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ANTIBACTERIAL ACTIVITIES OF *Lactobacillus* sp. GMP1 AND *Weisella* sp. GMP12 AGAINST SOME FOODBORNE DISEASE CAUSING-BACTERIA

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Abstract

Lactic acid bacteria (LAB) have been reported to have inhibitory activity against foodborne causative bacteria, some of which are generally recognized as safe (GRAS). The aim of this study was to isolate halotolerant lactic acid bacteria (HLAB) from fermented fish products, namely pakasam and wadi, and to determine their potential to inhibit the growth of contaminant bacteria and biogenic amine-producing bacteria. Isolation of HLAB was performed using De Man, Rogosa, and Sharpe agar (MRSA) supplemented with 1% $CaCO_3$ and 7% NaCl. Colonies that grew and showed clear zones continued to undergo halotolerant growth tests in MRS broth with several NaCl concentrations. Two selected isolates were identified as lactic acid bacteria: *Lactobacillus* sp. GMP1 and *Weisella* sp. GMP12. The isolation of antibacterial compounds targeting bacteriocin was carried out by fermentation in MRSB media at 37°C for 24 h, followed by separation of the supernatant and isolation of the antibacterial compounds by precipitation with ammonium sulfate and dialysis. Antibacterial activity tests showed that bacteriocins produced by *Lactobacillus* sp. GMP1 and *Weisella* sp. GMP12 is able to inhibit *Staphylococcus aureus* ATCC 6,538 with bacteriocin activity of 5,868.19 AU and 3,693.60 AU, respectively. Bacteriocins can also inhibit *Salmonella* spp.. 230C with bacteriocin activity respectively is 1,696.39 AU and 2,254.17 AU, respectively, whereas only *Weisella* sp. GMP12 inhibits *Klebsiella* sp. CK2 with bacteriocin activity is 3,165.51 AU. These results indicate that *Lactobacillus* sp. GMP1 and *Weisella* sp. GMP12 has the potential to be used as a starter culture in fermented products.

Keyword: bacteriocin, *Lactobacillus* sp., pakasam, wadi, *Weisella* sp.

Aktivitas Antibakteri *Lactobacillus* Sp. GMP1 dan *Weisella* Sp. GMP12 terhadap Beberapa Bakteri Pembawa Penyakit pada Makanan

Abstrak

Bakteri asam laktat (BAL) telah dilaporkan memiliki aktivitas penghambatan terhadap bakteri-bakteri kontaminan pangan dan di antaranya telah diakui sebagai *generally recognize as safe* (GRAS). Penelitian ini bertujuan untuk mengisolasi bakteri asam laktat halotoleran (BALH) dari produk fermentasi perikanan pakasam dan wadi serta mengetahui potensinya dalam menghambat pertumbuhan bakteri kontaminan dan juga bakteri penghasil amina biogenik. Isolasi BALH dilakukan dengan agar De Man, Rogosa dan Sharpe (MRSA) yang disuplementasi dengan 1% $CaCO_3$ dan 7% NaCl. Koloni yang tumbuh dan menunjukkan zona jernih di sekitarnya dilanjutkan ke uji pertumbuhan halotoleran dalam MRS cair

dengan beberapa konsentrasi NaCl. Dua isolat terpilih diidentifikasi sebagai bakteri asam laktat halotoleran yaitu *Lactobacillus* sp. GMP1 dan *Weisella* sp. GMP12. Isolasi senyawa antibakteri dengan target bakteriosin dilakukan dengan fermentasi pada media MRSB pada 37°C selama 24 jam, dilanjutkan dengan pemisahan supernatan dan isolasi senyawa antibakterinya dengan pengendapan amonium sulfat dan dialisis. Uji aktivitas antibakteri yang dilakukan dengan difusi cakram didapatkan bahwa senyawa antibakteri yang dihasilkan oleh *Lactobacillus* sp. GMP1 dan *Weisella* sp. GMP12 mampu menghambat *Staphylococcus aureus* ATCC 6538 dengan aktivitas penghambatan masing-masing sebesar 5.868,19 AU dan 3.693,60 AU. Senyawa antibakteri yang dihasilkan juga dapat menghambat *Salmonella* sp. 230C dengan aktivitas bakteriosin masing-masing adalah 1.696,39 AU dan 2.254,17 AU, namun hanya *Weisella* sp. GMP12 yang mampu menghambat *Klebsiella* sp. CK2 dengan aktivitas bakteriosin sebesar 3.165,51 AU. Hasil ini menunjukkan bahwa *Lactobacillus* sp. GMP1 dan *Weisella* sp. GMP12 memiliki potensi untuk digunakan sebagai *starter* pada produk fermentasi.

Kata kunci: bakteriosin, *Lactobacillus* sp., pakasam, wadi, *Weisella* sp.

INTRODUCTION

Fishery-fermented products are widely developed in Asia, especially in Southeast Asia and have become specialties in various regions. However, they are generally processed in a poor and non-controlled condition which might trigger foodborne diseases caused by contamination of pathogenic bacteria such as *Salmonella* sp., *Staphylococcus aureus*, *Eschericia coli,* and some biogenic amine producing bacteria (Marti-Quijal *et al.*, 2020). Effective methods to overcome those problems were by using bio preservatives such as lactic acid bacteria (LAB) which can be used as culture starter (Sivamaruthi *et al.*, 2018). Recent research has reported that LAB, such as *Lactobacillus* spp., *Bifidobacterium* spp., *Lactococcus* spp. *Leuconostoc* spp., *Pediococcus* spp., *Propionibacterium* spp., and *Streptococcus* spp. were reported to have an antibacterial activity against pathogenic bacteria which causing foodborne disease (Sivamaruthi *et al.*, 2018; Girma & Aleka, 2021; Zhang *et al.*, 2021).

Lactic acid bacteria have several antibacterial mechanisms such as producing acid and specifically producing proteinaceous antibacterial substances named bacteriocin (Reis *et al.*, 2012). Previous studies reported that bacteriocin from *Lactobacillus plantarum* succeeded in reducing biogenic main content and inhibiting the growth of pathogenic bacteria in fish sauce (Lim, 2016; Pasini *et al.*, 2018; Wang *et al.*, 2018; Sanpa *et al.*, 2019). Another study also reported bacteriocin from *Weissella confusa* were able to inhibit the growth of *Bacillus cereus* ATCC14579, *Escherichia coli* UT18, *Listeria monocytogenes* NCTC10890, *Pseudomonas aeruginosa* PA7, *Staphylococcus aureus* RF122, *Micrococcus luteus* ATCC10240, *Lactobacillus lactis* A1 (Goh & Philip, 2015). Moreover, they also give positive advantages on increase sensory properties (Adeniran *et al.*, 2012; Litwinek *et al.*, 2022; Nie *et al.*, 2022).

Pakasam and wadi are traditionally processed fish fermented products originating from Sumatra and Kalimantan, Indonesia respectively. The fermentation process in the manufacture of both products enables the growth of LAB in the product. In this study, the aims were to isolate LAB from Pakasam and Wadi products and determine their potential in inhibiting the growth of contaminant bacteria and also biogenic amine producing bacteria.

MATERIALS AND METHODS

Halotolerant Lactic Acid Bacteria Isolation and Screening

Isolation of halotolerant lactic acid bacteria (HLAB) from fermented products was carried out based on the method described by Thao *et al.* (2021). As much as 25 g of samples were homogenized in 225 of 7% mL NaCl solution followed by serial dilution up to 10^{-7}. The 0.1 mL from each dilution was then inoculated on MRSA media supplemented with 7% NaCl and 1% $CaCO_3$ then incubated aerobically at 37°C for 48 h. A single colony that grew and form a clear zone was re-inoculated on the same agar media and incubated in the same condition to get a pure culture. To confirm the lactic acid bacteria,

each colony undergoes initial characterization including Gram type by using 3% KOH and catalase by using 3% H_2O_2. All LAB suspected isolates were then stored in 25% glycerol stock and stored at -80°C for further analysis.

Halotolerant Growth Test

Each colony from the previous step were grown in MRSB with 0%, 7%, and 14% NaCl concentration (Morales *et al.*, 2011) at 37°C for 24 h. The growth of each colony was measured as optical density at 600 nm by spectrophotometer UV-Vis (Thermo Scientific Genesys 10s UV-Vis, USA).

Bacterial Identification

Colonies which possess growth two times higher at medium containing salt were further identified by a molecular technique by amplified 6S rRNA region using forward primers: 15F 5'-GCTCAGGAYGAACGCYGG-3' and reverse primer: 687R 3'-CACCGCTACACATGRADTTC-5' (Hou *et al.*, 2018). Amplification was done by PCR (Bio-Rad Laboratories Ltd, USA) with PCR mix (MyTaq™HS Red Mix, Bioline) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol primers, 0.2 mM deoxynucleotide triphosphates and 0.5 U Taq DNA polymerase (Applied Biosystem). The PCR condition was conducted according to the PCR mix manufacturer as follows: preliminary denaturation at 95°C for 2 min, 39 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 45 s, first extension at 72°C for 2 min, and final extension at 72°C for 7 min. The PCR was visualized with gel electrophoresis and then the corresponding band was extracted and sequenced at 1st Base (Singapore). The sequence was further analyzed using the BLASTn tool from National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

Determination of Bacterial Growth Curve and Bacteriocin Production

As much as 1% (v/v) of each isolate were grown at 200 mL MRS broth and incubated at 37°C for 48 h (Maulidayanti *et al.*, 2019). The growth curve was observed by taking 1 mL of culture every 6 h, and determination was carried out by the total plate count (TPC) spot test method by Whitmire & Merrel (2012) from liquid culture every 6 h for 48 h. The bacteriocin production was measured by antibacterial activity test on its supernatant (cell free supernatant/CFS) by paper disk method against selected target bacterial.

Bacteriocin Production and Partial Purification

Bacteriocin production and partial purification were done according to Zhang *et al.* (2018). Each lactic acid bacteria (LAB) isolate was inoculated on MRS agar supplemented with 7% NaCl and incubated at 37°C for 48 h. A single colony was pre-cultured on MRS broth and 1% (v/v) of it was cultured on the same media and incubated at 37°C for 24 h without shaking. Cell free-supernatant (CFS) were collected from 1L MRS broth by centrifugation at 12,000x *g* for 15 min at 4°C and the pH of CFS was adjusted to pH 6.5 using 1 M NaOH. CFS was then filtered through a syringe filter membrane of 0.22 µm (Himedia, India). Filtrate was continued to precipitation step by ammonium sulphate at 80%, while the precipitate was collected by centrifugation at 10,000x *g* for 10 min at 4°C. The precipitate was then diluted in phosphate buffer 0.2 M at neutral pH (1:1) and followed by dialysis with MWCO 1 kDa. Finally, the crude bacteriocin was lyophilized by freeze dry method.

Antibacterial Activity Test

Each targeted bacterial namely *Morganella morganii* (TK7), *Klebsiella pneumoniae* (CK2), *Citrobacter freundii* (CK1) *Eschericia coli* 563B, *Salmonella* sp. 230°C, and *Staphylococcus aureus* ATCC 6538 were grown on TSB (for histamine producing bacteria) or NB (other than histamine producing bacteria) at 37°C for 24 h and the final optical density (OD600) was adjusted to 0.1 (Farkas *et al.*, 2018). Antibacterial activity test was conducted by disc diffusion as follows: as much as 5 µl of each targeted bacterial culture (OD600 0.1) were inoculated on 5 mL of TSA or NA soft agar 0. 7% and poured into solid agar. As much as 20 µl of bacteriocin (final concentration

1 mg/mL) was applied to a 6 mm paper disc and placed in top of soft agar. Confirmation of bacteriocin was carried out by treating using 4% papain enzyme at its optimum enzyme activity (according to the maker) for 30 min. Kanamycin was used as a positive control. Bacterial inhibition activity was measured from the clear zone formed around the paper disc and calculated as AU (activity unit). One AU was the inhibition zone per volume of bacteriocin samples tested (mm^2/mL).

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

Notes:
Lz = Clear zone area (mm^2)
Lc = Disc area (mm^2)
V = Bacteriocin volume (mL)

Statistical Analysis

The bacterial growth and antibacterial activity data were subjected to a one-way analysis of Variance (ANOVA) followed by Duncan Multiple Range Rest (DMRT) with significance levels of 95%. The analysis was performed using SPSS ver.20 software.

RESULTS AND DISCUSSION

Screening and Isolation of Halotolerant Lactic Acid Bacteria

A total of 381 colonies were isolated from fermented fish products namely pakasam and wadi. The initial screening by Gram stain and catalase test resulted on only 12 isolates and 13 isolates of each possess Gram positive and catalase negative characteristics. The visual observation can be seen in Figure 1 showed that LAB isolated from pakasam and wadi were mostly rod shape either coccus shape, round with a convex elevation, yellowish white like milk color, flat and smooth edges similar to the other LAB which was characterized previously (Guevarra & Barraquio, 2015; Tilahun *et al.*, 2018).

Halotolerant Bacteria Growth at Various Salt Concentrations

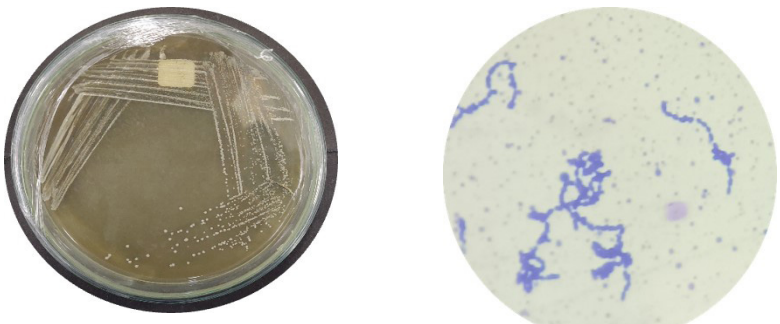
To observe the ability of each isolate to grow in various salt concentration, the isolates were grown in 0%, 7% and 15% NaCl concentration (Table 1). The choices of 7% were based on the research results from Rahman *et al.* (2017) which conclude that no LAB growth on media with salt concentration more than 8%.

Most of the isolates were growth well in 0% NaCl concentration and could not grow in 15% NaCl concentration (Table 1). To select which isolates were grouped as halotolerant LAB, we followed Rahman *et al.* (2017) with criteria grow better at 7% salt concentration than 0%. From that, GMP 1 and GMP 12 were classified as slightly halotolerant LAB which able to growth 1.96 times and 1.86 times higher in 7% NaCl then 0% NaCl respectively.

Molecular Identification of Selected Lactic Acid Bacteria

The 16S rRNA region of isolates GMP 1 and GMP 12 were amplified using LAB specific primers 15F and 687R. The PCR successfully amplified 750 bp sequence related gene fragment and depicted in Figure 2.

The sequences of 16S rRNA obtained



(A) (B)

Figure 1 Lactic acid bacteria isolated from pakasam and wadi; (A) purification step; (B) Gram staining result

Table 1 Growth test results (OD600) at various salt concentrations

Isolate	NaCl concentration (% w/v)		
	0	7	15
GMP1*	2.065±0.051[a]	1.555±0.009[b]	0.032±0.011[c]
GMP2*	1.509±0.049[a]	0.752±0.050[b]	0.044±0.034[c]
GMP3*	1.527±0.010[a]	0.905±0.046[b]	0.096±0.018[c]
GMP6*	1.471±0.038[a]	0.711±0.045[b]	0.054±0.052[c]
GMP7*	1.458±0.050[a]	1.000±0.089[b]	0.273±0.019[c]
GMP8*	1.341±0.034[a]	0.785±0.046[b]	0.055±0.042[c]
GMP9*	1.371±0.026[a]	0.837±0.079[b]	0.027±0.028[c]
GMP10*	1.247±0.240[a]	0.891±0.013[b]	0.062±0.012[c]
GMP11*	1.239±0.016[a]	1.075±0.004[b]	0.027±0.010[c]
GMP12*	0.494±0.052[a]	0.905±0.006[b]	0.069±0.030[c]
GMP13*	1.256±0.036[a]	1.067±0.017[b]	0.019±0.002[c]
GMP14*	1.273±0.003[a]	1.083±0.015[b]	0.022±0.006[c]
GMW 2.1*	0.671±0.053[a]	0.656±0.596[a]	0.060±0.127[b]
GMW 2.2*	0.892±0.201[a]	0.365±0.208[b]	0.088±0.006[c]
GMW 2.3*	0.766±0.009[a]	0.477±0.018[b]	0.087±0.006[c]
GMW 2.4*	0.711±0.055[a]	0.717±0.045[a]	0.124±0.011[b]
GMW 2.5*	0.535±0.037[a]	0.706±0.025[b]	0.109±0.008[c]
GMW 2.6*	0.115±0.017[a]	0.484±0.012[b]	0.121±0.019[a]
GMW 2.9*	0.977±0.003[a]	0.674±0.100[b]	0.146±0.004[c]
GMW 2.10*	0.017±0.004[a]	0.229±0.018[b]	0.088±0.106[a]
GMW 2.11*	0.730±0.043[a]	0.259±0.034[b]	0.027±0.010[c]
GMW 2.13*	0.861±0.090[a]	0.606±0.194[b]	0.151±0.032[c]
GMW 2.14*	0.661±0.031[a]	0.674±0.068[a]	0.132±0.033[b]
GMW 2.15*	0.406±0.003[a]	0.629±0.027[b]	0.081±0.010[c]
GMW 2.16*	0.358±0.030[a]	0.552±0.017[b]	0.042±0.001[c]

P indicate from Pakasam while W from Wadi; *Significant one-way ANOVA; different letters showed significant differences

were compared with the GenBank database using the BLAST search tool and the results suggested that GMP 1 was identified as *Lactobacillus plantarum* SN13T (accession number AP019815.1) with 99.71% identify while GMP 12 was identified as *Weisella* sp. JCM 10694 (accession number LC306848.1) with 99.55% identify and both isolates showed 100% query cover. From now on, GMP 1 will be mentioned as *Lactobacillus* sp. GMP 1 and GMP 12 will be mentioned as *Weisella* sp. GMP 12.

Lactobacillus sp. and *Weisella* sp. have been reported previously as LAB which isolated from fermented fish (Wang *et al.*, 2018) and milk products (Goh & Phillip,

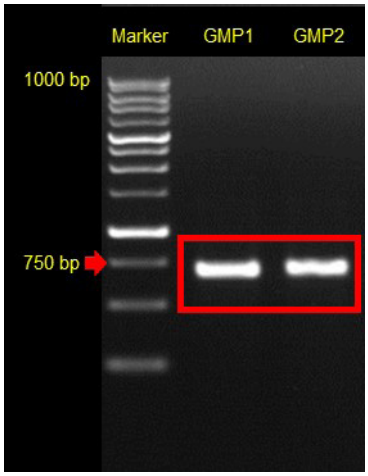


Figure 2 Amplification of 16s rRNA region

2015). *Lactobacillus plantarum* were reported to be able to produce plantaricin (Wang *et al.*, 2018), while Goh & Phillip (2015) reported that *Weisella confusa* produced bacteriocins with a molecular weight of 2.7 kDa which was able to inhibit the growth of food contaminants and pathogenic bacteria.

Optimization of Bacteriocin-like Substance Production

To optimize the bacterial growth and the production of bacteriocin, we conducted 48 h incubation and checked the colony forming unit and antibacterial activity every 6 h. The growth of both strains showed the same pattern that reached maximum exponential phase at 24 h (Figure 3 and 4). This result was in accordance with the growth of some bacteriocinogenic LAB reported by Yang *et al.* (2018).

The antibacterial of cell-free supernatant (CFS) against indicator bacteria namely *Morganella morganii* TK7, *Klebsiella pneumoniae* CK2, *Citrobacter freundii* CK1, *Escherichia coli* 563B, *Salmonella* sp. 230C, and *Staphylococcus aureus* ATCC 6538 indicated that CFS only active to several bacteria. CFS produced by *Lactobacillus* sp. GMP1 inhibit the growth of *Staphylococcus aureus* ATCC 6538, *Salmonella* sp. 230C, and *Klebsiella* sp. CK2. The largest inhibition zone was formed for the growth of *Staphylococcus aureus* ATCC

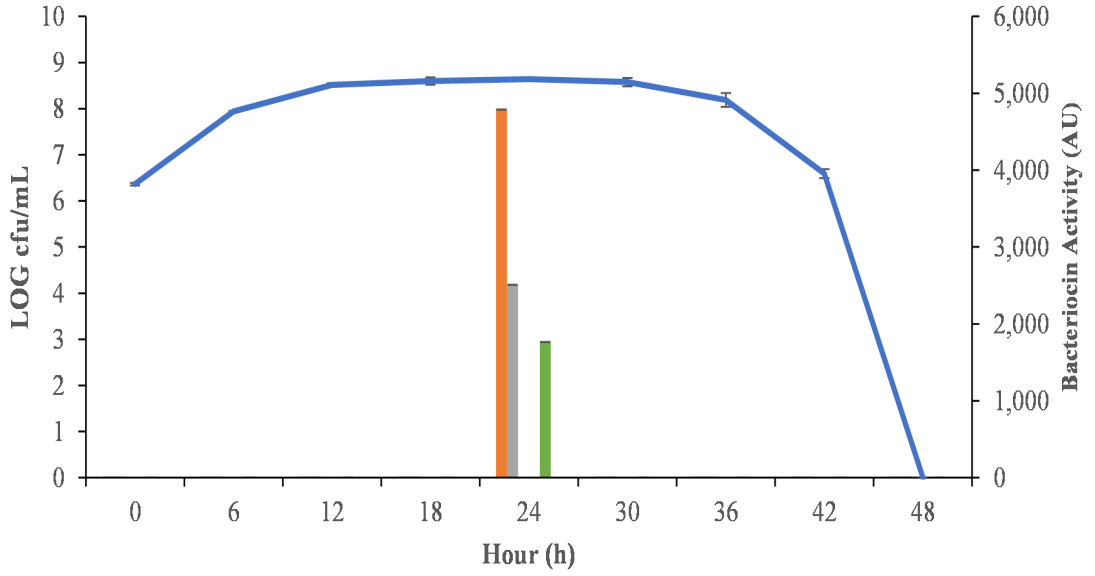


Figure 3 The growth curve and the antibacterial activity of *Lactobacillus* sp. GMP1, *Staphylococcus* aaureus ATCC 6538, *Salmonella* sp. 230C, *Klebsiella* sp. CK2

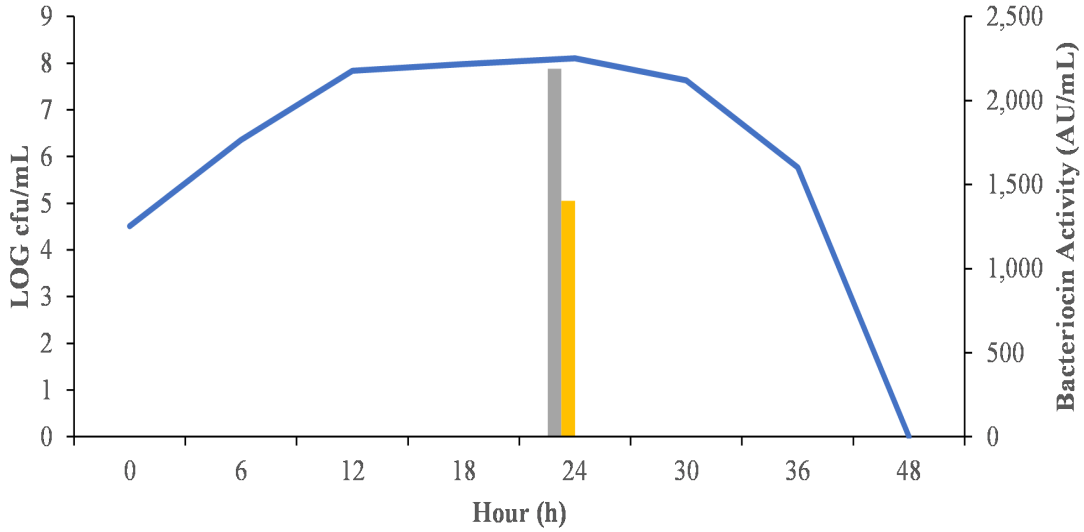


Figure 4 The growth curve and the antibacterial activity of *Weisella* sp. GMP12, *Salmonella* sp. 230C, *Escherichia coli* 563B

6538 with bacteriocin activity as 4,788.69 AU and the lowest for *Klebsiella* sp. CK2 with bacteriocin activity as 1,766.25 AU. On the other hand, CFS from *Weisella* sp. GMP12 inhibited the growth of *E. coli* 563B with activity of 2,189.37 AU and *Citrobacter freundii* CK1 of 1,402.97 AU. The inhibitory activity of Gram negative is relatively lower compared to Gram positive due to physicochemical differences in the outer cell membrane (Yoneyama *et al.*, 2011). The inhibitory activity was directly proportional to the production of bacteriocins, which were greatest at 24 h (Figures 3 and 4). Kusmarwati *et al.* (2013) also reported that LAB began to produce bacteriocins at the 12 h and optimally at the 24 h.

Bacteriocin-like Substance Antibacterial Activity

The bacteriocin-like substance from *Lactobacillus* sp. GMP1 and *Weisella* sp. GMP12 was partially purified by 80% precipitation of ammonium sulfate and followed by dialysis. The partially purified bacteriocin-like substance obtained was tested again against indicator bacteria to determine its antibacterial activity (Table 2).

Table 2 showed that bacteriocin-like substance produced by *Lactobacillus* sp. GMP 1 and *Weisella* sp. GMP 12 was able to inhibit the growth of Gram-positive bacteria, *Staphylococcus aureus* ATCC 6538, and Gram-negative bacteria, *Salmonella* sp. 230C and *Klebsiella* sp. CK2 ($p<0.05$). The bacteriocin activity of *Lactobacillus* sp. GMP 1 and *Weisella* sp. GMP 12 against *Staphylococcus aureus* ATCC 6538 were 5,868.19 AU and 3,693.60 AU respectively and was the highest bacteriocin activity. The lowest inhibitory activity was shown against *Salmonella* sp. 230C with activity 1,696.39 AU and 2,254.17 AU respectively. Recent research also reported that the bacteriocin produced by *Lactobacillus plantarum* was able to inhibit the

Table 2 Bacteriocin activity of *Lactobacillus* sp. GMP1 and *Weisella* sp. GMP12 against indicator bacteria

Indicator Bacteria	Bacteriocin Activity (AU)	
	Lactobacillus sp. GMP1	*Weisella* sp. GMP12
Staphylococcus aureus ATCC 6538	5,868.19[a]	3,693.60[a]
Salmonella sp. 230C	1,696.39[b]	2,254.17[b]
Klebsiella sp. CK2	0	3,165.51[ab]

*Another bacterial indicator which not listed in this table possessed 0 AU

growth of *Staphylococcus aureus* ATCC 25923 and *Salmonella* spp. (Peng *et al*., 2021; Jiang *et al*., 2016). The bacteriocin produced by *Weisella confusa* was able to inhibit the growth of *Staphylococcus aureus* (Goh & Phillip, 2015). This fact concludes that *Lactobacillus* sp. GMP 1 and *Weisella* sp. GMP 12 were potential to be used as bio-preservation.

CONCLUSION

The screening of halotolerant lactic acid bacteria successfully isolates two strains namely *Lactobacillus* sp. GMP 1 and *Weisella* sp. GMP 12. The production of bacteriocin-like substance from *Lactobacillus* sp. GMP1 and *Weisella* sp. GMP12 is optimum at 24 hours and is able to inhibit the growth of *Staphylococcus aureus* ATCC 6538, *Salmonella* sp. 230C, *Klebsiella* sp. CK2.

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