Screening of antimicrobial-producing lactic acid bacteria isolated from traditional fish fermentation against pathogenic bacteria

C Amarantinia), D Satwika, T Y Budiarso, E R Yunita, E A Laheba
Biology Department, Faculty of Biotechnology, Universitas Kristen Duta Wacana.
Jl dr Wahidin Sudirohusodo 5-25 Yogyakarta.55224.

Abstract. This study aims to obtain lactic acid bacteria (LAB) which have probiotic properties and inhibitory effects against pathogens. Peda fish fermented in 20% salt solution used as samples. Antimicrobial properties of LAB isolates against gram-positive and gram-negative pathogens (Salmonella typhi BPE 127.1.MC, Salmonella typhi BPE 122.4.CCA, Salmonella typhi NCTC 786, Salmonella typhimurium FNCC 0050, Pseudomonas putida FNCC 0071, Bacillus subtilis ATCC 6633 and Staphylococcus aureus ATCC 25923) were tested using agar well diffusion method. To exclude acid production, the cell-free culture supernatant (CFCS) solution was neutralized using 1 N NaOH before antimicrobial activity was tested. Likewise with bacteriocin tests, CFCS solutions that have been neutralized, were treated first by heating at 100ºC for 10 minutes in order to enzyme inactivation. A total of 26 out of 50 LAB isolates showed clear zones in the MRS-CaCO3 agar medium, gram positive, and negative catalase as the main characteristics of LAB. Sixteen of the 26 LAB isolates were known to be tolerant to pH 2 and 1% of bile salts. In addition, 92.3% of LAB isolates (15 of 16 LAB isolates) were homofermentative. The antimicrobial test results found that two of 26 LAB isolates (i.e. LAB Pr.3.4L and Pi.5.8 isolates) were shown to have very strong inhibitory effects against gram - positive pathogens S. aureus ATCC 25923 compared to S. typhi BPE 122.4.CCA. Thus the two strains of LAB are indicated as bacteriocin-producing probiotic strains, and need to be pursued further for identification and optimization of production.

1. Introduction
Lactic acid bacteria (LAB) isolated from traditional fermentation processes are known as antibacterial producers which are active against both gram positive and negative bacteria. This broad antibacterial spectrum causes LAB as potential natural preservative in various food products [1]. As reported elsewhere, many LAB could be recovered from traditional Indonesian fermented foods, including species from Lactobacillus, Pediococcus, Enterococcus, Weisella and Leuconostoc, as well as some fungi [2]. On that account, Indonesian fermented foods are a potential source of probiotics that benefit human health.

Studies on the antimicrobial potential of lactic acid bacteria in vitro have been carried out to assess the potential of LAB probiotics from fermented foods. The antimicrobial function of lactic acid bacteria (LAB) in fermented foods is mainly served by lactic acid and acetic acid. The production of organic acids is beneficial for nutritional quality, organoleptic properties, and shelf life characteristics.
Traditionally, lactic acid bacteria develop spontaneously during fermentation process, or in their industrial application, they are added intentionally in the food matrix as a natural preservative. Lactic acid bacteria also produce acetic acid, ethanol, aroma compounds, exopolysaccharide, and several important enzymes. In addition, antimicrobial production by probiotic LAB plays a role during in-vivo interactions in the gastrointestinal tract, thus contributing to the intestinal health. Many LAB strains also produce bacteriocins that show antibacterial activity. Some LABs are also capable of synthesizing other antimicrobial peptides that also contribute to food preservation and safety [3]. Thus, compounds such as bacteriocin and antifungal peptides produced by LAB have the potential for in depth studies as new antimicrobials that will be relevant in ensuring food quality and safety in the future. Considering this background, LAB producing bacteriocin, known as bacteriocinogenic strain, deserve further research for the improvement of food quality and its safety. It is then the purpose of this study to screened LAB strain(s) with the ability to produce bacteriocins having bacteriocin activity able to inhibit other bacteria.

2. Experimental

2.1. Samples
Twelve samples of Peda fish (Rastrelliger sp.) were purchased from local markets namely Demangan and Kranggan in Yogyakarta, Indonesia. The samples were divided into two types. The first type is dried peda fish. For this category, each sample purchased is immediately taken to the laboratory for isolation based on the established procedures. The second type was samples taken from a solution of dried peda fish soaked in 20% salt solution for 2 weeks.

2.2. Pathogenic strain
The pathogenic strains that represent gram negative bacteria used in this study were Salmonella typhi BPE 127.1.MC and Salmonella typhi BPE 122.4.CCA obtained from previous research collections [4][5]; Salmonella typhi NCTC 786 obtained from PT Biofarma; and Salmonella typhimurium FNCC 0050 and Pseudomonas putida FNCC 0071 obtained from the Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta. Whereas, for gram-positive bacteria, the indicator strains Bacillus subtilis ATCC 6633 and Staphylococcus aureus ATCC 25923 were used.

2.3. Isolation and selection of lactic acid bacteria isolates
Fifty grams of samples were cultured in 500ml Buffered Peptone Water (BPW) 0.1% and incubated at 37°C for 24 hours. Then, 25 ml of the culture was taken to be grown in 225 ml of de Man-Rogosa-Sharpe (MRS) broth medium and re-incubated at 37°C for 24 hours. Through a series of dilutions using 0.1% peptone solution, the culture was grown in an MRS-CaCO₃ agar medium using the spread plate method, and incubated at 37°C for 24–48 hours. Based on the characteristics of the LAB colonies, LAB isolates were then selected by picking creamy-white colonies with clear zones formed around it. The obtained isolates were collected as LAB candidates that are potential to produce antimicrobials. To ensure that these isolates are gram-positive bacteria as the main characteristic of LAB, the LAB isolates were observed microscopically for determining cell shape and gram properties to. All of the collections of LAB isolates were maintained routinely and stored in MRS agar medium containing 20% glycerol at -20°C.

2.4. Screening and characterization of antimicrobial-producing lactic acid bacteria.
Several tests were done to identify the antimicrobial-producing LAB strains by observing its morphology, gram staining, catalase formation, growth on MRS broth at different pH, the ability to grow on different concentrations of bile salts, and homofermentative or heterofermentative test.

**Growth of LAB strains at various pH.** To ensure the growth of LAB isolates at different pH, the MRS broth was adjusted at pH 2, pH 3, and pH 4 using 1 N HCL. The tested culture was prepared by growing it in an MRS broth for 48 hours. Cells were harvested by centrifugation at 13,500 rpm for 15 minutes. Cells were then washed twice using 100 µl Phosphate Buffer Saline (PBS) solution, after which the cell pellets were inoculated into 5 ml of MRS broth and incubated at 37 °C for 4 hours. Their growth was confirmed by plating the culture in the MRS agar [6].

**Growth of LAB isolates at different concentrations of bile salts.** To determine the growth of LAB isolates in various concentrations of bile salts, 1 ml of the test culture were grown in 5 ml MRS broth containing bile salts at concentrations of 0.3%, 0.5%, and 1%. Using the spread plate method, culture was grown in MRS agar and incubated at 37 °C for 24 hours. Growth in the MRSA medium was determined by observing the growth of colonies in the medium [6].

2.5. Determining antimicrobial activity
To determine the antimicrobial activity of the obtained LAB strains, the indicator strain was tested against the LAB strains using well-diffusion agar method.

**Cell-free culture supernatant (CFCS) preparation.** The antimicrobial LAB isolates were grown in MRS broth at 37 °C for 18 hours. The supernatant was neutralized at pH 6.5 with 1 N NaOH solution before used for antimicrobial testing. The cells were then harvested through a 10,000-rpm centrifugation process for 10 minutes. The supernatant was collected and stored in the refrigerator until it would be used [7].

**Preparation of bacteriocin-like substances.** The antimicrobial-producing LAB isolates were grown in a MRS broth at 37 °C for 18 hours. The supernatant was neutralized at pH 6.5 with 1 N NaOH solution before used for antimicrobial testing. The cells were then harvested through a 10,000-rpm centrifugation process for 10 minutes. The supernatant is heated at 100° C for 10 minutes, collected, and stored in the refrigerator until it would be used [8][9].

**Antimicrobial test.** The antimicrobial activity was tested visually using well diffusion agar method. Mueller-Hinton Agar (MHA) is used as a standard medium recommended in the guideline [10][11]. Indicator strains were grown in Brain Heart Infusion (BHI) broth at 37° C for 16–18 hours. Then, sterile cotton swabs are dipped in the standardized bacterial suspension. The excess inoculum fluid was removed by pressing a little swab on the tube wall above the liquid. The swab that contains a stained inoculum on the MHA medium was inoculated. This swab was repeated twice so that the test culture was evenly distributed on the surface of the MHA medium. A 8 mm-diameter well was cut into a compacted agar using a sterile blue tip and filled with 150µl of supernatant culture or a solution containing a bacteriocin-like substance. The plates were incubated at 37° C for 24 hours, after which the zone of the inhibition was measured [7][9][12].

3. Results and Discussion

3.1. Isolation of antimicrobial-producing lactic acid bacteria
As seen from the colonies that grew on the MRS-CaCO3 medium, it was found that the colonies of lactic acid bacteria could be single or group colonies with neat edges, smooth surfaces, and transparent or creamy yellow colors (Figure 1). The diameter of the colony varies from 1 mm to 5 mm. From each petri dish, 5 colonies were taken to be tested further and there was certainly no contamination. Fifty candidate isolates of lactic acid bacteria from peda fish that showed clear zones were selected from MRS-CaCO3 medium. Among the 50 isolates, 26 isolates showed the characteristics of gram-positive
and were negative catalase in nature. Based on the results of gram staining, under the microscope purple cells appeared in the form of basil without spores. These data follow the characteristics of lactic acid bacteria found in various fermented foods [6][8][13][14][15]. Thus, based on these characteristics, 26 isolates of lactic acid bacteria were obtained from peda fish and were potential to produce antimicrobial compounds (Table 1).

![Figure 1. Colonies of lactic acid bacteria that grow on the MRS-CaCO₃ medium.](image)

From microscopic observations, all of these isolates were found to be in the form of bacilli. Interestingly, most of the isolates obtained were known to be resistant to bile salts; 19 of the 26 isolates selected were even known to grow in 1% of bile salts. In addition, 16 of the 26 isolates were able to grow at pH = 2 and almost all (92.3%) were homofermentative (Table 1). These characteristics are important for assessing their potential as probiotics and as a producer of antimicrobial compounds.

To ensure that the lactic acid bacteria isolates obtained benefit human health, the bacteria must be able to reach the digestive tract alive. Although they only temporarily colonize the digestive tract, their ability to affect the mucous layer and the extracellular matrix components in the digestive tract are the main functions of LAB that give healthy effects, in addition to their role in terms of antibacterial activity and immunomudulators. In this case, lactic acid bacteria that act as probiotics play an important role in protecting host defenses against pathogenic infections in the intestinal lumen. With the fast formation of microbial communities in the digestive tract, pH will reduce, and adhesion competition will occur against pathogenic bacteria, preventing pathogenic from colonizing. In addition to competing in terms of adhesion, the production of antibacterial compounds by lactic acid bacteria such as acetic acid and bacteriocin also prevents the growth of pathogenic bacteria [6].
Table 1. Characteristics of morphology and physiology of lactic acid bacteria isolates as a result of screening from peda fish samples.

| Isolate code | Gram stain | Cell shape | Ability to grow in acidic conditions | Ability to grow in bile salts (%) | Homo-/Hetero-fermentative |
|--------------|------------|------------|--------------------------------------|-----------------------------------|---------------------------|
| Solution of dried peda fish: |            |            |                                      |                                   |                           |
| Pr.3.1 L     | +          | basil      | -                                    | +                                 | +                         | Homofermentative         |
| Pr.4.3 L     | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |
| Pr.4.2.      | +          | basil      | -                                    | +                                 | -                         | Homofermentative         |
| Pr.4.3.      | +          | basil      | -                                    | +                                 | -                         | Homofermentative         |
| Pr.4.4.      | +          | basil      | +                                    | +                                 | -                         | Homofermentative         |
| Pr.4.6.      | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |
| Pr.4.8.      | +          | basil      | -                                    | +                                 | -                         | Homofermentative         |
| Peda fish:   |            |            |                                      |                                   |                           |
| Pi.5.1.      | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |
| Pi.5.2.      | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |
| Pi.5.3.      | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |
| Pi.5.4.      | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |
| Pi.5.5.      | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |
| Pi.5.6.      | +          | basil      | +                                    | +                                 | +                         | Heterofermentative       |
| Pi.5.7.      | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |
| Pi.5.8.      | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |
| Pi.5.9.      | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |
| Pi.5.10.     | +          | basil      | -                                    | +                                 | +                         | Homofermentative         |
| Pi.6.1.      | +          | basil      | -                                    | +                                 | +                         | Homofermentative         |
| Pi.6.2.      | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |
| Pi.6.3.      | +          | basil      | -                                    | -                                 | +                         | Homofermentative         |
| Pi.6.4.      | +          | basil      | -                                    | +                                 | +                         | Homofermentative         |
| Pi.6.5.      | +          | basil      | -                                    | +                                 | +                         | Homofermentative         |
| Pi.6.6.      | +          | basil      | -                                    | +                                 | +                         | Homofermentative         |
| Pi.6.8.      | +          | basil      | -                                    | -                                 | +                         | Homofermentative         |
| Pi.6.9       | +          | basil      | +                                    | -                                 | -                         | Homofermentative         |
| Pi.6.10      | +          | basil      | +                                    | +                                 | +                         | Homofermentative         |

3.2. Screening for antimicrobial-producing lactic acid bacteria

At this stage, tests were performed to measure the ability of lactic acid bacteria in inhibiting pathogenic bacteria. These included testing the antimicrobial activity using the well diffusion assay method. To exclude the effects of acid production, antimicrobial activity was tested after the supernatant was neutralized with NAOH 1 N [16]. A total of 26 BAL isolates obtained from the isolation stage were tested for their ability as antimicrobial producers (Table 2). The results show that not all candidates for lactic acid bacteria have inhibitory effects against gram negative bacteria and against gram-positive bacteria at the same time. Overall, it was found that 21 of the 26 LAB isolates showed inhibitory effects on both strains of gram negative bacteria and gram-positive bacteria. Five LAB isolates (Pr.4.8, Pi.5.8, Pi.5.9, Pi.6.2, Pi.6.4) showed inhibitory effects only on gram-positive pathogens. *Bacillus subtilis* ATCC 6633 was found to be the most sensitive strain on the CFCS inhibitory effect of LAB isolates. The biggest inhibitory effect was seen in LAB Pr.4.3.L isolates.
against *Bacillus subtilis* ATCC 6633 and LAB Pi.5.8 isolates against *Staphylococcus aureus* ATCC 25923 (Figure 2). Both LAB isolates (Pr.4.3.L and Pi.5.8) also showed inhibitory effects on gram negative pathogens (Figure 3).

Figure 2. The inhibitory effect of antimicrobial compounds produced from LAB Pi.5.8 isolates against *Staphylococcus aureus* ATCC 25923 and LAB Pr.4.3.L isolates on *Bacillus subtilis* ATCC 6633.

![Figure 2](image)

Figure 3. The inhibitory effect of antimicrobial compounds produced from LAB Pr.4.3.L and LAB Pi.5.8 isolates against pathogen gram-negative bacteria.

The results of screening as shown in Table 2, Figure 1, and Figure 2 indicate that only 2 isolates were proved to be able to inhibit gram negative and gram positive pathogenic bacteria, namely LAB Pr.4.3.L and LAB Pi.5.8. Furthermore, these 2 isolates were selected for testing to detect bacteriocin activity against gram negative and gram positive pathogens (Table 3).

Table 3 shows that for the group of gram negative pathogens, both LAB Pr.4.3.L and Pi.5.8 isolates were able to inhibit all gram negative indicator strains. The biggest inhibition zone (19.5 ± 0.3 cm) was detected in *Salmonella typhi* BPE 122.4.CCA. The two LAB isolates also showed inhibition against *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633. Cytoplasm membranes of gram negative bacteria were easily damaged by organic acids, making gram negative bacteria sensitive to organic acids [14]. In this study, the inhibitory effect of acid production had been
neutralized with NaOH solution so that the pH of the CFCS solution becomes neutral. Therefore, it can be concluded that CFCS solutions from LAB Pr.4.3L and Pi.5.8 isolates contain antimicrobial compounds.

Table 2. The inhibitory effects of antimicrobial compounds produced by LAB isolates against indicator bacteria.

| Isolate code | Antimicrobial activity of LAB isolates against indicator bacteria |
|--------------|--------------------------------------------------------------------|
|              | Salmonella typhi NCTC 786                                           |
|              | Salmonella typhi BPE 127.1MC                                        |
|              | Salmonella typhi BPE 122.4CCA                                      |
|              | Salmonella typhimurium FNCC 0050                                    |
|              | Pseudomonas putida FNCC 0071                                        |
|              | Bacillus subtilis ATCC 6633                                         |
|              | Staphylococcus aureus ATCC 25923                                    |
| Pr.4.3 L     | +                                                                  |
| Pr.4.3.      | -                                                                  |
| Pr.4.5.      | -                                                                  |
| Pr.4.8.      | -                                                                  |
| Pi.5.3.      | +                                                                  |
| Pi.5.4.      | -                                                                  |
| Pi.5.5.      | +                                                                  |
| Pi.5.6.      | -                                                                  |
| Pi.5.8       | -                                                                  |
| Pi.5.9       | -                                                                  |
| Pi.5.10      | -                                                                  |
| Pi.6.1       | -                                                                  |
| Pi.6.2       | -                                                                  |
| Pi.6.3       | -                                                                  |
| Pi.6.4       | -                                                                  |
| Pi.6.5       | +                                                                  |
| Pi.6.10      | +                                                                  |

Note: + (presence) - (absence)

Table 3. Diameter of inhibitory zone (mm) of LAB strains Pr.4.3L and Pi.5.8, and control (without LAB) against pathogenic bacteria based on antimicrobial activity test and bacteriocin activity test with well diameter of 8 mm.

| Pathogenic Strains     | Clear zone (mm) based on antimicrobial activity test | Clear zone (mm) based on bacteriocin-like substance activity test | Clear zone (mm) of control (without LAB) |
|------------------------|------------------------------------------------------|-------------------------------------------------------------------|------------------------------------------|
|                        | Pr.4.3L  | Pi.5.8 | Pr.4.3L  | Pi.5.8  | Pr.4.3L  | Pi.5.8        |
| Salmonella typhi NCTC 786 | 13.3 ± 0.8 | 14.1 ± 0.2 | 13.6 ± 0.4 | 14.1 ± 0.2 | 0 ± 0.0 | 0 ± 0.0 |
| Salmonella typhi BPE 127.1MC | 14.0 ± 0.0 | 13.0 ± 0.7 | 14.8 ± 0.8 | 14.0 ± 1.3 | 0 ± 0.0 | 0 ± 0.0 |
| Salmonella typhi BPE 122.4CCA | **19.5 ± 0.3** | **19.0 ± 0.7** | **17.3 ± 0.9** | **17.7 ± 1.1** | 0 ± 0.0 | 0 ± 0.0 |
| Salmonella typhimurium FNCC 0050 | 13.7 ± 0.3 | 13.3 ± 1.1 | 14.8 ± 0.8 | 13.6 ± 0.4 | 0 ± 0.0 | 0 ± 0.0 |
| Pseudomonas putida FNCC 0071 | 14.7 ± 0.9 | 14.3 ± 0.4 | 15.3 ± 0.8 | 15.7 ± 1.1 | 0 ± 0.0 | 0 ± 0.0 |
| Bacillus subtilis        | 14.7 ± 0.4 | 13.0 ± 0.3 | 14.3 ± 1.7 | 13.3 ± 0.9 | 0 ± 0.0 | 0 ± 0.0 |
Bacteriocin is a protein compound synthesized by bacteria. It can be a peptide that gives a bactericidal effect. This antimicrobial peptide is acid resistant, heat-stable, but sensitive to the action of protease enzymes and inactivated by digestive enzymes [14][17]. Therefore, to observe the effect of heat treatment on the CFC S inhibitory effect on the indicator strain, the compounds were heated at a temperature of 100°C for 10 minutes for inactivating the enzyme. Overall, it was found that the bactericidal compounds contained in CFCS were resistant to heat, as evidenced by the inhibitory effect of bacteriocin compounds on gram positive and gram negative pathogens (Table 3).

Bacteriocin has especially caught the attention of microbiologists for its ability to control pathogenic bacteria. Bacteriocin from Lactobacillus fermentum was known to have antimicrobial potential against the methicillin-resistant Staphylococcus aureus. Likewise, it was also found that bacteriocin from L. acidophilus inhibits Salmonella and Escherichia, although it is not unusual to find that gram negative bacteria are sensitive to bacteriocins. Another important finding was the inhibition zone produced by L. fermentum that was greater than L. acidophilus [18]. This finding supports the conclusion that bacteriocin antimicrobial activity has a broad spectrum. Table 3 shows that the strongest inhibitory effect was known against Staphylococcus aureus ATCC 25923 compared to Salmonella typhi BPE 122.4CCA.

The Lactobacillus group is known for its use as probiotics and food preservatives. They can be used as food preservative due to the production of inhibiting compounds such as organic acids, hydrogen peroxide, diacetyl, and bacteriocin. Bakteriocin is a cationic protein produced by ribosomes that block other bacteria that live in the same ecological niche. This ability to inhibit other bacteria living in the same ecological niche generally causes bacteriocin to have a narrow inhibitive spectrum activity or, in other words, only inhibit closely related species [19]. However, the terminology still causes debate and by far has not been precisely defined. It has also been reported that bacteriocins that have broad spectrum have been found to have inhibitory effects on gram positive and gram negative inhibitory, and give antifungal activity [20][21]. Bacteriocin-producing strains isolated from various food fermentation processes such as yogurt, milk, sour milk, and milk cream are proved to have a very strong inhibition zone against Bacillus subtilis [13]. LAB strains isolated from meat are known to have inhibitory effects on gram-positive bacteria such as Staphylococcus aureus, Listeria monocytogenes, Clostridium perfringens, C. botulinum, Enterococcus faecalis, and Bacillus, in addition to LAB species. The inhibiting mechanism occurs by forming pores on the cytoplasmic membrane [14][22]. This opinion affirms the findings in this study where the LAB strain obtained are found to have a stronger inhibitory effect on gram-positive pathogens than that on gram-negative pathogens and is indicated as bacteriocin because of its resistance to pH and heat.

4. Conclusion
All isolates obtained from the screening in MRS-CaCO3 medium were lactic acid bacteria (LAB) based on its characteristic as gram-positive, did not have catalase activity and did not form spores, with basil or cocci form. Two out of twenty six (26) isolates have the potential as antimicrobial producers, namely LAB Pr.4.3L and LAB Pi.5.8 isolates. Both isolates were shown to have broad-spectrum inhibitory effects, with stronger inhibitory effect on gram-positive pathogenic strains. The CFCS solution used for the antimicrobial test has been neutralized, therefore the inhibitory effect on pathogenic indicator strains is caused by antimicrobial compounds produced by the LAB. The antimicrobial compound was stable after heat treatment, giving the strongest inhibitory effect against Staphylococcus aureus ATCC 25923 compared to Salmonella typhi BPE 122.4CCA. Based on this
finding, these antimicrobial compounds are assumed to be bacteriocin. Further research is still need to be done to optimize bacteriocin production and to identify it.

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