 but was not active against *Streptococcus mutans* GEJ11, *Lactococcus lactis* ATCC 11454 and *Corynebacterium* spp. GH17 **(Table 1).**

Bacteriocin-cell wall association assay

Of the total activity of 6.9 X 10^4 AU, 66.67 % (4.6 X 10^4 AU) was detected in the cell extract while 33.33 % (2.4 X 10^4 AU) was found in the CFS **(Table 2)**.

Bacteriocin stability test

Stability of pentocin MQ1 under different conditions of heat, enzyme and pH are shown in **Table 3**. Its stability when exposed to different chemicals (1 % Tween 80, Tween 20, SDS and triton X-100) are not shown in **Table 3** because it retained 100 % residual activity. Residual activities of 99.82 %, 97.99 %, 91.32 %, 90.78 % and 83.11 % were obtained after heating at 40 ^{0}C, 60 ^{0}C, 80 ^{0}C, 100 ^{0}C and 121 ^{0}C revealing its high thermal stability. Proteinase K, pepsin and proteinase significantly reduced its activity **(Table 3)**. There was a complete loss of activity when it was treated with trypsin, α-chymotrypsin and protease. Pentocin MQ1 retained its activity after exposure to lyticase, catalase and hyaluronidase **(Table 3)**. pH variation had effect on its activity. It had higher activity in the pH range of 2-5 than at pH value of 8. There was no activity at pH value of 10 **(Table 3)**.

Plasmid isolation

This experiment was done to ascertain if genes encoding pentocin MQ1 production are plasmid-borne. After agarose gel electrophoresis, clear bands were observed for the 1 kb molecular ladder but no band was seen for *Lactobacillus pentosus* CS2. This indicates the absence of plasmids in *Lactobacillus pentosus* CS2 **(Supplementary material 1)**.

Regulatory mechanism

This assay was done to investigate the regulatory mechanism of pentocin MQ1 production by *Lactobacillus pentosus* CS2. A bacteriocin-negative (bac$^-$) phenotype of *Lactobacillus pentosus* CS2 was produced. Addition of ammonium sulphate precipitate, amberlite XAD-16 and pure pentocin MQ1 to the bacteriocin-negative (bac$^-$) *Lactobacillus pentosus* CS2 restored pentocin MQ1 production.

Minimum inhibitory concentration

Pentocin MQ1 exhibited strong inhibitory effect against *Listeria monocytogenes* NCTC 10890, *Micrococcus luteus* ATCC 10240 and *Bacillus cereus* ATCC 14579. MIC value for *M. luteus* and *L. monocytogenes* and *B. cereus* were 1.66 µM, 1.66 µM and 3.32 µM respectively.

Mode of action

Time-killing

Pentocin MQ1 caused a decline in the $\log_{10}$ CFU/ml of *L. monocytogenes* and *B. cereus* **(Figure 3).** After 120 minutes the Log_{10} viable cell count for *L. monocytogenes* had decreased from 10.27 to 1.80 (82.47 % reduction) while that of *B. cereus* had decreased from 9.27 to 3.10 (66.56 % reduction).

Membrane permeabilization

Treatment of *Micrococcus luteus* with pentocin MQ1 caused an increase in fluorescence intensity over the course of the study indicating pore formation. Similar observation was made for nisin although

higher fluorescence intensity was observed. Fluorescence intensity of the untreated bacterial cells remained stable **(Figure 4).**

Biopreservation of banana

Total surface bacterial count and LAB count of 4.00 x 10^7 CFU/ml and 2.10 x 10^3 CFU/ml (0.005 % of total bacterial count), 3.70 x 10^5 CFU/ml and 9.4 x 10^2 CFU/ml (0.254 % of total bacterial count), 2.14 x 10^4 and 1.76 x 10^3 (8.22 % of total bacterial count), 7.30 x 10^2 and 3.20 x 10^2 (43.84 % of total bacterial count) were obtained for nonbacteriocin-treated sample stored at ambient condition, nonbacteriocin-treated sample stored at refrigeration condition, pentocin MQ1-treated sample stored at ambient condition and pentocin MQ1-treated sample stored at refrigeration condition respectively **(Table 4)**. The shelf life of nonbacteriocin-treated sample stored at ambient condition, non-bacteriocin-treated sample stored at refrigeration condition, pentocin MQ1-treated sample stored at ambient condition and pentocin MQ1-treated sample stored at refrigeration condition are 3 days, 5 days, 7 days and 11 days respectively. Total surface bacterial count and shelf had a Pearson correlation coefficient (r) value of -0.779 indicating a strong inverse relationship between the two parameters. An r value of 0.863 was obtained for Pearson correlation analysis between percentage LAB and shelf life of banana suggesting a strong direct relationship between the two parameters. Changes in organoleptic characteristics of nonbacteriocin-treated banana occurred much earlier than in bacteriocin-treated samples (**Figure 5**).These results show that treatment of banana with pentocin MQ1 extended its shelf. The microbiological quality and shelf life of pentocin MQ1-treated banana stored at refrigeration condition was better than that of pentocin MQ1-treated banana stored at ambient condition.

DISCUSSION

Bacteriocin-producing lactic acid bacteria confer various beneficial effects (such as improvement of quality and shelf life extension) on dairy products (Sultan *et al*., 2017). As such, the presence of *Lactobacillus pentosus* CS2 in coconut shake suggests it has bioprotective role. Although *Lactobacillus pentosus* CS2 has been isolated from vagina (Okkers *et al*., 1999), fermented Xuan-Wei ham (Zhang *et al*., 2008) and fermented shrimp (Watthanasakphuban *et al*., 2016), this is the first report of its isolation from coconut shake considered as a dairy product. Purification of pentocin MQ1 by sequential use of adsorption-desorption method and RP-HPLC proved successful. At low pH bacteriocins are released into the culture medium but when pH is increased to around 5.8-6.0 they become adsorbed onto the producer cells. This phenomenon was observed in this study due to the fact that no activity was detected in the CFS after the adsorption process. Adsorption-desorption approach has been used previously in the purification of some bacteriocins (Siying *et al*., 2000). Adsorption-desorption method has some advantages over traditional approaches such as ammonium sulphate precipitation. These include reduced time of processing, purer crude bacteriocin and cheap running cost (Jia-qi *et al*., 2011; Mu-xu and Zhi-jiang, 2009). Based on the retention time of pentocin MQ1 which corresponds to high concentration of acetonitrile (high hydrophobicity), it can be deduced that it contains slightly more hydrophobic amino acid residues than polar or hydrophilic ones.

MALDI-TOF mass spectrometry revealed that the molecular weight of pentocin MQ1 is 2110.672 Da. There are only a few reports on purification of bacteriocin from *Lactobacillus pentosus*. To date pentocins that have been successfully purified to homogeneity and molecular weight accurately determined are pentocin TV35b (3929·63 Da) (Okkers *et al*., 1999), pentocin 31-1 (5,592.225 Da) (Zhang *et al*., 2008), and bacteriocin K2N7 (2.017 kDa) (Watthanasakphuban *et al*., 2016). The molecular weight of pentocin MQ1 does not match with any of the reported pentocins. Hence, it is a

novel pentocin. Molar extinction coefficient is an important biophysical parameter that can facilitate the quantitation and future industrial application of pentocin MQ1.

Pentocin MQ1 displayed a broad spectrum of antibacterial activity. This attribute has been observed in cerein 7 (Oscáriz *et al*., 1999), enterocin P (Cintas *et al*., 1997) and enterocin LR/6 (Kumar and Srivastava, 2010). It was reported that pentocin TV35b is not inhibitory towards *Bacillus cereus* (Okkers *et al*., 1999). Lui *et al*. (2008) also reported that pentocin 31-1 is a broad spectrum bacteriocin with inhibitory activity against *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli*. Watthanasakphuban *et al*. (2016) reported that bacteriocin K2N7 has a narrow spectrum of antibacterial activity and was not inhibitory against *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecium*. Pentocin MQ1 is different from pentocin TV35b, pentocin 31-1 and bacteriocin K2N7 in that in addition to the aforementioned bacterial targets it is also inhibitory against *Micrococcus luteus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Enterococcus faecium*. It also showed inhibitory activity against closely related species *Lactobacillus plantarum* K25. Bacteriocins from many LAB strains have been found to inhibit the growth of both closely related and distantly-related bacterial strains (Müller *et al*., 2009). Broad spectrum of antibacterial activity is one of the important criteria for selection of bacteriocins for use in the biopreservation of foods (Johnson *et al*., 2017; Kaškonienė *et al*., 2017). The broad antibacterial spectrum of pentocin MQ1 well positions it as a good candidate for preservation of various types of foods.

Investigating the association of pentocin MQ1 with the cell wall of the producer is important because it can reveal whether the bacteriocin abound in the supernatant or on the cell wall. In this study more activity was detected on the cell wall than the supernatant indicating the cell wall-binding characteristic of pentocin MQ1. This finding shows that for a better recovery of pentocin MQ1 produced by *Lactobacillus pentosus* CS2, an adsorption-desorption approach facilitated by pH modifications should be employed. In this study, an adsorption-desorption approach suitable for purification of pentocin MQ1 was demonstrated. Association of bacteriocin with the cell wall of the producer is thought to enhance niche competition. Cell-wall associated bacteriocins have also been described in *Lactobacillus crispatus*, *Streptococcus salivarius* and *Streptococcus bovis* HC5 (Barbour and Philip, 2014; Mantovani *et al*., 2002; Tahara and Kanatani, 1997).

Pentocin MQ1 was highly stable to all chemical treatments investigated. This is evidenced by its retention of 100 % residual antibacterial activity. It exhibited high thermal and pH stability. Higher activity was detected in the acidic pH range (2-5) while moderately alkaline pH (pH 8) caused a drastic reduction in activity. Lack of activity at pH 10 (high alkaline pH) indicates severe denaturing of pentocin MQ1. Pentocin MQ1 is a proteinaceous biomolecule due to its susceptibility to proteinases. Retention of high antibacterial activity after exposure to lyticase, catalase and hyaluronidase provides more evidence on its proteinaceous nature. Pentocin TV35b was active in the pH range of 1-10 and after heating at 60 ^{0}C-100 ^{0}C (Okkers *et al*., 1999). Pentocin 31-1 was active at pH 2 to 10 and at 60 ^{0}C-121 ^{0}C but sensitive to SDS (Liu *et al*., 2008). Bacteriocin K2N7 retained activity at pH 2-12 but unlike pentocin MQ1 it was inactive at 121 ^{0}C (Watthanasakphuban *et al*., 2016). The combined attributes of chemical, pH and thermal stability of pentocin MQ1 favors its future application in food systems subjected to harsh processing conditions (Hemu *et al*., 2016; Yi *et al*., 2016). Its sensitivity to proteases is a desirable characteristic in that its chances of inhibiting beneficial components of the gut microbiota is reduced thereby, enhancing its safety (Hemu *et al*., 2016; Zacharof and Lovitt, 2012). Moreover, degradation of bacteriocin by proteases reduces the time of interaction between fragments of a given bacteriocin and its target thereby decreasing the possibility of resistance development (Perez *et al*., 2014). Its application in the treatment of gut infection would require encapsulation in

nanoparticles or bioengineering to make it resistant to protease of the gut (Arthur *et al*., 2014; Cavera *et al*., 2015; Zhang *et al*., 2010).

Genetic element harboring *Lactobacillus pentosus*-derived bacteriocin has not been reported. Genes encoding bacteriocin production have been detected on plasmids and chromosomes (Garcia *et al*., 2010). Absence of plasmids in *Lactobacillus pentosus* CS2 suggests that genes encoding pentocin MQ1 production are chromosome-borne. It is thought that chromosome-encoded bacteriocin genes are more stable than plasmid-encoded bacteriocin genes because plasmids, being small and mobile genetic elements can be lost by leaking out of bacterial cells (Sengupta and Austin, 2011). Hence, bacteriocin-producing LAB strains harboring chromosome-borne bacteriocin genes have an edge over those with plasmid-borne bacteriocin genes. Thus, *Lactobacillus pentosus* CS2 is genetically stable. Chromosome-borne bacteriocins include enterocin A (Aymerich *et al*., 1996) and ABP-118 (Flynn *et al*., 2002) and acidocins LF221(Majhenič *et al*., 2003).

Pentocin production was restored in this study when ammonium sulphate precipitate, amberlite XAD-16 and pure pentocin MQ 1 were added separately to bac$^-$ cultures of *Lactobacillus pentosus* CS2. This shows that pentocin MQ1 production is auto-inducible suggesting its regulation by a three-component quorum sensing mechanism involving an inducing peptide, a histidine protein kinase and a response regulator. Pentocin 31-1 production is also controlled by quorum sensing (Zhang *et al*., 2012). Regulation of bacteriocin production via quorum sensing mechanism is commonly found among class II bacteriocins. (Di Cagno *et al*., 2011; Di Cagno *et al*., 2010; Straume *et al*., 2007).

Pentocin MQ1 was strongly inhibitory against *Listeria monocytogenes* NCTC 10890, *Micrococcus luteus* ATCC 10240 and *Bacillus cereus* ATCC14579 at micromolar concentrations. High activity at low concentration is a desirable property of natural biopreservatives (Bali *et al*., 2016). Although MIC values for nisin A (Mota-Meira *et al*., 2000) are lower than that of pentocin MQ1 its broad spectrum of antibacterial activity suggests wider food and medical applications. Pentocin MQ1 exhibits a bactericidal mode of action against *L. monocytogenes* and *B. cereus*. After 120 minutes the viable cell count for *L. monocytogenes* and *B. cereus* had been reduced significantly. This shows the quick-acting characteristic of pentocin MQ1 against these pathogens. Pentocin 31-1 was also shown to exert a bactericidal effect against *Listeria monocytogenes* (Liu *et al*., 2008). Pentocin TV35b had a bactericidal activity against *Listeria innocua* (Okkers *et al*., 1999).

Pentocin MQ1 caused membrane permeabilization of *Micrococcus luteus* leading to leakage of intracellular DNA and consequently death of the bacteria. This is the first report on membrane permeabilization as a mechanism of action of *Lactobacillus pentosus*-derived bacteriocins. It is thought that pore formation also led to loss of other valuable intracellular molecules such as ATP contributing to the rapid death of the bacterial target. Pentocin MQ1 was quick-acting against its target. It is thought that resistance to a quick-acting antimicrobial agent is less likely to occur compared to a slow-acting one. Pore formation has been reported for several LAB bacteriocins (Perez *et al*., 2014; Snyder and Worobo, 2014)

Banana is one of the most consumed fruit in the tropics and subtropics (Huang *et al*., 2014). It is a good source of antioxidants, carbohydrates, calcium and potassium (Mohapatra et al., 2011). As a perishable and climacteric crop it has a short shelf life. Preserving fresh banana is quite challenging (Mohapatra *et al*., 2010). Various chemical and physical approaches are employed in the preservation of banana (Kudachikar *et al*., 2011; Mohapatra *et al*., 2011; Zaman *et al*., 2007). However, consumer inclination towards food containing biopreservatives and less of chemical preservatives triggered the search for natural products that can be used for biopreservation (Barbosa *et al*., 2017). In a recent study, combined

application of phenylurea and gibberellins was effective at extending the shelf life of banana (Huang *et al*., 2014). Although the bioprotective capabilities of several bacteriocins have been reported (Abriouel *et al*., 2010; Bhatia *et al*., 2016; Galvez *et al*., 2008), no report has been made for banana. Only one report has been made on biopreservation of food using pentocin. In that study, the potential of pentocin 31-1 for preserving pork meat was demonstrated (Zhang *et al*., 2009). Topical application of pentocin MQ1 extended the shelf of banana in this study. Shelf life extension was due to decrease in total bacterial count and increase in the percentage LAB compared to the other microflora **(Table 4)**. It can be deduced that pentocin MQ1 decreased the population of pathogenic and spoilage bacteria on the surface of banana. Moreover, it had a positive effect on the population dynamics of the surface microflora such that decrease in spoilage bacteria enhanced the growth of beneficial LAB stains leading to shelf life extension. These results reveal the biopreservative potential of pentocin MQ1. Furthermore, bacteriocin treatment and refrigeration had a synergistic effect on the microbiological quality of banana resulting in extension of shelf life. These findings pave the way for future *ex situ* application of pentocin MQ1 in the biopreservation of banana.

In conclusion, this is the first report on the presence of bacteriocinogenic strain of *Lactobacillus pentosus* in coconut shake. *Lactobacillus pentosus* CS2 produces a novel bacteriocin (pentocin MQ1) with a broad spectrum of antibacterial activity, high chemical, thermal and pH stability but sensitive to proteolytic enzymes. It is cell-wall associated and possesses a bactericidal mode of action. Pentocin MQ1 acted against its target through pore formation. Genes encoding pentocin MQ1 production are not plasmid-borne. Its biosynthesis is regulated by a quorum sensing mechanism. Its ability to preserve fresh banana was demonstrated in this study. The characteristics of pentocin MQ1 show its potential for the biopreservation of food.

AUTHOR CONTRIBUTIONS

KP designed, supervised execution of the experiments and wrote the manuscript. KP also edited the manuscript. SW designed the experiments, performed it and wrote the manuscript.

CONFLICT OF 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.

ABBREVIATIONS

CFS: Cell-free supernatant; LAB: Lactic acid bacteria; MALDI-TOF: Matrix-assisted laser desorption ionization time-of-flight; MIC: Minimum inhibitory concentration; MRS: De Man, Rogosa and Sharpe; RP-HPLC: Reversed-phase high performance liquid chromatography; SDS: Sodium dodecyl sulfate

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FIGURE LEGENDS

FIGURE 1 Reversed-phase HPLC chromatogram of crude cell extract from *Lactobacillus pentosus* CS2. Bacteriocin released into MRS broth was adsorbed onto the cell wall of the

producer by increasing the pH of the medium to 5.8 followed by desorption from the cell wall by lowering the pH to 2. Total crude bacteriocin obtained was subjected to RP-HPLC. The vertical lines indicate the retention time.

FIGURE 2 MALDI-TOF mass spectrum of purified pentocin MQ1. Purified pentocin MQ1 was subjected to MALDI-TOF mass spectrometry.

FIGURE 3 Time-killing assay for pentocin MQ1 (A) against *Listeria monocytogenes* NCTC 10890 **(B)** against *Bacillus cereus* ATCC 14579**.** Different preparations of pentocin MQ1 (5 X MIC) was added to cultures of baterial targets.

FIGURE 4 Pore-formation in the cell membrane of *Micrococcus luteus* ATCC 10240 by pentocin MQ1. Pentocin MQ1 was added to *M. luteus* stained with SYTOX green dye and increase in fluorescence as a result of leakage of intracellualr DNA was monitored using Real-Time PCR.

FIGURE 5 Appearance of banana after 5 days of storage at different conditions. (A) Nonbacteriocin-treated stored at ambient condition **(B)** Nonbacteriocin-treated stored at refrigeration condition **(C)** Pentocin MQ1-treated stored at ambient condition **(D)** Pentocin MQ1-treated stored at refrigeration condition.

LIST OF TABLES

TABLE 1 Antibacterial spectrum of pentocin MQ1.

Indicator Strain	Zone of inhibition
Streptococcus pyogenes ATCC 12344	+++
Streptococcus mutans GEJ11	-
Lactococcus lactis ATCC 11454	-
Staphylococcus aureus RF122	+++
MRSA	+
Listeria monocytogenes NCTC 10890	++++
Bacillus cereus ATCC 14579	+++
Pseudomonas aeruginosa PA7	+++
Corynebacterium spp. GH17	-
Escherichia coli UT181	++
Micrococcus luteus ATCC 10240	++++

Enterococcus faecium ATCC 19434	++
Enterococcus faecium ATCC 27270	++
Enterococcus faecium ATCC 27273	++
Enterococcus faecium ATCC BAA-2318	++
Enterococcus faecium ATCC BAA-2127	++
Enterococcus faecium ATCC 6569	++
Enterococcus faecium ATCC 25307	+++
Enterococcus faecium ATCC 349	++
Lactobacillus plantarum K25	++

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

TABLE 2 Pentocin MQ1 recovered from the cell-free supernatant and cell extract of *Lactobacillus pentosus* CS2.

Bacteriocin preparation	**Activity (AU)**	**Activity (%)**
Cell-free supernatant	2.3 X 10^4	33.33
Cell extract	4.6 X 10^4	66.67
Total	6.9 X 10^4	100.00

TABLE 3 Stability tests for pentocin MQ1.

Test	Zone of inhibition (mm)	Residual Activity (%)

Heat			
Control	15.95	100.00
40	15.93	99.82
60	15.73	97.99
80	15.00	91.32
100	14.94	90.78
121	14.17	83.11
Enzyme		
Control	15.18	100.00
Proteinase K	9.13	40.28
Lysozyme	15.18	100
Pepsin	7.87	28.19
Lyticase	14.95	97.74
Catalase	15.18	100.00
Trypsin	0.00	0.00
α-Chymotrypsin	0.00	0.00
Protease	0.00	0.00
Proteinase	10.23	51.38
Hyaluronidase	15.18	100.00
pH		
Control	15.80	100.00
2	16.60	107.40
3	16.53	106.76
5	16.00	101.85
8	9.10	37.96
10	0.00	0.00

TABLE 4 Effect of bacteriocin application on surface bacterial count and shelf-life of banana.

Bacteriocin	Bacterial count (CFU/ml) after 5 days of storage				Shelf-life (Days)	
	A		R		A	R
	Total	LAB	Total	LAB		
P	2.14×10^4	1.76×10^3	7.30×10^2	3.20×10^2	7	11
C	4.00×10^7	2.10×10^3	3.70×10^5	9.4×10^2	3	5

[2] P: pentocin MQ1, C: negative control, A: ambient condition, R: refrigerated

Figure 1.JPEG

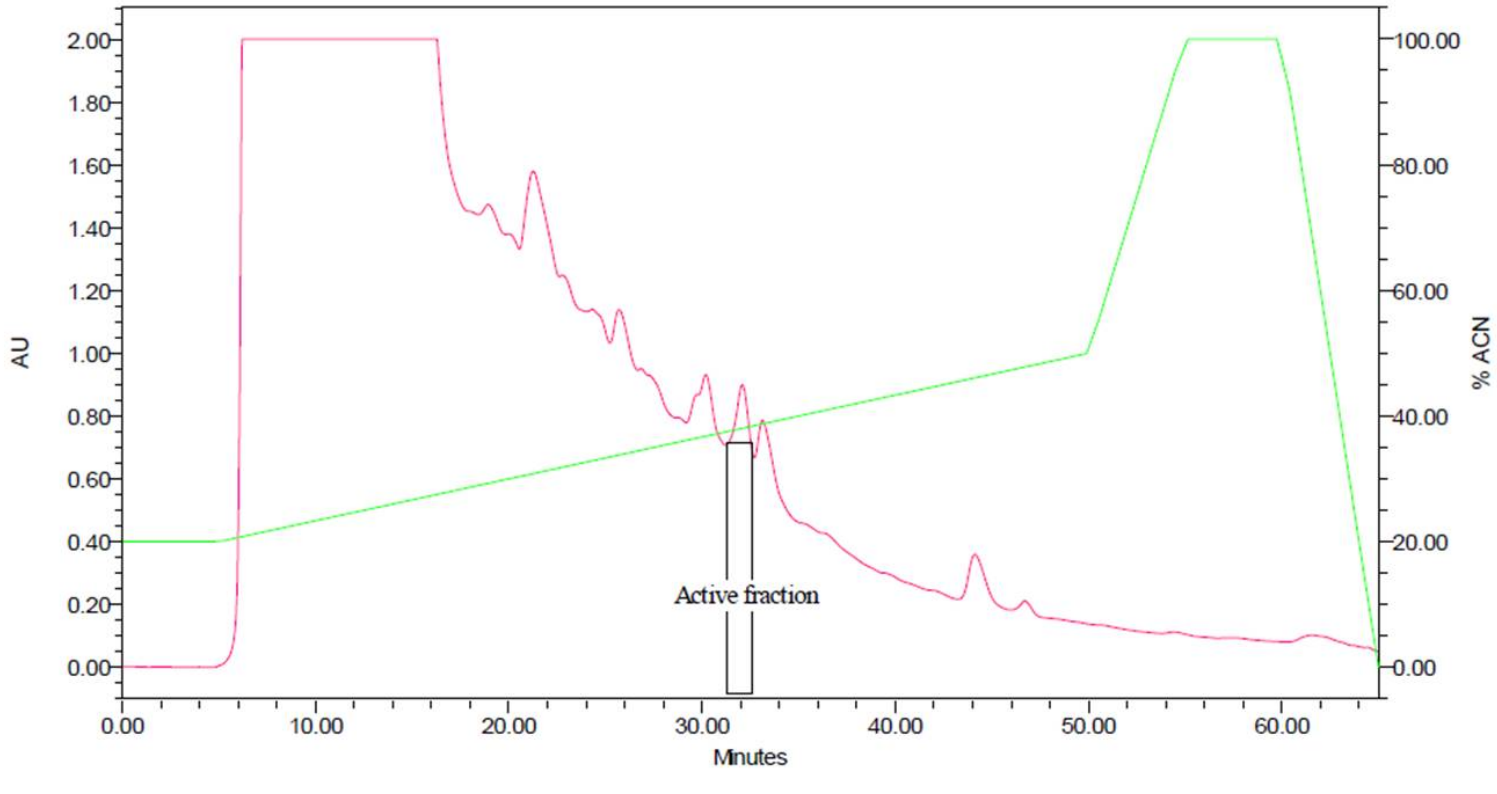


Figure 2.JPEG

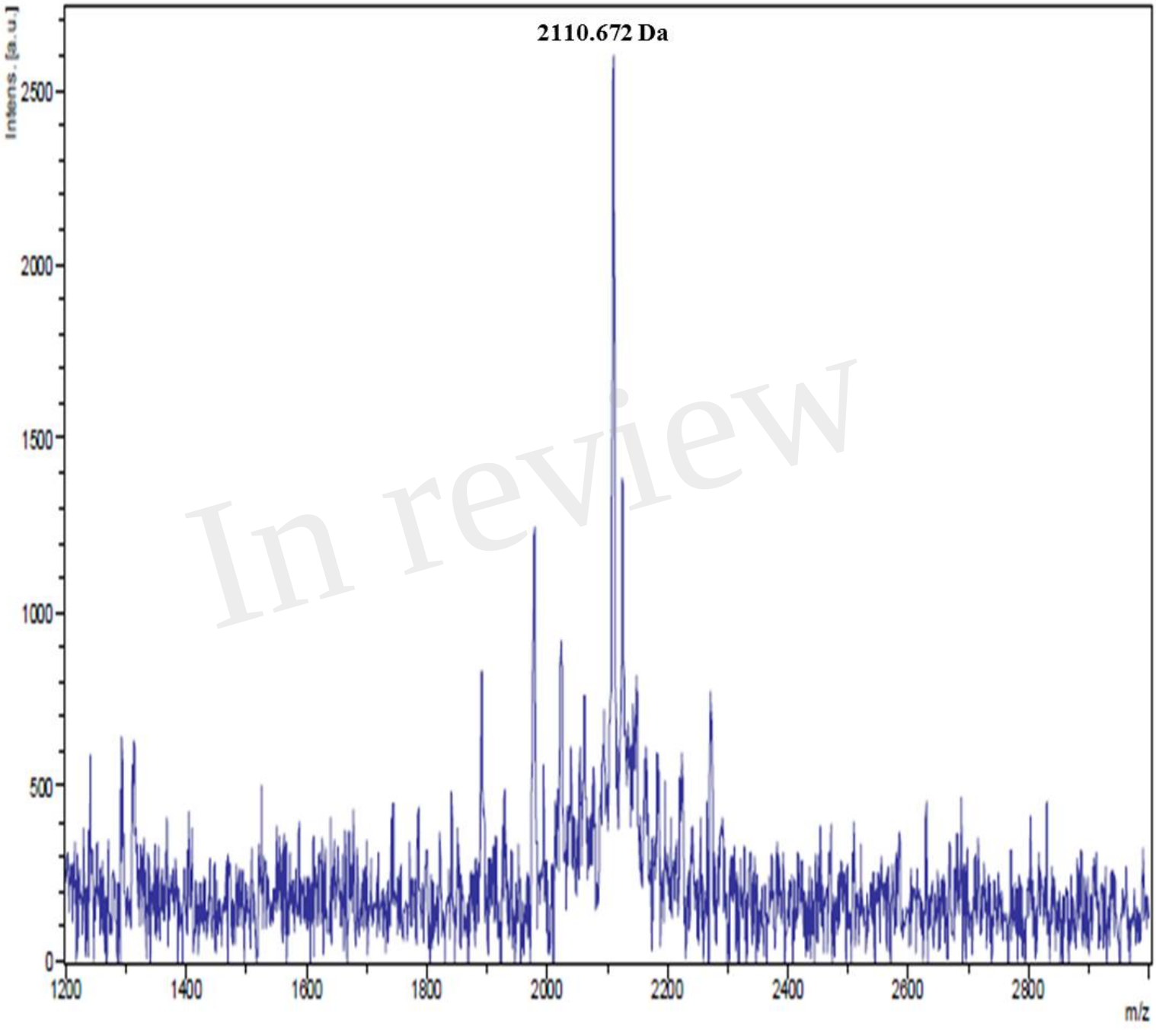
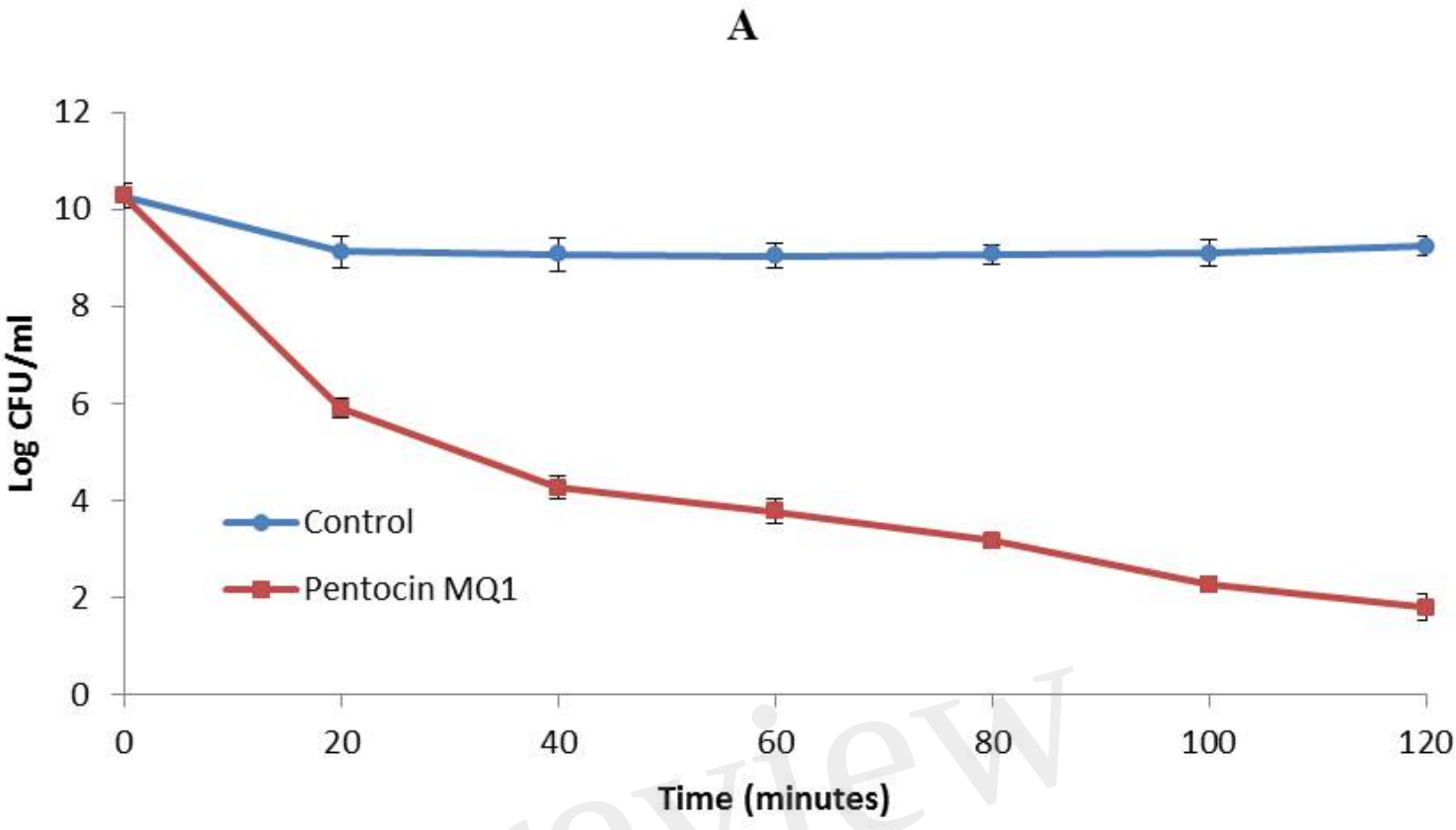


Figure 3.JPEG



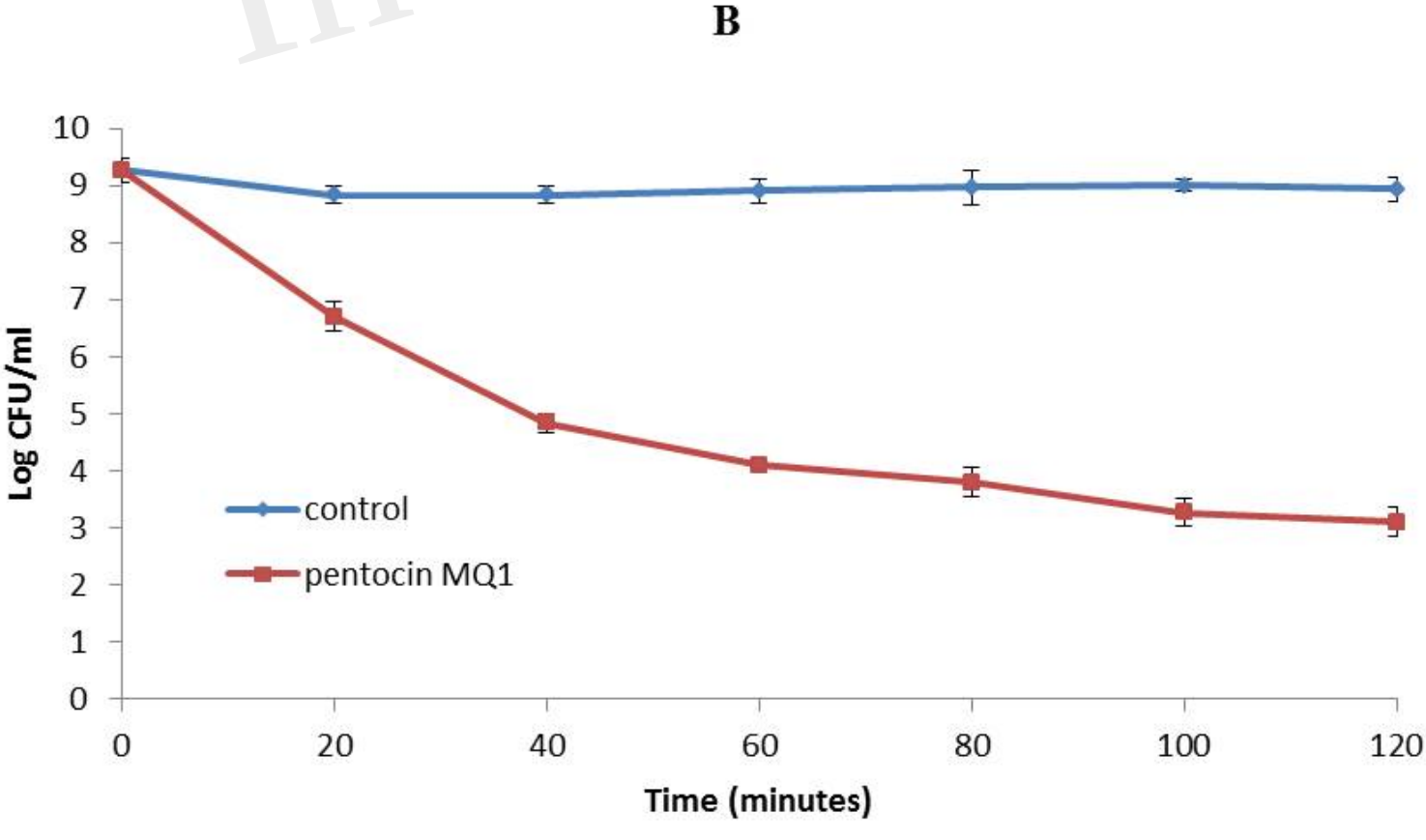


Figure 4.JPEG

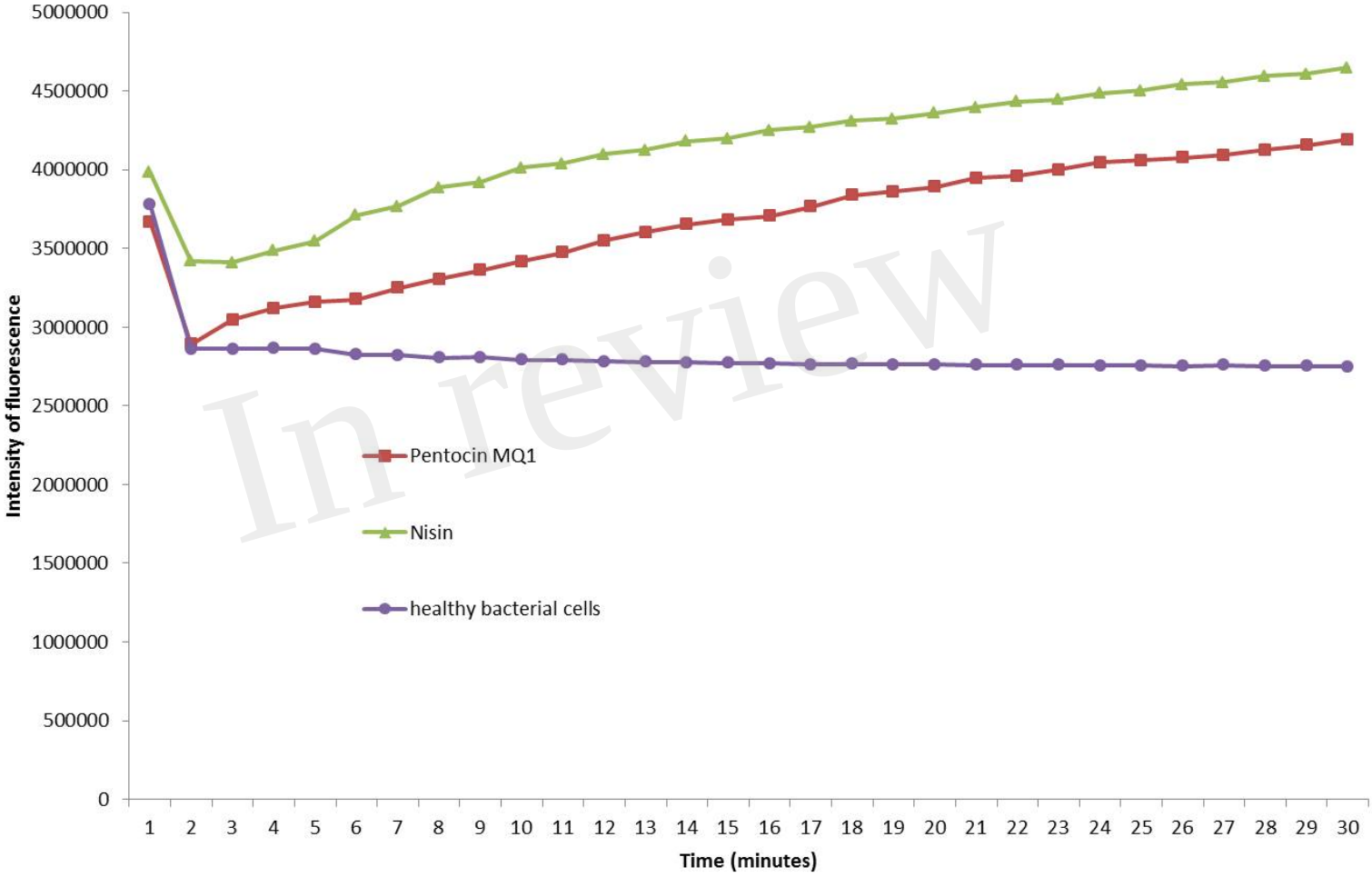


Figure 5.JPEG

A

B

C

D