



RESEARCH ARTICLE

Potential of bacteriocins produced by probiotic bacteria isolated from tiger shrimp and prawns as antibacterial to *Vibrio*, *Pseudomonas*, and *Aeromonas* species on fish [version 1; referees: 2 approved, 1 approved with reservations, 1 not approved]

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Abstract

Backgrounds: Bacteriocin has been used widely in industry as a biopreservative agent. The objective of the present study was to investigate the potency of Bacteriocin isolated from tiger prawn *Penaeus monodon* and freshwater shrimp *Macrobrachium rosenbergii* as an anti-bacterial on fish.
Methods: A total of ten candidates of probiotic bacteria consisted of five isolates from tiger shrimps (H1, H2, H3, H4, H5) and five isolates from freshwater prawns (W1, W2, W3, W4, W5) were evaluated. Bacteriocin was produced by centrifugation at a speed of 150 rpm and at 37 °C for 24 hours. The bacteriocin extract was purified by adding sulphate ammonium salt {(NH₄)₂SO₄} at 80% of the saturation level. Bacteriocin activity was determined using a diffusion method against pathogenic bacteria *Vibrio alginolyticus*, *Aeromonas hydrophilla* and *Aeromonas hydrophilla* and *Pseudomonas stutzeri*. Bacteriocins were analyzed using High Performance Liquid Chromatography (HPLC) and Fourier Transform Infra-Red (FTIR). The data were subjected to analysis of variance (ANOVA) and followed with Duncans multiple range test.
Results: Bacteriocins produced by bacteria isolate H4 from tiger prawn indicated the highest bacteriocin activity against bacteriocin activity against *Pseudomonas stutzeri* at the diameter of inhibition zone of 887.10 ± 409.24 mm²/mL. While isolate W2 from freshwater shrimp indicated inhibition zone of 1466.96 ± 127.62 mm²/mL. Both bacteriocins were purified by chromatography column using Sephadex LH-20.
Conclusions: This finding showed that bacterial isolates H4 and W2 have the potential to produce bacteriocins which inhibit the pathogenic bacteria. FTIR analysis showed an amide group at wave number 1652cm⁻¹ contained in the bacteriocins of isolates H4 and W2.

Keywords

potency, bacteriocin, probiotic, tiger shrimp, prawn, antibacterial, pathogen

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Referee Status:

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Introduction

Probiotics are beneficial microbes for living organisms and, in certain quantities, have a positive impact on health^{1,2}. Probiotic bacteria play a vital role in suppressing the growth of pathogenic microbial populations. Probiotic bacteria including lactic acid bacteria have an ability to produce several antimicrobial compounds such as lactic acid, diacetyl, hydrogen peroxide, carbon dioxide and bacteriocins^{3,4}.

Bacteriocins are antimicrobial peptides which act as antibacterial compounds against bacterial pathogens⁵. Bacteriocins can inhibit Gram positive and Gram negative bacteria such as *Salmonella* species (sp.), *Escherichia coli*, *Vibrio* sp., *Shigella* sp., *Aeromonas* sp. and *Pseudomonas* sp.⁶. Bacteriocins have an important role against microbes as a bactericidal as they are resistant to heat and maintain activity in an acidic environment, and low temperature during storage does not affect bacteriocin activity⁵. Bacteriocins can be damaged by degradation from proteolytic enzymes⁷.

Lately the use of bacteriocins has become of interest due to their potential use as a preservative in the food industry, especially in fermented foods. Nisin, for example, is one of the bacteriocins produced by lactic acid bacteria of *Lactococcus lactis* that has been known and recognized as safe to use, and can degrade pathogenic and spoilage microbes. Therefore, it could improve the quality and shelf life of food products⁸. This study examines extracts of bacteriocins produced by probiotic bacteria isolated from tiger shrimp and prawn which have an ability to inhibit pathogenic bacteria. The findings in this study will help researchers to inform fish farmers in preparing food for fish and shrimp populations in order to prevent pathogens, improve food efficiency and to increase the animals' immune system.

Methods

Sample collection

A total of ten isolates of probiotic bacteria were collected, consisting of five isolates from tiger shrimp and five isolates from prawn⁹, which were from the collection of the Marine Microbiology Laboratory of the Faculty of Fisheries, University of Riau, Indonesia. ID numbers for each isolates are (GenBank): H1: KY995544, H2: KY995545, H3: KY995546, H4: KY995547, H5: KY995548, W1: KY995549, W2: KY995550, W3: KY995551, W4: KY995552 and W5: KY995553. This study was conducted from April-September 2016. Pathogenic gram-negative bacteria, *Vibrio alginolyticus* was obtained from Brackish water Research Institute in Jepara, Indonesia, while *Aeromonas hydrophila* and *Pseudomonas stutzeri* were purchased from Quarantine Centre in Pekanbaru, Indonesia.

The Nutrient Agar (NA; Merck, Kenilworth, NJ, USA, Cat. No. 1.05450.0500), Nutrient Broth (NB; Thermo Scientific Oxoid, Waltham, MA, USA, Cat.No.CM0001), Thiosulfate Citrate Bile Sucrose (TCBS; Merck, Cat.No.1.10263.0500), Trypticase Soy Agar, (TSA;Merck, Cat.No. 1.05458.0500), Trypticase Soy Broth (TSB, Merck, Cat.No. 1.05459.0500) were used in this study.

Glycerol, glasswool, paper disc 6mm (Macharey-Nagel, Düren, Germany, MN827ATD), gel filtration chromatography using Sephadex LH-20 (Sigma-Aldrich, St-Louis, MO, USA - Offer sigma-GE17-0090-01), methanol and other chemicals were used in accordance with the laboratory procedures.

Refreshment of probiotic bacteria

Probiotic bacteria can grow in acidic conditions so each process was performed in physiological acidic pH solutions. In this study, probiotic bacteria were grown at pH 4. The probiotic bacteria was cultured in the following manner: a tube filled with the 500µL physiological solution of pH 4 was inoculated with probiotic bacteria aseptically at concentration of 10⁸ cfu/mL. The mixture was homogenized and left to stand for 5 minutes. One loop full of the mixture was streaked on NA media. Media containing the bacteria were incubated at 37 °C for ± 24 hours.

Production of bacteriocins

Liquid medium, nutrient broth of 300 mL volume was used for the production of bacteriocins¹⁰. The Medium was sterilized in an autoclave at a pressure of 15 psi, at 121°C for 15 minutes. After one day at room temperature, the medium was inoculated with 5% of probiotic bacteria that had previously been incubated overnight at the optical density (OD) at 600nm ~ 0.1 (v/v) which was equivalent to 10⁷ CFU/mL (OD measured with Thermo Scientific GENESYS 10S Uv-Visible)¹¹. The inoculated media was then fermented in a shaking incubator at a speed of 150 rpm for ± 24 hours at 37 °C. After incubation, the fermented medium was cooled in a refrigerator at 5–10°C for ± one hour. The crude bacteriocin extract from the medium by centrifugation (Hitachi, Tokyo, Japan – CS150FNX) at 10,000 rpm for 10 min at 4°C. The supernatant was then separated by filtration through glasswool. The cell-free supernatant (extracted bacteriocins) produced, was tested for activity against pathogenic bacteria by using a disc diffusion method. Some of the supernatants was precipitated by the addition of 80% salt ammonium sulfate [(NH₄)₂SO₄], and then was tested for activity in the same manner, and finally was analyzed by using High-performance liquid chromatography (HPLC).

Purification of bacteriocins

Bacteriocin was precipitated from the crude extract by the addition of 80% salt ammonium sulfate [(NH₄)₂SO₄] in a cold condition (temperatures of 5 °C to 10 °C) while stirring gently to achieve 80% saturation and was then left overnight. The precipitate was then separated from the filtrate by centrifugation (Hitachi – CS150FNX) in a cold state at 13,000 rpm for 10 minutes. After centrifugation, the precipitate was added with 0.05 M phosphate buffer solution at pH 7.0, and it was ready to be used for the bacteriocins activity test. The rest was put in microtubes and was stored in a freezer (-20 °C).

Bacteriocin activity test

Bacteriocin activity was tested by the diffusion agar method by using a paper disc of 6 mm pore size against pathogenic bacteria *V. alginolyticus*, *A. halophyla*, and *P. stutzeri*. One mL of

each of pathogen inoculum (OD 600nm ~ 0.1, which is equivalent to 10^7 CFU/mL¹¹) was inoculated into a test tube containing 15 mL of NA medium and was then vortexed. The inoculated medium was poured into a Petri dish and was allowed to solidify. A total of 50 mL of bacteriocins (bacteriocin extract both before and after precipitation) was dropped on paper discs and was allowed to dry. Amoxsan® 30µg from local pharmacy in Pekanbaru Indonesia was used as positive control and sterile liquid medium as the negative control. The dried paper discs were placed on pathogenic-inoculated NA media. After incubation for ± 24 hours, the bacteriocin activities were indicated by clear zone formed around the discs. The diameter of the clear zones was measured by using calipers. Inhibitory activity against pathogenic bacteria of extracellular fluid was calculated as AU (Activity Unit). One AU/mL was the area of inhibition zone per unit volume of bacteriocin samples tested (mm²/mL). Bacteriocin activity can be calculated using the following equation³:

$$\text{Bacteriocin Activity (mm}^2\text{/mL)} = \frac{\text{Lz} - \text{Lc}}{\text{V}}$$

Where:

Lz = diameter of clear zone area (mm²/ml)

Lc = Disc diameter (mm²/ml)

V = Sample Volume (mL)

Gel filtration chromatography

Gel filtration or gel permeation is a protein separation technique based on molecular size¹². This technique used a column measuring 2.5 × 50 cm (Sephadex LH-20) as steady phase and methanol as mobile phase. A total of 9 g Sephadex LH-20 was weighed and then was added to 50 mL of methanol. The mixture was stirred gently until dissolved. The dissolved Sephadex was poured into the column until the marked limit. The eluent was collected in a beaker containing a mixture of Sephadex, it was then poured again into the column until Sephadex expanded and solidified for 20 minutes. Afterward, the bacteriocin was inserted into the column through the column wall carefully and waited until the sample penetrated through pores of the gel Sephadex

LH-20 pores. The eluate was then collected in a marked vial as the first fraction, and the next eluent was collected in vials containing 20 drops until final-clear eluent reached. Small molecules will enter the pores of the gel Sephadex and move slowly, while the large molecules will move faster because it cannot enter and retaining the gel. Thus, the large molecules will emerge as the initial component. Products of each of separated bacteriocin fractions were finally analyzed by using FTIR spectroscopy.

HPLC (high-performance liquid chromatography) analysis

Bacteriocins produced after precipitation in ammonium sulphate was then analyzed using HPLC. A spectrophotometer ultraviolet (UV) detector (Thermo Scientific Genesys 10S) was used for the analysis of bacteriocins at a wavelength of 210 nm and 250 nm. Wavelength selection was based on preliminary measurement using UV spectrophotometer in The Research Laboratory of Enzymes, Fermentation, and Biomolecular, following the research performed by Masuda *et al.*,¹³. The analysis used Shimadzu HPLC system of UFLC Shim Pack C18 series with column size of 4.6 mm × 250 mm (Shimadzu, Kyoto, Japan).

Statistical analysis

The data were subjected to one-way analysis of Variance (ANOVA)¹⁴ followed by Duncan multiple range test at significance levels of 95%. The analysis was performed using a SPSS ver.20 software.

Results

Bacteriocins activities

Inhibitory activity of bacteriocins was expressed as the inhibition zone per unit volume of samples tested (mm²/mL). **Table 1** shows the activity of bacteriocins extract of tiger shrimp against pathogenic bacteria *V. alginolyticus*, *A. hydrophila* and *P. stutzeri*. Statistical analysis indicated that the activity was not significantly different ($P > 0.05$), the highest activity was indicated by bacteria isolate H1 against *V. alginolyticus*. However, the activity was not significantly different ($P > 0.05$) from isolates H2 and H5, which was 674.65 mm²/mL, but it was

Table 1. Summary of crude extract of bacteriocins of probiotic bacteria isolated from black tiger shrimp before being precipitated in ammonium sulphate [(NH₄)₂SO₄].

Probiotics	Activities of bacteriocin crude extract (mm ² /mL)		
	<i>V. alginolyticus</i>	<i>A. hydrophila</i>	<i>P. stutzeri</i>
H1	(674.65±369.34) ^{ab}	(-5.59±530.69) ^b	(287.078±127.834) ^{bc}
H2	(409.38±56.01) ^{ab}	(193.31±130.33) ^{ab}	(262.073± 79.677) ^{bc}
H3	(244.66±175.62) ^b	(208.44±101.49) ^{ab}	(338.348±60.722) ^{bc}
H4	(117.00±66.13) ^b	(319.91±101.03) ^{ab}	(178.791±61.842) ^b
H5	(329.93±98.57) ^{ab}	(222.30±115.91) ^{ab}	(361.823±33.006) ^a

Note: H = Bacterial Code. Superscript of the same letters indicates no significant differences in the level of 5%. Values were average of triplicate samples ± standard deviation.

significantly different ($P > 0.05$) from isolates H3 and H4 (Table 1). The highest activity of bacteriocin crude extract against *A. hydrophila* was found at 319.91 mm²/mL from isolate H4, which was not significantly different ($P > 0.05$) from isolates H2, H3, and H5. However, it was significantly different ($P > 0.05$) from isolate H1 ($P < 0.05$). The highest inhibitory activity against *P. stutzeri* was indicated by isolate H5 which was significantly different ($P < 0.05$) from four other bacteria.

After precipitation with ammonium sulphate [(NH₄)₂SO₄], the activity of each of bacteriocins tends to change. The highest activity was performed by isolate H3 against *V. alginolyticus*, that was 896.50 mm²/mL (Table 2). Meanwhile, the lowest activity was indicated by isolate H1 that was 321.47 mm²/mL. This condition was different from that which was before been precipitated with ammonium sulphate. The highest activity was indicated by isolate H1 against *V. alginolyticus* (Table 1). This concluded that precipitation process resulted in the different effect on bacteriocin produced by each of tested bacteria.

The activity of the bacteriocins-crude extract of bacterial isolates from prawns against pathogens *V. alginolyticus*, *A. hydrophila* and *P. stutzeri* was not significantly different ($P > 0.05$), and the highest activity was indicated by isolate W4 against *A. hydrophila* (746.95 mm²/mL). In comparison to bacteriocin produced by bacteria isolated from black tiger shrimp, of which the highest was indicated by isolate H1 against *A. hydrophila*, that was 674.65 mm²/mL. Therefore, the activity of bacteriocin produced by bacteria from prawns was higher than that of black tiger shrimp.

Values for W4 were not significantly different from isolates W2 and W5 ($P > 0.05$), but it was significantly different from W1 and W3 ($P < 0.05$). The highest activity of the bacteriocins-crude extract against *V. alginolyticus* was shown by isolate W4 (645.76 mm²/mL), which was significantly different ($P < 0.05$) from other four isolates. Meanwhile, the highest activity of bacteriocins-crude extract against *P. stutzeri* was indicated by isolate W2 (412.73 mm²/mL), which was significantly different ($P < 0.05$) from the other four isolates.

Bacteriocin activities of bacterial isolates from prawns against all pathogens (*V. alginolyticus*, *A. hydrophila*, and *P. stutzeri*) were not significantly different ($P > 0.05$). The highest activity was produced by isolate W2 against *P. stutzeri*, and the highest inhibitory activity was 1466.96 mm²/mL which was significantly ($P > 0.05$) different from other four bacteria isolates (Table 4). However, the activity of isolate W1 was not significantly different from isolate W5 ($P > 0.05$) against *P. stutzeri* (Table 4). Overall, the activity of bacteriocin precipitated in ammonium sulphate {(NH₄)₂SO₄} was higher than bacteriocin before precipitation in ammonium sulphate.

A quantity of 270 mL of bacteriocin-crude extract was obtained after centrifugation, and then it was precipitated with ammonium sulphate salt [(NH₄)₂SO₄]. In the deposition process at salt saturation level of 80%, a total of 139.32 g of salt ammonium sulphate [(NH₄)₂SO₄] was added to the crude extract of bacteriocins. The precipitated bacteriocin was added with buffer in order to reach a volume of 3,375 mL bacteriocins.

HPLC analysis. High-Performance Liquid Chromatography (HPLC) is a chromatographic method that uses a reversed-phase system as its working system. This method was used to determine the purity level of a compound to be analyzed. Bacteriocins of tiger shrimp and prawns with high bacteriocin-activity values (H4 and W2), were then analyzed by HPLC as shown in Figure 1 and Figure 2. The figures showed that bacteriocin produced by each of probiotic bacteria was not a pure product indicated by the number of chromatogram peaks.

Gel filtration chromatography

Bacteriocins as a group of proteins, were separated by column chromatography on Sephadex LH-20 using methanol. The resulted eluate was initially appeared light brown in color in the column, and was collected in a vial which was then marked as fraction 1. After that, as the sample was in the middle of the column, the next eluate was collected as fraction 2, and followed by collecting every 20 drop samples until the sample appeared faded, and finally clear in vial 25. This indicates that the protein samples in the column Sephadex LH-20 have been

Table 2. Activities of bacteriocins-crude extract of probiotic bacteria isolated from black tiger shrimp after precipitation in ammonium sulphate [(NH₄)₂SO₄].

Probiotics	Bateriocins Activities (mm ² /mL)		
	<i>V. alginolyticus</i>	<i>A. hydrophila</i>	<i>P. stutzeri</i>
H1	(321.47±80.75) ^{cd}	(321.47±80.75) ^{bc}	(516.28±145.10) ^{ab}
H2	(854.07±55.25) ^a	(542.69±26.37) ^{abc}	(406.82±149.97) ^{ab}
H3	(896.50±375.14) ^a	(642.86±279.28) ^{abc}	(759.39±304.51) ^a
H4	(610.28±257.32) ^{abc}	(872.93±170.02) ^{ab}	(887.10±169.08) ^a
H5	(702.26±156.45) ^{ab}	(669.03±79.56) ^{abc}	(586.14±409.24) ^{ab}

Note: H = Bacterial Code. Superscript of the same letters indicates no significant differences in the level of 5%. Values were average of triplicate samples ± standard deviation.

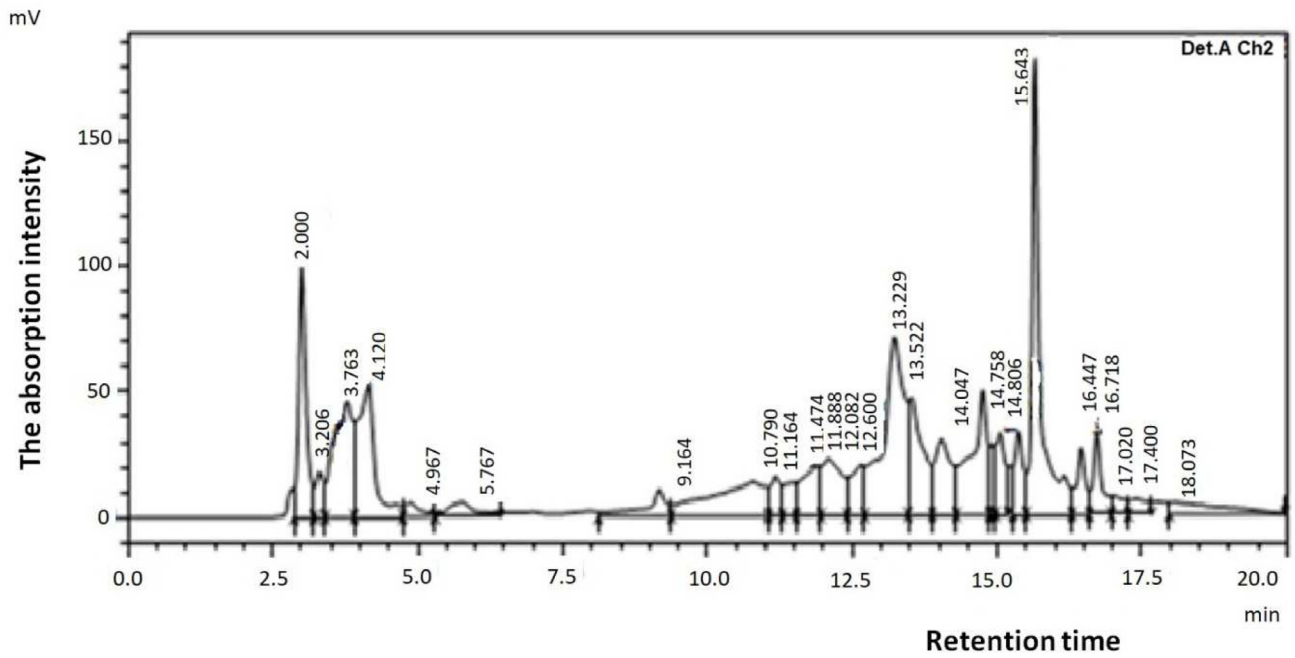


Figure 1. Chromatogram bacteriocins H4 of probiotic bacteria isolated from black tiger shrimp.

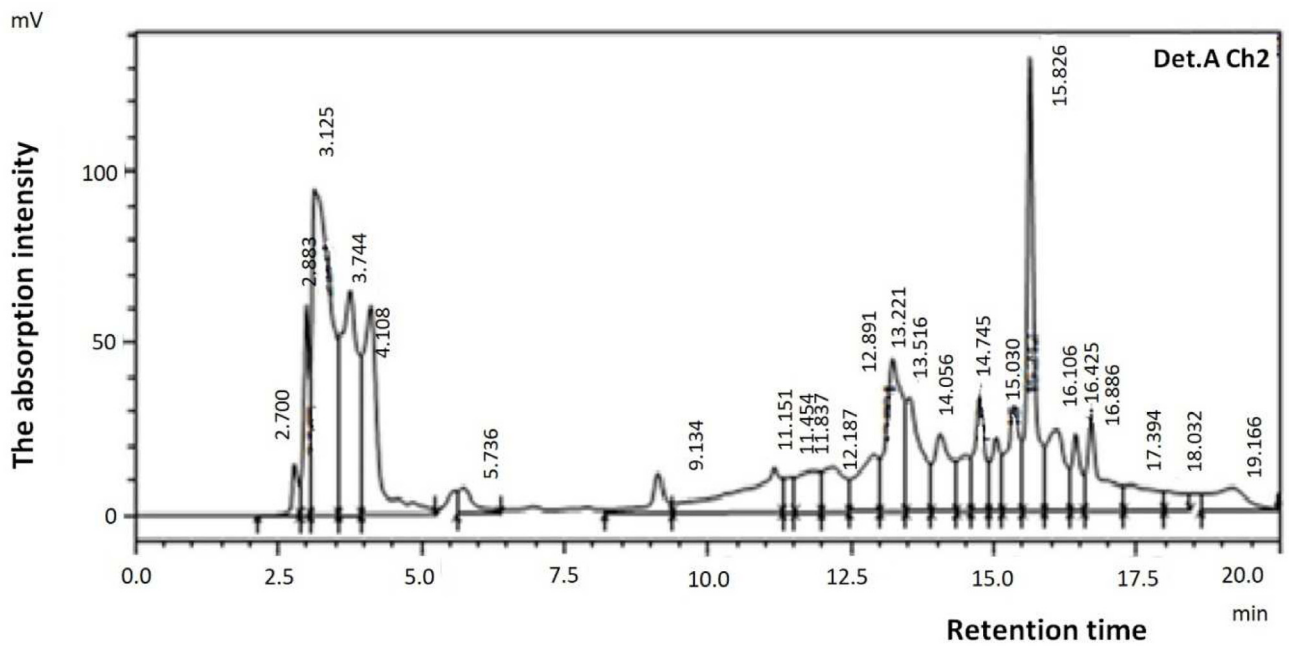


Figure 2. Chromatogram of bacteriocins W2 from probiotic bacteria isolated from prawns.

completely eluted by methanol. The eluate of 25 fractions was allowed to evaporate at room temperature to remove the solvent. The solids obtained were observed to look like thin films in some of the fractions. Five fractions contained thin films of 25 fractions produced, those were fractions 6, 9, 14, 18 and 25, which were then analyzed using FTIR spectroscopy to observe the functional groups containing the fraction.

FTIR analysis

Fourier Transform Infra-Red (FTIR) was used to find out bonding vibration and functional groups contained by bacteriocins. FTIR analysis results from one of the bacteriocins produced by bacteria H4 are shown in Figure 3. The spectrum figure shows the comparison of fractions that produced films (fractions 6, 9, 14, 18 and 25) as the separation products of bacteriocins H4 using gel filtration column, and after being precipitated in ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$. Each fraction showed absorption bands at similar wave numbers.

The infrared spectrum of bacteriocins for fraction 6 shows absorption at wave numbers (cm^{-1}), 978, 1340, 1355, 1648, 2831, 2921, 3420 and 3595. Fraction 9 shows absorption at wave numbers (cm^{-1}) of 977, 1089, 1339, 1363, 1404, 1457, 1647, 3239, and 3587. Fraction 14 showed absorption at wave numbers (cm^{-1}) of 978, 1097, 1312, 1363, 1405, 1457, 1647, 2836,

and 3502. Fraction 18 shows absorption at wave numbers (cm^{-1}) of 988, 1334, 1358, 1400, 1460, 1649, 2835, 2954, 3415 and 3588. Finally, fraction 25 shows absorption at wave numbers (cm^{-1}) of 971, 1082, 1312, 1339, 1355, 1416, 1457, 1647, 2834, 2988, 3336, 3443 and 3593. Meanwhile, bacteriocins precipitated by salts of ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$ shows an absorption band at wave number (cm^{-1}) 979, 1339, 1355, 1418, 1456, 3336, 3445, and 3592.

Dataset 1. Word document containing the following data tables

<http://dx.doi.org/10.5256/f1000research.13958.d198458>

Crude extract of bacteriocins of probiotic bacteria isolated from black tiger shrimp before being precipitated in ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$. The activities of bacteriocins-crude extract of probiotic bacteria isolated from black tiger shrimp after precipitation in ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$. The activities of bacteriocins-crude extract of probiotic bacteria isolated from prawns before precipitation in ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$.

Dataset 2. The bacteriocins activities of probiotic bacteria isolated from prawns after precipitation in ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$

<http://dx.doi.org/10.5256/f1000research.13958.d198459>

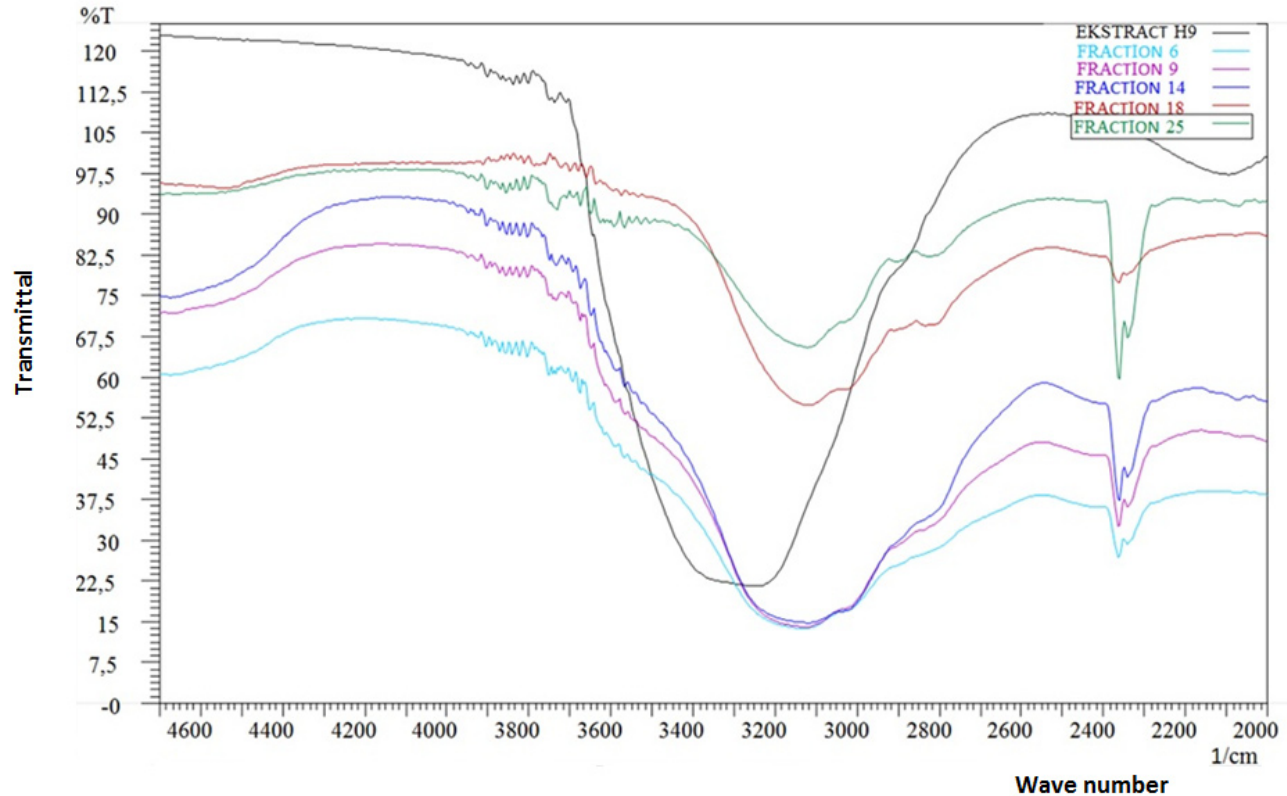


Figure 3. FTIR spectrum bacteriocin H4 precipitated in ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$ in comparison to the purification product of bacteriocins of some fractions (fraction 6,9,14,18 and 25).

Discussion

Bacteriocins are secondary metabolites in the form of proteins that act as antimicrobial compounds. The activity of bacteriocin-crude extracts of bacteria from tiger shrimp before precipitation in ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$ was as presented in Table 1, and after precipitation with the same salt was shown in Table 2. Protein precipitation using 80% ammonium sulphate salt was a partial protein purification technique (partial purification) that is frequently applied as it has high solubility and is easy to obtain. Bacteriocins can be concentrated through the application of methods of salting out using ammonium sulfate as a natural protein¹⁵. Salting out is a mechanism of protein precipitation as a result of reduction in solvent molecules required to dissolve the protein. This occurs due to the increase of salt concentration which can decrease protein solubility. When ammonium sulphate is added to the protein solution, the salt ions of ammonium sulphate will attract water molecules away from protein. This is due to the competition between the binding of ions of ammonium sulphate and water which causes the protein precipitates¹⁶.

Bacteriocin inhibitory activity against bacterial indicators was indicated as AU (Activity unit). One AU/mL was inhibition area per unit volume of bacteriocin samples tested (mm^2/mL).

The method used was similar to the antibacterial assay method using agar diffusion method and three Gram-negative bacteria as pathogenic indicators (*V.alginolyticus*, *A. hydrophila*, and *P. stutzeri*).

The bacteriocins-crude extract before being precipitated in ammonium sulphate showed inhibitory activity against three different indicators. The activity of bacteriocin-crude extract of bacterial isolate H4 from tiger shrimp was the highest (Table 1) against *V. alginolyticus* ($117.00 \pm 66.13 \text{ mm}^2/\text{mL}$), *A. hydrophila* ($319.91 \pm 101.03 \text{ mm}^2/\text{mL}$) and *P. stutzeri* ($178.79 \pm 61.84 \text{ mm}^2/\text{mL}$). After precipitation in ammonium sulphate, bacteriocin-activities (Table 2) increased against *V. alginolyticus* ($610.28 \pm 257.32 \text{ mm}^2/\text{mL}$), *A. hydrophila* ($872.93 \pm 170.02 \text{ mm}^2/\text{mL}$), and *P. stutzeri* ($887.10 \pm 169.08 \text{ mm}^2/\text{mL}$). The H4 isolate showed the greatest inhibitory activity due to its high inhibitory activity against the three bacterial indicators. The better the bacteria inhibit pathogenic bacteria, the better the ability to produce bacteriocins.

Activities of the bacteriocins-crude extract of bacteria from prawns before and after the salt precipitation in ammonium sulphate were shown in Table 3 and Table 4, respectively. Similar to the inhibitory activity of probiotics bacteria from tiger

Table 3. Activities of bacteriocins-crude extract of probiotic bacteria isolated from prawns before precipitation in ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$.

Probiotics	Activity of crude extract bacteriocins(mm^2/mL)		
	<i>V. alginolyticus</i>	<i>A. hydrophila</i>	<i>P. stutzeri</i>
W1	(310.69±223.32) ^{bc}	(238.41±197.97) ^{ab}	(314.35±238.86) ^{ab}
W2	(345.90±306.16) ^{bc}	(455.02±169.59) ^a	(412.73±209.53) ^a
W3	(343.00±158.96) ^{bc}	(279.41±112.59) ^{ab}	(342.36±257.02) ^{ab}
W4	(645.76±315.20) ^{ab}	(746.95±227.01) ^a	(92.21±59.37) ^{ab}
W5	(293.20±197.16) ^{bc}	(625.10±296.59) ^a	(346.00±81.58) ^{ab}

Note: W = Bacterial Code. Superscript of the same letters indicates no significant differences in the level of 5%. Values were average of triplicate samples \pm standard deviation.

Table 4. Bacteriocins activities of probiotic bacteria isolated from prawns after precipitation in ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$.

Probiotics	Bacteriocins activities(mm^2/mL)		
	<i>V. alginolyticus</i>	<i>A. hydrophila</i>	<i>P. stutzeri</i>
W1	(581.03±425.55) ^{ab}	(490.88±400.68) ^{bc}	(770.87±570.42) ^b
W2	(1043.2±357.10) ^a	(1113.91±423.02) ^a	(1466.96±127.62) ^a
W3	(607.73±13.94) ^{ab}	(531.90±179.18) ^{bc}	(461.59±341.75) ^{bc}
W4	(367.01±86.39) ^b	(917.90±186.53) ^{ab}	(238.42±35.42) ^c
W5	(706.18±365.07) ^{ab}	(666.49±268.47) ^{ab}	(888.76±360.27) ^b

Note: W Bacterial Code. Superscript of the same letters indicates no significant differences in the level of 5%. Values were average of triplicate samples \pm standard deviation.

shrimp, all probiotic bacteria from prawns produced bacteriocins which indicated the active role in inhibiting pathogenic bacteria.

The average activity of bacteriocins-crude extract from bacteria W2 before salt-precipitation in ammonium sulphate (Table 4) against *V. alginolyticus*, *A. hydrophila* and *P. stutzeri* were 345.907 ± 306.160 mm²/mL, 455.022 ± 169.591 mm²/mL and 412.735 ± 209.537 mm²/mL, respectively. After the salt-precipitation in ammonium sulfate, the bacteriocin activities increase against *V. alginolyticus*, *A. hydrophila* and *P. stutzeri*, which were 1043.228 ± 357.102 mm²/mL, 1113.914 ± 423.026 mm²/mL and $1466, 127.626 \pm 962$ mm²/mL, respectively. Activities of bacteriocins produced by bacteria W2 demonstrated the greatest potential to inhibit all three pathogenic indicator bacteria. Ponce *et al.*,¹⁷ found that bacteriocins of *Lactococcus lactis* have inhibitory activity against *L. monocytogenes* which was 83.33 mm²/mL. This value was lower than the bacteriocin activity of bacteria W2 (1043.228 mm²/mL) against *V. alginolyticus*. This is likely due to the inhibition ability of bacteria W2 being closely related to the bacterial cell growth. The better the cell growth, the number of cells increases, and this will further increase the production of bacteriocins.

Antimicrobial compounds such as bacteriocins are proteins produced as secondary metabolites. The production of secondary metabolites is encoded by DNA-containing genes. Formation of secondary metabolites was affected by several conditions, among which are limitations of available nutrients in the media, the addition of inducer compounds and decrease in the growth rate¹⁸.

Comparing the activity of bacteriocins produced by both bacteria (H5 and W2), probiotic bacteria W2 from prawns had the highest activity against the three bacterial indicators. The significant difference ($P > 0.05$) in the inhibition of the three bacterial indicators showed that the active protein compounds (bacteriocins) were also different¹⁹. The sensitivity of bacteria to bacteriocins is determined by the specific characteristics possessed by each bacteria. Thus, inhibition mechanism of bacteriocins against indicator bacteria depends on the specific receptors possessed by the bacteria.

The high inhibitory activity caused by bacterial bacteriocins is closely related to the bacterial indicators which belong to Gram-negative bacteria as shown in Table 5. Bacteriocins produced by Gram-positive bacteria have low inhibitory activity, or none at all, against Gram-negative bacteria²⁰. For example, the activity generated by *Lactobacillus lactis* against *E. coli* is very low²¹. Bacteriocins can inhibit or kill pathogenic bacteria when the bacteria have a close relationship¹⁹. Such as bacteriocins produced by *Lactobacillus* sp. SCG 1223 has a high inhibitory activity against *L. monocytogenes* (1648.500 mm²/mL)²². High activity was also obtained from bacteriocins produced by *Lactobacillus lactis* against *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus cereus*, *Staphylococcus aureus* and *Enterococcus faecalis*²¹.

Table 5. Gram stain test the probiotic bacteria of tiger shrimp and prawns.

Code isolates of tiger shrimp	Gram	Code isolates of tiger shrimp	Gram
H1	(+)	W1	(+)
H2	(-)	W2	(-)
H3	(-)	W3	(-)
H4	(-)	W4	(-)
H5	(+)	W5	(+)

Bacteriocins analysis was performed by HPLC system after purified with salt ammonium sulfate [(NH₄)₂SO₄]. This analysis used a wavelength of 210 nm and 250 nm because, at these wavelengths, bacteriocins produced by *Lactococcus mesenteroides* show high purity¹³. The mobile phase used was methanol while the steady phase was silica gel. Bacteriocins analysis results were shown as in Figure 1 and Figure 2. Bacteriocins produced by all the probiotic bacteria after analysis cannot be considered as the pure product because many peaks appear in the chromatogram analysis. This proves that the new protein sample was just partially purified using ammonium sulfate salt [(NH₄)₂SO₄]. Bacteriocins were protein compounds that the separation should be done with a variety of purification methods as has been done by Smaoui *et al.*,²³ bacteriocins with code BacTN635 initially purified by ammonium sulfate [(NH₄)₂SO₄], followed by gel filtration chromatography, HPLC, and SDS-PAGE electrophoresis. Nisin isolated from *Lactococcus lactis* was also purified by various purification methods, which was initially precipitated with ammonium sulphate salt [(NH₄)₂SO₄], and was continued by purification using various chromatographic methods^{8,24}. Nisin was the first type bacteriocin allowed as biopreservation on food⁵.

Bacteriocins were produced by probiotic bacteria H4 from tiger shrimp and probiotic bacteria W2 from prawns which were purified by gel filtration chromatography using Sephadex LH-20. The results of the sample separation were characterized by FTIR spectroscopy to identify functional groups contained in the functional group of the bacteriocins based on the literature of various types of bacteriocins by Sakhamuri *et al.*,²⁴ and Selvendran and Babu²⁵ as shown in Table 6.

The infrared spectrum of bacteriocins H4 at wave numbers (cm⁻¹) of 971, 977, 978, 979, and 988 indicated the presence of bending vibration of amide group (O= oxygen, C= carbon, N= nitrogen), while the wave numbers (cm⁻¹) of 1082, 1089 and 1079 indicated a bond phosphodiester (P = O), and the wave number (cm⁻¹) of 1647, 1648, 1649 and 1652 indicated a stretching vibration of carbonyl (C = O, amide I). The infrared spectrum bacteriocins obtained relate to specific functional groups of nisin which was similar to that reported by Sakhamuri *et al.*²⁴. The wave numbers (cm⁻¹) 3336, 3415, 3420, 3443, and 3445

Table 6. FTIR characteristic wave numbers of different types of bacteriocins by Sakhamuri et al.,²⁴, Selvendran and Babu²⁵.

Phase Number	Functional groups
976	OCN vibration buckling
1079	P=O Fosfodiester
1180-1360	C-N peptide bond
1650	C=O carbonyl stretching vibration
3336	N-H
3500	OH

indicated the presence of N-H stretching vibration. Bacteriocins produced was the result of probiotic bacteria isolated from black tiger shrimp and prawns, one of which has 99% homologous with the bacteria *Bacillus* sp.²⁶.

Conclusions

Bacteriocins had been explored from probiotic bacteria isolated from black tiger shrimp and prawns. The bacteriocins show potential as anti-pathogens in shrimp culture or in fish farming. Bacteriocins of from H4 isolate from tiger shrimp and bacteriocins of W2 isolate from prawns were two antimicrobial compounds which had the greatest inhibitory activity against all three pathogens, *Vibrio alginolyticus*, *Aeromonas hydrophila* and *Pseudomonas stutzeri*. HPLC analysis of the bacteriocins produced by 18 of probiotic bacteria did not show a high degree of purity. FTIR analysis of the purified products of H4 bacteriocins showed an amide bond at a wavelength of 1652 cm⁻¹ which indicated that the compound was a protein. Bacteriocins

produced from the laboratory isolates may be useful as a food preservative for controlling microbial deterioration, enhancing the hygienic quality, and extending the self-life of fish and seafood products²⁷.

Data availability

Dataset 1: Word document containing the following data tables: [10.5256/f1000research.13958.d198458](https://doi.org/10.5256/f1000research.13958.d198458)²⁸

Crude extract of bacteriocins of probiotic bacteria isolated from black tiger shrimp before being precipitated in ammonium sulphate [(NH₄)₂SO₄].

The activities of bacteriocins-crude extract of probiotic bacteria isolated from black tiger shrimp after precipitation in ammonium sulphate [(NH₄)₂SO₄].

The activities of bacteriocins-crude extract of probiotic bacteria isolated from prawns before precipitation in ammonium sulphate [(NH₄)₂SO₄].

Dataset 2: The bacteriocins activities of probiotic bacteria isolated from prawns after precipitation in ammonium sulphate [(NH₄)₂SO₄]. [10.5256/f1000research.13958.d198459](https://doi.org/10.5256/f1000research.13958.d198459)²⁹

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

Grateful thanks goes to the Ministry of Technology, Research and Higher Education of Indonesia who has supported this research.

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[Data Source](#)

Open Peer Review

Current Referee Status:



Version 1

Referee Report 10 October 2018

doi:10.5256/f1000research.15172.r38187



Eddy Afrianto 

Faculty of Fisheries and Marine Sciences, Padjadjaran University, Jatinangor, West Java, Indonesia

In the field of fisheries, the attack of pathogenic microbes and the use of formaldehyde are some of the main issues. The use of formalin as a food preservative has reached dangerous stage, but its more effective substitute compounds have not been successfully developed. Bacteriocin is a compound that has great potential to be developed as a prevent attacks of pathogenic bacteria and preservative inhibitor to replace formaldehyde.

Bacteriocin is a secondary metabolite product from lactic acid bacteria (BAL). These bacteria are often found in various places, including the digestive tract of fish and shrimp. There have been many efforts made to get bacteriocin-producing microbes. Increased mass production of bacteriocin is very helpful for fish cultivation activities in preventing pathogenic microbial attack and processing of fishery products as preservatives replacing formalin.

This research is the initial stage of the effort to develop bacteriocin as a deterrent to the activity of pathogenic microbes and decay. Bacterial isolates that have been found need to go through several stages in order to produce bacteriocins, including isolation processes to produce pure cultures and optimal bacteriocin separation technology from microbial culture media.

1. In the abstract several words are repeated, reducing the use efficiency of the word.
2. The reasons put forward to carry out this research are quite relevant. It is hoped that bacteriocin can be found that can overcome the problem of pathogens that attack fish and shrimp.
3. The method used has been presented in sufficient detail so as to facilitate future replication. Some parts still need to be detailed further, for example need further explanation on how to get bacteriocins using centrifugation, how many bacteriocin samples are used in the HPLC method, what and how many solvents are used?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 20 September 2018

doi:[10.5256/f1000research.15172.r38186](https://doi.org/10.5256/f1000research.15172.r38186)



Tati Nurhayati

Department of Aquatic Product Technology, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Bogor, Indonesia

The manuscript describes the isolation of bacteriocin derived from probiotic bacteria isolated from shrimp and lobster. This compound is tested against pathogenic bacteria. However, there are some improvement notes in some parts.

Abstract

1. The research objective in the abstract has not been clearly written. Are bacteriocins obtained from shrimp or from bacteria that live on shrimp?
2. There is a typo in the method section in the abstract. Bacteriocin was written 2 times.
3. In the abstract: the sentence Bacteriocin activity against is written 2 times.
4. The results of chromatographic column analysis and HPLC have not been written in the results section of the abstract.

Methodology

1. Assay of bacteriocin activity, need to be re-examined. Need to be written clearly, how many bacteriocins are inserted into each well? In order to be able to track how to calculate the inhibition per ml. Unit diameter should be mm NOT mm².
2. In methodology purification methods, the use of ammonium sulfate, only one concentration is 80%. What is the basis? If there have been previous studies, it is necessary to mention here referring to whose research? Use of the term the purification of proteins with ammonium sulfate is inappropriate.
3. There is a discrepancy in the designation term. In the abstract, it is stated that purification is done by chromatography column. But in the methodology, protein purification is carried out with ammonium sulfate.
4. In the methodology, there is step gel filtration. What is the use of gel filtration for? For purification only, or for specific analysis. Need to write a clear title in this method.

5. In the methodology, analysis with HPLC is used to analyze what. Need to be written in the methodology.

Discussion

1. Especially for the discussion of gel filtration, only the stages of the method are explained. The method stage should be written on the methodology not in the results and discussion.
2. In sub discussion, it is better not to repeat mentioning the actual data already in the results section. In this sub-chapter, what should be written is the discussion of the data in the results section.

Conclusion

1. The last sentence from the conclusion is incorrect, because the researchers did not test bacteriocin activity against spoilage bacteria but against pathogenic bacteria.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 07 September 2018

doi:[10.5256/f1000research.15172.r37670](https://doi.org/10.5256/f1000research.15172.r37670)



Sriram Seshadri 

Institute of Science, Nirma University, Ahmedabad, India

1. Lot of words are repeating in the same sentence in the abstract.
2. How do you produce bacteriocin by centrifugation?

3. The species name of shrimp and prawn is missing in the whole test.
4. How was the isolates obtained? What was the sample used?
5. How was the health status of the shrimp and prawn confirmed?
6. Characterization of probiotic features are completely missing.
7. Have the authors confirmed whether the isolates by itself had antibacterial potentials?
8. If so, have they determined the mode of action before moving on to bacteriocin isolation and purification?
9. How do the authors justify selection of only Amoxicillin as the test antibiotics?
10. There are no citations provided for Gel filtration chromatography.
11. There is no mention of standard probiotic strain which are known to produce bacteriocins. How did the authors compare the bacteriocin production and its efficacy?
12. The chemical formula of Ammonium Sulphate is $(\text{NH}_4)_2\text{SO}_4$ not $(\text{NH}_4)_2\text{SO}_4$. It has to be rectified throughout the text.
13. Bacteriocin inhibitory activity is indicated as AU. AU does not stand for Activity Unit by Arbitrary Unit.
14. Discussion is very poorly written. It has to be re-written and there is no mention of any papers on bacteriocins isolated from prawn/shrimps/fish sources.

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Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Metabolic disorder, gut microbiota, liver inflammation, probiotic characterization

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Referee Report 24 April 2018

doi:10.5256/f1000research.15172.r32653



Hazel Monica Matias-Perala 

Department of Technology and Heritage, Faculty of Science, Technology and Human Development, Tun Hussein Onn University of Malaysia (UTHM), Batu Pahat, Malaysia

The manuscript contained important results from a research activity aimed to isolate potential probiotic bacteria which produce potent bacteriocin. Upon isolation of bacteriocins, they were tested for potential antibacterial activity against pathogenic bacteria, through the utilization of disc diffusion method/ assay. After which, the bacteria that perform the best in the disc diffusion method was chosen (one from the tiger shrimp (H) and another one from freshwater prawn (W)) was further purified and subjected to HPLC and FTIR analysis.

- The Introduction section clearly stated the importance of doing this research which is the utilization of bacteriocin to help in alleviating the problems of pathogens in aquaculture particularly the fish and shrimp culture. The authors also cited recent references to indicate the timeliness of this work.
- The methods presented are clear with enough details for easy replication in the future. However, few details were left out in the HPLC analysis which has to be added to the manuscript: (Please indicate in the HPLC method the information on how much (microliter) of the bacteriocin samples were injected, what is the solvent used, how many percents of solvent are added and etc.
- The statistical analysis is appropriate. Although it is suggested to add up which dataset were subjected to ANOVA and DMRT.
- There are few things to clear up in the results section.
 - Please check the results which the author considered "significantly different" throughout the results. Similar letters indicate no significance, therefore as an example, "ab", "bc", and "b" are not significantly different.
 - Table 1, H1 - under A. hydrophila, indicate the real value (there seemed to be a problem).
 - Table 3 was not cited in the text.

- Why is Table 5 not presented in the "Results" section, but is discussed in the "Discussion" section? If the data tabulated in Table 5 is from your own research, it is more appropriate to present in the results section, rather than only cite in the Discussion section.
- The results are considered only partially discussed in the Discussion section. Therefore it will be best to add more appropriate justifications. Following are suggested in the discussion section for improvement:
 - Tables and figures should only be cited minimally in the discussion section since they have already been presented in the results section.
 - Explain more the implication of increasing inhibitory activity against pathogens after precipitation in ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$. Will it help in terms of increased efficiency of bacteriocin? How about in terms of the economics?
 - It will be best to expound on the result of the inhibitory activity of the bacteriocin comparing the current results to that of those previously been used. In the methodology, there was a mention of positive and negative control but the result was not used in the argument on how efficient the currently isolated and purified bacteriocin in this study.
 - There should also be a clear explanation of the reason why H4 and W2 were chosen among other isolates. In here the statistical results will become handy in explaining why.
 - Include more explanation on the FTIR results for the two chosen isolate which are potentially good source of potent bacteriocin. What were the implications of the FTIR results having the specific wave numbers as in the current results? Do they mean more potent? better than others?
- As for the conclusion section: It is not advisable to cite any reference in the conclusion section since conclusion should contain the authors' own interpretation of the results collected from the study.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Discuss this Article

Version 1

Referee Response 14 Aug 2018

Leila Ariyani Sofia, university of lambung mangkurat, Indonesia

The results of this study are needed to overcome disease attacks that often occur in aquaculture businesses in several countries with tropical climates, especially in the Asian region.

Manuscript management is complete and very good.

Therefore, it is a very worthy manuscript for indexing.

Competing Interests: No competing interests were disclosed.

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