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Antagonistic activities against selected pathogens of a bacteriocin-producing lactic acid bacteria isolated from Balao-balao, a fermented rice-shrimp mixture

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Abstract

Lactic acid bacteria from a fermented rice-shrimp mixture traditionally known in the Philippines as Balao-balao were isolated in the present study. Screening of lactic acid-producing strains revealed that ten isolates showed promising inhibitory activities against test microorganisms namely *Staphylococcus aureus* BIOTECH 1634, *Escherichia coli* BIOTECH 1582, *Bacillus subtilis* BIOTECH 1679 and *Vibrio harveyi* SEAFDEC 010. Of interest was isolate PL12, a bacteriocin-producing strain which showed the highest inhibitory activity against the pathogens tested. Isolate PL12 was identified as *Pediococcus pentosaceus* (GenBank accession number MF353992) with 100% similarity by 16S rDNA sequence analyses. Excluding the effects of organic acids and hydrogen peroxide, the cell-free supernatant (CFS) of PL12 isolate exhibited strong antagonistic activities against test pathogens in an agar well diffusion assay. These results confirmed the isolate's proteinaceous nature and are indicative of typical characteristics of bacteriocins. To further concentrate the proteins present in the CFS, ammonium sulfate precipitation followed by column purification (Sep-Pak C_{18} cartridge column) was performed. Positive antagonism of PL12 bacteriocin was observed in both Gram-positive and Gram-negative bacteria tested. The highest inhibitory activity was found against *E. coli* in every purification step. These results suggested that bacteriocin-producing PL12 isolate can be a promising preservative in the food industry and as probiotics in aquaculture since it possesses antagonistic activities against *V. harveyi*.

1. Introduction

Lactic acid bacteria (LAB) are among the most important groups of microorganisms used in food fermentation, contributing to the unique taste and texture of fermented products. Their production of lactic acid maintains the acidic conditions of fermented food which is lethal to food spoilage bacteria (Rhee *et al*., 2011). Aside from lactic acid, LAB can also produce a variety of bioactive compounds with antimicrobial activity such as bacteriocins or bacteriocin-like inhibitory substances during their growth (Hwanhlem *et al*., 2011; Aarti *et al*., 2016).

Bacteriocins are ribosomally synthesized antimicrobial peptides with bactericidal and bacteriostatic effects (Zimina *et al*., 2020). LAB-derived bacteriocins have attracted attention as natural preservatives to control microorganisms primarily responsible for food spoilage and poisoning. Moreover, bacteriocins produced by LAB have several advantages such as bio preservatives. They are inactive and nontoxic on eukaryotic cells, are pH and heat-tolerant, and have a relatively broad antimicrobial spectrum against many food-borne pathogenic and spoilage bacteria (Sharma *et al*., 2018). Moreover, bacteriocins are generally recognized as safe (GRAS) substances and can be used as starter cultures for controlled fermentation processes (Zacharof and Lovitt, 2012).

With consumer demands on natural preservatives, screening and identification of LAB-producing novel bacteriocins from fermented products with a broad antimicrobial spectrum have gained interest worldwide. Particularly in fermented fish products, several bacteriocin-producing LAB strains were isolated and identified since the fish viscera is naturally rich in beneficial LAB. For instance, a bacteriocin discovered from *Weissella cibaria* 110 named Weissellicin 110 was isolated from Plaa-Som, a fermented fish product from Thailand and showed inhibitory activity against some

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Gram-positive bacteria (Srionnual *et al*., 2007). Moreover, five LAB strains identified as *Pediococcus acidilactici* MCL11, *Leuconostoc mesenteroides* MCL12, *Enterococcus faecium* MCL13, *Lactobacillus sakei* MCL14, and *Lactobacillus acidophilus* MCL15, isolated from *Myeolchi-jeot*, a traditional Korean salted and fermented seafood, were also found to produce an antibacterial compound with inhibitory activity against the tested histamine-producing bacteria (Lim, 2016). Enterocin, derived from *Enterococcus faecium* NKR-5-3, and isolated from a Thai fermented fish product was also shown to exhibit a broad antimicrobial spectrum and have high activity against *Listeria innocua* (Ishibashi *et al*., 2012). Similarly, Peng *et al*. (2017) characterized an anti-listerial bacteriocin produced by LAB isolated from Cambodian fermented fish and shrimp. Four LAB isolates confirmed bacteriocin production and demonstrated antimicrobial activity against *Listeria monocytogenes* NCTC 11994.

Fermented rice-shrimp mixture or Balao-balao is a popular traditional Philippine fermented food where shrimp and rice are mixed in the presence of salt and allowed to ferment for 10 - 12 days (Elegado *et al*., 2003). It is characterized by an acidic flavor and aroma with free-flowing consistency where the shrimp shell becomes red and soft, and the rice starch is saccharified. It is popularly consumed as a sauce or main dish after sautéing with garlic and onion (Sanchez, 1999). The microbial diversity of *Balao-balao* as well as the succession of microorganisms in every stage of fermentation has been previously documented (Sanchez, 1999; Elegado *et al*., 2003). However, to date, there has been no report on bacteriocin-producing LAB isolated from Balao-balao. The objectives of this study were to isolate, identify and determine the antimicrobial activities of a bacteriocin-producing LAB, *Pediococcus pentosaceus* PL12 from Balao-balao.

2. Materials and methods

2.1 Balao-balao preparation

Balao-balao was prepared using the method of Sanchez (1999) with some modifications. Briefly, Pacific whiteleg shrimp *(Litopenaeus vannamei)* with an average weight and length of 8.23±0.15 g and 10.82±0.07 cm, respectively, were purchased from a local market in Miagao, Iloilo, Philippines. Samples were washed thoroughly with tap water and 20% rock salt was added based on the weight of the shrimp. The shrimp was then allowed to stand for 30 mins before the mixture was drained. Cooked rice (short grain white rice) was prepared and cooled, then mixed with shrimp in a ratio of 5:1. A total of 3% salt (w/v) was further added to the mixture. The rice shrimp mixture was mixed thoroughly, then packed in sterilized glass bottles and allowed to ferment for 12 days at room temperature (23 - 25°C).

2.2 Isolation and screening of bacteriocin-producing lactic acid bacteria with antimicrobial activities

Approximately 25 g of the prepared sample was added to 225 mL of 0.05 M phosphate buffer containing 1% sodium chloride (NaCl) (pH 7.2). Appropriate serial dilutions were prepared. For the LAB isolation, 0.1 mL was spread plated on a sterile petri dish with de Man, Rogosa and Sharpe (MRS) agar (Difco, Detroit, USA) containing 0.3% (w/v) calcium carbonate ($CaCO_3$). Plates were then incubated at 37°C for 48 hrs. Total bacterial colonies and those which exhibited clearing zones were counted and expressed as $\log_{10}$ colony forming units (CFU)/g. Those colonies that exhibited clear zones were randomly selected and streaked on MRS agar containing 0.3% (w/v) $CaCO_3$. Repeated streaking was done to purify the isolates. All bacterial isolates were tested for Gram-staining reaction and catalase production. Only those isolates which were catalase-negative and Gram-positive were selected as presumptive LAB. For routine analysis, strains were subculture twice in MRS broth for 24 hrs at 37°C.

The agar spot method was used to determine the antagonistic activity of the selected isolates. The bacterial strains which include *Escherichia coli* (BIOTECH 1634)*, Staphylococcus aureus* (BIOTECH 1582), and *Bacillus subtilis* (BIOTECH 1679) were obtained from the Philippine National Collection of Microorganisms (PNCM), University of the Philippines Los Baños, Laguna, Philippines. *Vibrio harveyi* was provided by the Southeast Asian Fisheries Development Center (SEAFDEC 010), Tigbauan, Iloilo, Philippines. All pathogens were grown in tryptic soy broth (Difco, Detroit, USA), and incubated at 37°C. Approximately 10^6 CFU/mL indicator strains were inoculated into soft agar (nutrient broth with 0.7% bacteriological agar) and then overlaid onto the MRS agar plates with LAB spots. Plates were incubated at 37°C for 24 hrs. Isolates which exhibited clear inhibition zones surrounding the colonies indicated antimicrobial activity. The result of the inhibition zone according to intensity was scored as follows: +, 8.00-12.00 mm; ++, 12.00-16.00 mm; +++, more than 16.00 mm; -, no inhibition zone (Gao *et al*., 2010).

2.3 Strain identification

One prospective strain, PL12, with the highest antimicrobial activity was identified according to morphology, physiology, and biochemical characteristics. The isolate was subjected to Gram-staining, catalase, oxidase, production of acid and gas

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from glucose, nitrate reduction, arginine hydrolysis, growth at 15°C and 45°C, methyl red and Voges-Proskauer (MRVP) tests (Kandler and Weiss, 1986).

For molecular identification, genomic DNA was extracted using PureLink™ Genomic DNA Kit (Invitrogen, USA) from a 24-hour culture grown in MRS agar plate. Gene amplification was performed using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The genomic DNA (25 µL reaction mix) was subjected to the following polymerase chain reaction (PCR) conditions as described by Adimpong *et al*. (2012), 5 min of initial denaturation at 94°C followed by 30 cycles at 94°C for 90 s, 52°C for 30 s, 72°C for 90 s and final elongation step of 72°C for 7 mins. The PCR product was visualized in 1% agarose stained with gel red (0.5 ug/mL) and was sequenced using capillary sequencing (AIT Biotech, Singapore). Basic Local Alignment Search Tool (BLAST) was used to identify sequence similarity. Molecular Evolutionary Genetics Analysis (MEGA) X software (Kumar *et al*., 2018) was used to construct a phylogenetic tree following the neighbor-joining method. A partial sequence of the isolate was deposited in the NCBI GenBank database with accession number MF353992.

2.4 Detection of antagonistic activity of the cell-free supernatant

LAB isolate PL12 was grown in MRS broth and the cell-free supernatant (CFS) was collected by centrifugation (Hsiangtai CN-2060, China) at 8000 rpm for 10 min at 4°C. Antagonistic activity was carried out using well diffusion assay (Hwanhlem *et al*., 2011). Briefly, CFS was collected and diluted two-fold with normal saline (0.85% w/v). Twenty microliters of each diluted CFS were dispensed into each well. The diluted CFS was allowed to diffuse for about 2 hrs at room temperature before overlaying with 3 mL of soft nutrient agar containing 1% (v/v) indicator strains (*S. aureus, E. coli, B. subtilis* and *V. harveyi*). Bacteriocin activities were quantified by serial two-fold dilutions and expressed in arbitrary units per milliliter (AU/mL). Arbitrary units are defined as the reciprocal of the highest dilution which gives a distinct inhibition zone (Saeed *et al*., 2004).

$$\frac{AU}{mL} = \frac{1000}{V} \times D$$

where *D* is the dilution factor and *V* is the volume of CFS.

2.5 Testing for the presence of bacteriocin

To eliminate the effect of hydrogen peroxide, CFS was added with catalase (300 U/mL in PBS, pH 7). The CFS was adjusted to pH 6 with 1 M hydrochloric acid (HCl) or sodium hydroxide (NaOH) and filtered through a 0.45 µm membrane filter to eliminate the inhibition activity of organic acids (Hwanhlem *et al*., 2011). With the inactivation of the effects of hydrogen peroxide and organic acids, crude bacteriocin was obtained. To deactivate the effect of bacteriocin, proteolytic enzymes trypsin and proteinase K dissolved in 0.1 mg/mL in PBS (pH 7) were added and incubated at 37°C for 2 hrs (Kaktcham, 2012). Antagonistic activity was determined using well diffusion assay as described above.

2.6 Purification of crude bacteriocin

The supernatant obtained from the culture of LAB isolate PL12 was subjected to 80% ammonium sulfate precipitation to obtain the crude bacteriocin and then subjected to overnight stirring at 150 rpm at 4°C. Then, the bacteriocin was pelleted by centrifugation at 10000 rpm at 4°C for 1 hr and dissolved in 20 mM phosphate buffer, pH5.5 (Xiraphi *et al.*, 2006). Purification was done using the protocol of Millette *et al.* (2008) with some modifications. Briefly, batch purification was performed using Sep-Pak C_{18} cartridge column (Waters, USA). The column was first equilibrated with 20 mL of 100% acetonitrile, followed by 10 mL of water. The sample was applied at a flow rate of 2 drops per second and successively washed with 10 mL of 0%, 10%, 20%, 30%, 40%, 50% acetonitrile and final elution was done with 30 mL of 60% acetonitrile. Approximately 2 mL of sample eluted in every acetonitrile concentration was collected. The absorbance of the fractions was obtained using a spectrophotometer at 220 nm (BMG Labtech, Germany).

2.7 Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test and expressed as mean±standard deviation. All statistical analyses were performed using R software version 4.1.2 (R Core Team, 2014). *P* values of 0.05 or less were considered statistically different.

3. Results and discussion

3.1 Antimicrobial activities of selected lactic acid bacteria isolates

Among the colonies that showed clear halo zones, ten isolates were chosen and screened for antimicrobial properties. As shown in Table 1, PL12 showed the highest inhibitory activity against test microorganisms, particularly the Gram-negative *E. coli*. It has moderate antimicrobial activity against Gram-positive bacteria namely *S. aureus* and *B. subtilis*. The antagonistic activity of LAB towards pathogenic microorganisms can

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be attributed to the production of organic acids and bactericidal substances such as bacteriocins (Zacharof and Lovitt, 2012). The cell outer membrane of Gram-negative bacteria such as *E. coli* contains lipopolysaccharide (LPS) which is highly specific thus, the outer membrane (OM) becomes an efficient permeable barrier that can exclude hydrophobic substances and macromolecules (Schär-Zammaretti and Ubbink, 2003). However, lactic acid from LAB is small and water soluble so it can gain entry to the cell periplasm through the hydrophilic porin proteins of the OM (Haghighatafshar *et al*., 2021). Furthermore, lactic acid as an important metabolite produced by the isolates in this study may have caused the release of the LPS found in the OM of Gram-negative bacteria, disrupting the permeable barrier of the OM. Permeabilizers or OM perturbants such as lactic acid cause disruption of the OM barrier allowing for the entry of antimicrobials into Gram-negative pathogens, thus sensitizing the bacteria (Alakomi *et al*., 2000; Wang *et al*., 2022).

Table 1. Antimicrobial activities of primarily selected lactic acid bacteria isolated from Balao-balao against test microorganisms using agar spot method.

Isolate	Zone of Inhibition			
	S. aureus	*B. subtilis*	*E. coli*	*V. harveyi*
PL2	+	+	-	+
PL3	+	+	-	+
PL5	+	+	+	+
PL8	++	+	+++	+
PL12	++	++	+++	+
PL15	++	+++	-	-
LAB1	+	+	-	++
LAB7	+	+	+	-
LAB8	+	+	++	+
LAB9	+	++	-	+

Diameter of inhibition zone = +: 8.00-12.00 mm, ++: 12.00-16.00 mm, +++: more than 16.00 mm, -: no inhibition zone.

3.2 Strain identification

Phenotypic characterization of LAB isolate PL12 showed Gram-positive cocci clustered in tetrads, catalase and oxidase negative and non-spore-forming. While taxonomic information of LAB isolates PL12 was further confirmed using 16S rDNA amplification. Partial nucleotide sequence revealed that PL12 has 1129 base pairs and the BLAST tool showed 100% similarity to *Pediococcus pentosaceus* (GenBank Accession No. AJ305321). The sequence was deposited at GenBank with accession number MF353992. The closest type of strain to PL12 isolate was also *P. pentosaceus* based on nucleotide homology and phylogenetic analysis (Figure 1).

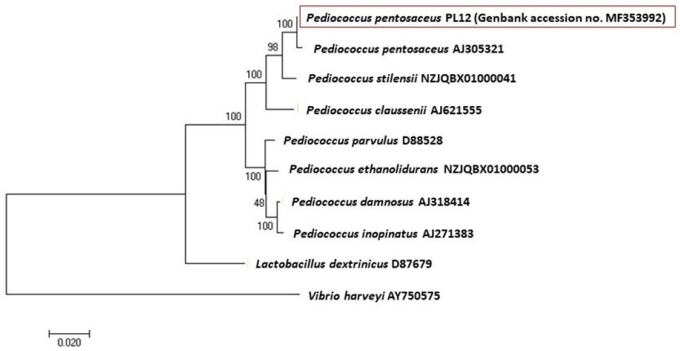

Figure 1. Phylogenetic tree derived from the 16s rDNA sequence of LAB isolate PL12 and the phylogenetically closest LAB type strains. The GenBank accession numbers of the type strains and the studied strain are shown following species names. The number at the nodes indicated bootstrap values on neighbor-joining analyses of 1000 resampled data sets. Bar 0.02 represents sequence divergence. *Vibrio harveyi* served as an outgroup of this tree.

3.3 Antagonistic activities of bacteriocin in PL12 lactic acid bacteria isolate

The cell-free supernatant (CFS) of *P. pentosaceus* PL12 was treated with different substances to determine whether the ability to inhibit the growth of the test microorganisms was due to its proteinaceous nature as in bacteriocin. As shown in Table 2, the antagonistic activities of *P. pentosaceus* PL12 against the test microorganisms were evident even after the addition of catalase and NaOH, indicating that the activity was not related to the production of either organic acids or hydrogen peroxide. Moreover, a significant increase (p<0.05) in antimicrobial activities was observed after the addition of proteases namely trypsin and proteinase-K in the CFS. Thus, it can be inferred that the inhibitory activity of the CFS from PL12 isolate can be attributed to the presence of other metabolites present in the medium. However, when CFS was subjected to all treatments, no antagonistic activity was observed. These results confirmed the proteinaceous nature of the antimicrobial component present in the CFS and were responsible for inhibiting the growth of the test microorganisms. Several studies confirmed that antagonistic activities of bacteriocin-producing LAB isolates decreased after the addition of different proteases and other treatments. For instance, Hwanhlem *et al*. (2014) observed that treatments with proteinase K, a-amylase and a-chymotrypsin resulted in substantial decreases in antibacterial activity of bacteriocin-producing LAB isolates from mangrove forests. Furthermore, in a study by Marie *et al*. (2012), when bacteriocin Lp6SH was treated with α-amylase and lipase, these enzymes did not affect its antimicrobial activity.

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Table 2. Antagonistic activities of the cell-free supernatant (CFS) of *Pediococcus pentosaceus* PL12 against test microorganisms when subjected to different treatments.

Treatment	Antimicrobial Activity (mm)			
	S. aureus	*B. subtilis*	*E. coli*	*V. harveyi*
CFS + catalase	$9.10\pm0.20^{b;y}$	$11.00\pm0.30^{a;x}$	$12.40\pm0.80^{b;x}$	$12.50\pm0.33^{b;x}$
CFS + NaOH/ HCl	$8.00\pm0.33^{b;y}$	$2.00\pm0.66^{c;z}$	$13.50\pm0.30^{b;x}$	$14.00\pm0.66^{a;x}$
CFS + catalase + NaOH/HCl	$8.67\pm0.20^{b;x}$	$7.00\pm0.33^{b;y}$	$8.00\pm0.33^{c;x}$	$9.50\pm0.33^{c;x}$
CFS + proteinase K + trypsin	$15.00\pm0.67^{a;x}$	$12.00\pm0.33^{a;y}$	$15.00\pm0.88^{a;x}$	$12.00\pm0.66^{b;y}$
CFS + catalase + NaOH/ HCl + proteinase K + trypsin	-	-	-	-

Values are presented as mean±SD of triplicates. -: not detected.
[a-c]Different superscripts in the same column indicate significant differences ($p<0.05$).
[x-z]Different superscripts in the same row indicate significant differences ($p<0.05$).

3.4 Purification and antagonistic activities of bacteriocin from PL12 lactic acid bacteria isolate

Among the acetonitrile fractions tested, the highest inhibitory activity against *S. aureus* as the test microorganism was obtained at a 30% fraction. Thus, subsequent purification steps made use of this concentration. For various purification techniques, the yield, activity and purification fold of bacteriocin from *P. pentosaceus* PL12 obtained were summarized in Table 3. The column chromatography technique provided a fairly pure preparation (6.89-fold), while the ammonium sulfate precipitation gave comparatively low purification (1.27-fold). Specific activity was highest in column chromatography at 24.40 AU/mg protein and this activity was confirmed when the antagonism was tested against test microorganisms.

As shown in Table 4, the metabolites obtained from each purification step of *P. pentosaceus* PL12 exhibited inhibitory activities against both Gram-positive (*S. aureus* and *B. subtilis*) and Gram-negative (*E. coli* and *V. harveyi*) bacteria. The highest inhibitory activity was found against *E. coli* in every purification step. Notably, antagonistic activities of *P. pentosaceus* PL12 were also observed in *V. harveyi*, an important pathogen in aquaculture. There is evidence of the potential of bacteriocin-producing LAB as probiotics for shellfish including shrimp (Naiel *et al*., 2021). Metabolites produced by LAB including bacteriocin can either kill or inhibit the growth of bacteria including those that may be potentially pathogenic to the host or modulate intestinal microbiota in shrimp resulting to improved resistance to pathogens (Thompson *et al*., 2022).

Bacteriocins from LAB typically restrict their activity to strains of species related to the producing species and particularly to strains of the same species (Luchansky, 1999). *P. pentosaceus* is a Gram-positive, homofermentative LAB and this antagonism against Gram-negative bacteria was not frequently observed in bacteriocins produced from LAB (Gao *et al*., 2010). However, some studies showed that bacteriocins from *Lactobacillus plantarum* and C*arnobacterium maltaromaticum* can widely inhibit the growth of Gram-negative bacteria like *E. coli* (Todorov and Dicks, 2005; Martin-Visscher *et al*., 2011). Several types of bacteriocins were produced by *P. pentosaceus* isolated from different food matrices like kimchi (Shin *et al*.,

Table 3. Purification of bacteriocin from *Pediococcus pentosaceus* PL12.

Purification step	Volume (mL)	Protein (mg/mL)	Specific activity (AU/mg protein)	Yield (%)	Purification fold
CFS	1000	451.59	3.54	100.00	1.00
Ammonium sulfate	50	530.44	4.51	5.87	1.27
Column chromatography (C_{18})	2	655.00	24.40	4.94	6.89

CFS: cell-free supernatant

Table 4. Antagonistic activities of the metabolites obtained from each purification step of bacteriocin from *Pediococcus pentosaceus* PL12 against test microorganisms.

Purification step	Antagonistic activity (mm)			
	S. aureus	*B. subtilis*	*E. coli*	*V. harveyi*
CFS	$20.30\pm0.33^{a;x}$	$20.00\pm0.50^{a;x}$	$20.00\pm0.50^{c;x}$	$18.00\pm0.50^{a;y}$
Ammonium sulfate	$15.50\pm0.24^{c;z}$	$18.30\pm0.66^{b;y}$	$22.10\pm0.33^{b;x}$	$16.20\pm0.66^{b;z}$
Column chromatography (C_{18})	$18.50\pm0.66^{b;y}$	$19.40\pm0.66^{a;y}$	$25.00\pm0.33^{a;x}$	$15.40\pm0.33^{c;z}$

Values are presented as mean±SD of triplicates. CFS: cell-free supernatant.
[a-c]Different superscripts in the same column indicate significant differences ($p<0.05$).
[x-z]Different superscripts in the same row indicate significant differences ($p<0.05$).

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2008), sausages (Bungenstock *et al*., 2020) and milk cheese (Todorov *et al*., 2020) with observed antagonistic activities against pathogens. But this is the first report of a bacteriocin-producing *P. pentosaceus* isolated from a fermented fish product with positive antagonism against both Gram-positive and Gram-negative bacteria.

4. Conclusion

Pediococcus pentosaceus PL12, isolated from Balao-balao, a fermented rice-shrimp mixture, was found to produce a bacteriocin with high inhibitory activities against the tested Gram-positive and Gram-negative bacteria namely *Staphylococcus aureus* BIOTECH 1634*, Escherichia coli* BIOTECH 1582*, Bacillus subtilis* BIOTECH 1679 and *Vibrio harveyi* SEAFDEC 010. The antagonistic activities of *P. pentosaceus* PL12 were evident at every purification step: crude, ammonium sulfate precipitation and column chromatography. The results of this study may serve as groundwork for future studies on indigenous fish products of the Philippines and broaden the application of LAB in the food and fish industry. In the case of *P. pentosaceus* PL12, it is a promising candidate as a bio-preservative in the food industry. It can also be applied as probiotics in aquaculture as it possesses antagonistic activities against *V. harveyi*. Further work is to determine the type of bacteriocin found in *P. pentosaceus* PL12, its DNA and amino acid sequences, as well as its stability and modes of action.

Conflict of interest

The authors declare no conflict of interest.

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