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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan  
Mob: +92 300 3008585, Fax: +92 41 8815544  
E-mail: [editorpjn@gmail.com](mailto:editorpjn@gmail.com)



## Research Article

# Isolation and Identification of Lactic Acid Bacteria from Okara and Evaluation of Their Potential as Candidate Probiotics

<sup>1</sup>Salam N. Aritonang, <sup>1</sup>Elly Roza, <sup>2</sup>Evy Rossi, <sup>1</sup>Endang Purwati and <sup>1</sup>Husmaini

<sup>1</sup>Department of Animal Production, Faculty of Animal Science, Universitas Andalas, Padang, Indonesia

<sup>2</sup>Department of Agricultural Technology, Faculty of Agriculture, Universitas Riau, Indonesia

## Abstract

**Background:** Okara is an industrial waste product resulting from the manufacture of soy milk or tofu. This waste is a pulp consisting of the insoluble parts of the soybean that remain after pureed soybeans are filtered during the production of soy milk and tofu. Okara is not widely used and is dumped into the environment, causing pollution. However, this waste contains nutrients and may be a good medium for the growth and development of microorganisms such as Lactic Acid Bacteria (LAB). **Objective:** The purpose of this study was to isolate and identify probiotic LAB from okara and determine their antimicrobial activity against pathogenic bacteria (*Escherichia coli* 0157, *Staphylococcus aureus* ATCC25923 and *Listeria monocytogenes* EP01). **Methodology:** Isolation and purification of LAB were carried out on MRS agar supplemented with 0.2% CaCO<sub>3</sub>. Isolates were identified by morphological and physiological tests. Antibacterial activity was tested using the well diffusion method and the obtained data were analyzed descriptively. Twenty-four isolates were grown on MRS agar. **Results:** Sixteen isolates that exhibited growth at the clear zone were identified as gram positive, catalase-negative homofermentative rod and coccus cells. The results showed that the LAB isolated from okara had the ability to inhibit pathogenic bacteria, causing various clear zone diameters. The survival rates of these isolates under acidic conditions and their tolerance to 0.5% bile salt were varied. Based on the antimicrobial activity and probiotic properties of the LAB isolated from okara, these isolates belong to the species *Lactobacillus spentosus* and *Lactobacillus plantarum*. **Conclusion:** The results showed that the LAB isolated from okara belonged to the species *Lactobacillus pentosus* and *Lactobacillus plantarum*. Both species exhibited properties attributed to probiotics.

**Key words:** Okara, lactic acid bacteria, antibacterial activity, pathogenic bacteria, bile salt

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**Corresponding Author:** Salam N. Aritonang, Department of Animal Production, Faculty of Animal Science, Universitas Andalas, Padang, Indonesia

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Soy milk is a popular Indonesian beverage made by grinding soy beans that have been soaked in water, filtered, cooked until boiling and then supplemented with sugar and essence to enhance the flavor. The process of making soy milk produces solid waste such as soybean pulp (okara)<sup>1</sup> that is rarely consumed by humans and therefore has low economic value<sup>2</sup>. Okara is made up of 16.1% crude proteins, 52.6% carbohydrates and 2.2% crude lipids as well as vitamin B and the amino acids lysine and methionine<sup>3</sup>. The high nutrient content of okara supports the growth of microorganisms such as Lactic Acid Bacteria (LAB)<sup>2</sup>. Okara also contains isoflavones (22%) is a potential source of antioxidants, has prebiotic characteristics and can lower cholesterol and blood sugar levels<sup>4</sup>.

The LAB have been isolated and identified as probiotics from many sources in nature, including Turkish pastirma, a dry-cured meat product<sup>5</sup>, the heaps and boxes used in the cocoa fermentation process<sup>6</sup>, human milk<sup>7</sup>, Brazilian kefir grains<sup>8</sup>, forage paddy rice silage<sup>9</sup> and some Nigerian cereal-based-fermented foods<sup>10</sup>. For LAB to be considered probiotics, they must be proven safe for the consumer, exhibit tolerance to gastric acid and bile<sup>11</sup> and display antibacterial activity<sup>12</sup>.

Lactic acid bacteria are beneficial microorganisms used as starter cultures for the processing of functional food<sup>13,14</sup>. These LAB are not toxic to the host and have the ability to kill pathogenic bacteria<sup>15</sup>. Researchers have developed LAB that can be used for food fermentation, for probiotic effects and for food preservation<sup>16</sup>. The primary metabolic product of LAB is lactic acid<sup>17</sup>; however, LAB also produce secondary metabolites that can inhibit the growth of spoilage and pathogenic microbes because these metabolites function as antimicrobial compounds<sup>18</sup>. One of the antimicrobial compounds produced by LAB is bacteriocin. This compound is effective, safe and able to prevent or inhibit the growth of pathogenic bacteria compared to other antimicrobial compounds<sup>19</sup>.

The antimicrobial activity of LAB is a subject of intense study. Ammor *et al.*<sup>20</sup> studied the antibacterial activity of LAB against spoilage and pathogenic bacteria isolated from the same small-scale meat facility. Arques *et al.*<sup>21</sup> evaluated the antimicrobial activity of LAB in dairy products and the gut. Rosa *et al.*<sup>22</sup> characterized the antimicrobial activity of LAB isolated from cassava processing waste water. Previously, Siroli *et al.*<sup>23</sup> found that combining selected strains with natural antimicrobials further increased in the shelf-life of these products without detrimental effects on their

organoleptic qualities. Additionally, Li<sup>24</sup> evaluated the antimicrobial activity of lactic acid bacteria isolated from fermented taro skins against the pathogenic bacteria *Salmonella typhimurium* and *Listeria monocytogenes*.

Antimicrobial compounds produced by LAB provide several advantages. These compounds can extend food storage time, can suppress the growth of undesirable microorganisms and are safe for humans to consume because they are not toxic. The activity of lactic acid bacteria isolated from fermented taro skins against *Salmonella typhimurium* and *Listeria monocytogenes* has been demonstrated<sup>24</sup>. Developing a natural food preservative is a focus of current research. This natural preservative can replace the synthetic additives that are currently used often as food preservatives<sup>16, 25</sup>.

Developing LAB as food preservatives is of great interest as they can be an alternative to synthetic (non-food) food additives, which are often misused. The purpose of this study was to isolate and identify the LAB of okara and evaluate their antimicrobial activity against pathogenic bacteria such as *Escherichia coli* 0157, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* EP01.

## MATERIALS AND METHODS

**Isolation of lactic acid bacteria from okara:** Okara, the material used in this study, was taken from three locations of soybean milk production in Taman Karya District, Pekanbaru, Indonesia. Each sample (1 mL) of spontaneously fermented (48 h) okara was homogenized with 9 mL of 0.85% sterile NaCl. The mixture was then serially diluted ( $10^{-1}$  to  $10^{-5}$ ) for bacterial enumeration. One milliliter samples of each dilution were spread directly on the surface of MRS agar. To differentiate acid-producing bacteria from other bacteria, 1% CaCO<sub>3</sub> was added to the MRS agar. Plates were incubated at 37°C for 48 h and colonies exhibiting a surrounding clear zone were collected. The colonies that formed a clear zone on MRS agar were randomly selected as acid-producing bacteria. The selected colonies were purified using the streak plate technique. Lactic Acid Bacteria (LAB) isolation was carried out according to the method described by Chen *et al.*<sup>26</sup>. The purified LAB isolates were stored at -20°C in 15% glycerol and 85% MRS broth.

**Morphological and physiological identification of lactic acid bacteria:** The selected LAB isolates were examined using the following methods as described by Felten *et al.*<sup>27</sup>. (a) Gram staining was performed, where Gram-positive bacteria retain crystal violet and safranin in the cell membrane

and Gram-negative bacteria are unable to retain the crystal violet stain. (b) Cell shape was also determined using Gram staining followed by microscopic observation. Holzappel and Wood<sup>28</sup>, previously determined that LAB consist of two families, Lactobacillaceae and Streptococcaceae. The genera *Lactobacillus* and *Bifidobacterium* are found within the rod-shaped Lactobacillaceae family and the round-shaped Streptococcaceae cells include the genera *Streptococcus*, *Leuconostoc* and *Pediococcus*. (c) Catalase production was observed by placing one dose of the bacterial suspension on a glass object, adding 1-2 drops of 3% hydrogen peroxide and then observing the production of a bubble or gas. Roberts *et al.*<sup>29</sup> suggested that a positive reaction in the catalase test is indicated by the appearance of bubbles (gas) caused by the enzymatic activity of catalase, which is an enzyme known to break down H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O and CO<sub>2</sub>. If this reaction does not generate gas, it can be concluded that the bacteria do not have the catalase enzyme and the bacteria are then categorized as catalase negative. (d) Gas production by LAB was observed by inoculating 1 mL suspension of bacterial cells in a test tube containing MRS broth (Merck) and an inverted Durham tube. After incubation at 37°C for 48 h, production of gas in the Durham tube indicates that the bacteria ferment glucose to CO<sub>2</sub> and acid. Salminen *et al.*<sup>30</sup> stated that homofermentative LAB only produce lactic acid through the glycolysis pathway, whereas heterofermentative LAB produce not only lactic acid but also acetic acid, ethanol and carbon dioxide (CO<sub>2</sub>). (e) Growth observation at various temperatures was performed to determine the optimal temperature conditions for LAB isolates. One milliliter of LAB cell suspension was inoculated into a test tube containing MRS broth medium and incubated at 10, 60 and 70°C for 48 h. The sample was then poured into MRS agar and incubated at 37°C for 48 h before colony growth was observed.

### Potential evaluation of lab isolates as probiotics

#### Antimicrobial activity

**Bacterial strains and media:** Sixteen LAB isolates were preserved in MRS broth (Merck) with 20% (v/v) glycerol at 20°C. All isolates were propagated in MRS broth and incubated at 37°C for 24 h to obtain active cultures. The pathogenic indicator strains, *Escherichia coli* 0157, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* EP01 were inoculated into a test tube containing 5 mL nutrient broth (Merck) and incubated at 37°C for 24 h to obtain active cultures. Active cultures were marked by media turbidity. The pathogenic bacteria

were obtained from the collection of the Animal Food Technology Laboratory, Universitas Andalas, Padang, Indonesia.

**Preparation of LAB cell-free supernatant:** Each LAB isolate from okara was inoculated into MRS broth and then incubated at 37°C for 24 h. Afterwards, these isolates were centrifuged at a speed of 8,000 rpm at 4°C for 10 min to separate the cells and supernatant. The cell-free supernatant was then harvested by centrifugation.

#### Antimicrobial activity of lab against pathogen bacteria:

The antimicrobial activity of LAB was determined by the well diffusion method<sup>31</sup>. Using a sterile pipette, 0.1 mL of indicator bacteria culture was placed on Nutrient Agar (NA) media<sup>32</sup> and swabbed using a hockey stick. To create 5 mm wells, the agar was drilled using the bottom of a sterile blue pipette tip and the bottom of the wellbore was covered with sterile agar. Furthermore, 50 µL of each LAB isolate (an overnight culture of each LAB isolate grown in MRS broth at 37°C that had been standardized to an optical density of 0.5 at 600 nm) was added to the previously made wells and incubated at 37°C for 24 h. After incubation, the clear zone diameter was measured three times at different positions and averaged. Isolates that displayed antimicrobial activity (a clear zone larger than 5 mm) were selected for survival assays, including survival under conditions of pH 3 for 3 h and of 0.3% bile salt (w/v) for 5 h. Total viable colonies were enumerated for 0 and 5 h using the pour plate technique.

#### Assay of the antimicrobial activity of the lab cell-free supernatant against various bacteria:

This test was previously described by Bromberg *et al.*<sup>31</sup> and used 50 mL of the supernatant in previously prepared wells. Each petri dish and its contents were placed in the refrigerator for 2 h to allow the agar to adsorb the supernatant. Then, the plates were incubated at 37°C for 24 h. The formation of a clear zone demonstrates that the supernatant could prevent the growth of bacteria. The diameter of the clear zone was measured using calipers three times at different positions and averaged.

**Survival rate under low pH and bile salt conditions:** Lactic acid bacteria that exhibited a clear zone diameter of more than 6 mm against pathogenic bacteria were used for survival tests. Three samples of each isolate were assayed as replicates. The initial population of the isolate was

determined. The responses of LAB isolates to pH 3, the pH of the stomach was used to evaluate their survival rates using a modified method described by Oluwajoba *et al.*<sup>33</sup>. The MRS broth solutions at pH 6.5 (control), 2.5 and 3 were inoculated with a 1% (v/v) LAB isolate and then incubated at 37°C for 1.5 h. The surviving LAB were plated onto MRS agar. The number of CFU mL<sup>-1</sup> was calculated and compared to the CFU mL<sup>-1</sup> at time 0.

The effect of bile salt on the growth of LAB isolates was evaluated by inoculating 1 mL of LAB isolate into MRS broth containing 0.3% bile salts (Sigma). After incubation at 37°C for 5 h, the surviving LAB were counted by plating onto MRS agar and incubating for 24 h at 37°C. The number of CFU mL<sup>-1</sup> was calculated and compared to the CFU mL<sup>-1</sup> at time 0.

**Initial identification of lactic acid bacteria strains using an api 50 CHL kit:**

The selected isolates were identified using an API 50 CHL Kit (bioMerieux, France), which evaluates LAB based on their carbohydrate fermentation activity. During anaerobic incubation at 30°C, LAB fermented the 49 carbohydrates on the API 50 CHL strip, which produced a decrease in pH that was detected by the change in the color as an indicator. The results of biochemical tests and carbohydrate fermentation were determined after 24 and 48 h of incubation. All of the results were confirmed by observing the biochemical profile using API web™ identification software to identify the strain.

**Statistical analysis:** All experiments were carried out in quadruplicate. Statistical analysis was carried out using SPSS Ver. 15 for Windows. Antibacterial activities and survival

rates under low pH and bile salt exposure were analyzed for statistical significance using one way analysis of variance (ANOVA). Duncan’s multiple range test was used to determine significant (p<0.05) differences among the mean values.

**RESULTS AND DISCUSSION**

**Isolation and identification of lactic acid bacteria:**

Isolation of LAB from okara in MRS agar medium supplemented with 0.2% CaCO<sub>3</sub> yielded 24 isolates. Sixteen isolates exhibited a clear zone in the medium, while the remaining eight isolates did not produce a clear zone. The CaCO<sub>3</sub> served as a buffer and was used for the initial selection of lactic acid-producing bacteria. Seeley *et al.*<sup>34</sup> suggested that lactic acid production would cause the formation of a clear zone around the colony and it was used as an early marker of LAB colony formation. Candidate LAB can be found in okara due to the retained nutritional content of the dregs. According to Lu *et al.*<sup>35</sup> okara still retains nutritional components such as protein, fat, dietary fiber, minerals, monosaccharides and oligosaccharides. This nutrition allows the growth of microorganisms such as LAB.

The genera of the candidate probiotic LAB isolated from okara were identified by tests such as Gram staining, cell morphology, gas production and catalase production. The identification results are shown in Table 1.

Table 1 indicates that all isolates have either round (cocci) or rod (*Bacillus*) cell shapes. Holzapfel dan Wood<sup>36</sup> stated that based on the shape of LAB cells, they consist of two families: (a) Lactobacillaceae, which are rod shaped and consist of the genera *Lactobacillus* and *Bifidobacterium* and (b) Streptococcaceae, which are round and consist of the

Table 1: Morphological, biochemical and physiological profiles of LAB isolates

Isolate code	Gram stain	Cell shape	Catalase test	Gas production	Growth at		
					15°C	60°C	70°C
A.11.3	+	Coccus	Negative	Negative	+	+	+
A.11.4	+	Coccus	Negative	Negative	+	++	+
A.12.4	+	Coccus	Negative	Negative	+	+	+
A.13.1	+	Bacillus	Negative	Negative	+	++	+
A.13.2	+	Bacillus	Negative	Negative	+	++	+
A.13.3	+	Bacillus	Negative	Negative	++	+	-
A.13.4	+	Bacillus	Negative	Negative	+	+	-
A.21.1	+	Bacillus	Negative	Negative	+	++	++
A.21.2	+	Bacillus	Negative	Negative	+	++	++
A.21.3	+	Bacillus	Negative	Negative	+	+	+
A.22.1	+	Coccus	Negative	Negative	+	+	-
A.22.2	+	Coccus	Negative	Negative	+	+	+
A.22.2	+	Coccus	Negative	Negative	+	++	+
A.22.4	+	Bacillus	Negative	Negative	+	++	++
A.23.2	+	Bacillus	Negative	Negative	+	+	+
A.23.4	+	Bacillus	Negative	Negative	+	++	++

+: <10×10<sup>-5</sup> CFU mL<sup>-1</sup>, ++: >10×10<sup>-5</sup> CFU mL<sup>-1</sup>

Table 2: LAB inhibition zone diameter against pathogenic bacterial isolate LAB

	Diameter of inhibition zone (mm)		
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Listeria monocytogenes</i>
A.11.3	6.25 <sup>abc</sup>	5.06 <sup>a</sup>	7.90 <sup>abcd</sup>
A.11.4	5.68 <sup>ab</sup>	5.13 <sup>a</sup>	8.49 <sup>de</sup>
A.12.4	7.58 <sup>cde</sup>	7.15 <sup>defg</sup>	8.41 <sup>de</sup>
A.13.1	7.27 <sup>cde</sup>	6.43 <sup>bcde</sup>	9.44 <sup>e</sup>
A.13.2	9.28 <sup>fg</sup>	8.00 <sup>gh</sup>	7.42 <sup>abc</sup>
A.13.3	6.34 <sup>bc</sup>	5.49 <sup>abcd</sup>	8.45 <sup>bcd</sup>
A.13.4	7.14 <sup>cd</sup>	7.02 <sup>cdef</sup>	7.20 <sup>a</sup>
A.21.1	7.32 <sup>cde</sup>	6.93 <sup>cdef</sup>	8.15 <sup>abcd</sup>
A.21.2	6.54 <sup>bc</sup>	5.37 <sup>ab</sup>	8.37 <sup>cd</sup>
A.21.3	8.74 <sup>ef</sup>	7.44 <sup>efg</sup>	8.35 <sup>abcd</sup>
A.22.1	8.57 <sup>ef</sup>	7.90 <sup>fgh</sup>	12.85 <sup>f</sup>
A.22.2	6.73 <sup>bc</sup>	6.26 <sup>abcde</sup>	8.42 <sup>cd</sup>
A.22.3	9.90 <sup>g</sup>	6.43 <sup>bcde</sup>	7.31 <sup>ab</sup>
A.22.4	8.48 <sup>def</sup>	5.77 <sup>abc</sup>	14.49 <sup>g</sup>
A.23.2	7.12 <sup>cd</sup>	8.33 <sup>gh</sup>	13.56 <sup>g</sup>
A.23.4	9.11 <sup>g</sup>	9.10 <sup>h</sup>	8.22 <sup>abcd</sup>

Values in the same columns followed by different letters (a-h) are significantly different ( $p < 0.05$ )

genera *Streptococcus*, *Leuconostoc* and *Pediococcus*. None of the isolates demonstrated catalase activity, indicating that all observed isolates did not produce the enzyme catalase. Roberts and Greenwood<sup>37</sup> stated that positive reactions in catalase test are indicated by the appearance of bubbles (gas) due to the activity of the enzyme catalase, which breaks down  $H_2O_2$  into  $H_2O$  and  $CO_2$ . Taken together, these results indicate that all the isolates were LAB.

The ability of the LAB isolates to produce gas from glucose was then assayed to determine the types of fermentation that the LAB isolates could perform. The results in Table 1 show that all the LAB isolates are homofermentative, i.e., glycolysis only produced lactic acid<sup>38</sup>. The LAB demonstrating homofermentative include *Streptococcus*, *Pediococcus* and *Lactobacillus*, while some LAB are known to be heterofermentative, namely, *Leuconostoc* and some species of *Lactobacillus*.

Further identification of LAB isolates was performed through growth tests at 15, 60 and 70°C for 48 h. All LAB isolates demonstrated growth at 15 and 60°C, whereas only eight LAB isolates showed growth at 70°C. Bacteria that can grow at 60°C are defined as thermophilic bacteria. Garbutt<sup>39</sup> stated that thermophilic bacteria can grow at temperatures up to 70°C.

**Antibacterial activity of lactic acid bacteria:** Antibacterial compounds produced by microorganisms can interfere with the growth and metabolism of other microorganisms. Antibacterial-producing LAB have the potential to be used as probiotics. Probiotics are live microorganisms that when consumed, can provide health benefits. A probiotic

microorganism is capable of producing antimicrobial compounds that may inhibit the growth of pathogenic microbes. Allen *et al.*<sup>40</sup> stated that the main requirement for strains to be used as probiotic agents are resistance to acid and bile and the production of antimicrobial compounds. The presence of antibacterial compounds produced by LAB can be identified by inhibition of the growth of pathogenic bacteria.

The LAB isolated from okara demonstrate various abilities to inhibit the growth of pathogenic bacteria such as *Escherichia coli* 0157, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* EP01; this inhibitory ability is characterized by the formation of a clear zone around the wells. Table 2 shows that the average antimicrobial activity of LAB isolates against *Listeria monocytogenes* EP01 was higher than those of *Escherichia coli* 0157 and *Staphylococcus aureus* ATCC 25923. The highest antimicrobial activity of LAB was against *Listeria monocytogenes* EP01 as demonstrated by the formation of a greater clear zone around the wells that were filled with LAB isolate cells from okara. However, the antibacterial activity of LAB isolates on *Escherichia coli* was similar to their antimicrobial activity against *Staphylococcus aureus* ATCC 25923. The average inhibition zone diameter of LAB isolates against *Escherichia coli* 0157 ranged from 5.68-9.90 mm, *Staphylococcus aureus* ATCC 25923, from 5.06-9.10 mm and *Listeria monocytogenes* EP01, from 7.31-12.85 mm (Table 2). Most antimicrobial activities were in the range from 5.06-9.90 mm, indicating moderate antimicrobial activity; only a few isolates have demonstrated high antimicrobial activity<sup>41</sup>.

Table 3: Diameter of the inhibition zone of lactic acid bacteria cell-free supernatant against bacterial pathogens

LAB cell-free supernatant	Diameter of the inhibition zone of LAB cell-free supernatant (mm)		
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Listeria monocytogenes</i>
A.11.3	6.30 <sup>a</sup>	4.94 <sup>b</sup>	7.97 <sup>d</sup>
A.11.4	2.40 <sup>a</sup>	7.93 <sup>f</sup>	7.46 <sup>de</sup>
A.12.4	7.77 <sup>figh</sup>	7.87 <sup>f</sup>	7.53 <sup>de</sup>
A.13.1	6.79 <sup>def</sup>	6.80 <sup>de</sup>	7.63 <sup>de</sup>
A.13.2	5.00 <sup>bc</sup>	3.43 <sup>a</sup>	11.74 <sup>e</sup>
A.13.3	6.37 <sup>bcde</sup>	9.65 <sup>g</sup>	5.60 <sup>abc</sup>
A.13.4	6.37 <sup>bcde</sup>	5.83 <sup>c</sup>	6.03 <sup>bc</sup>
A.21.1	5.64 <sup>bcd</sup>	9.91 <sup>g</sup>	12.26 <sup>e</sup>
A.21.2	7.15 <sup>ef</sup>	7.46 <sup>ef</sup>	6.59 <sup>cd</sup>
A.21.3	5.39 <sup>bc</sup>	7.62 <sup>ef</sup>	6.16 <sup>c</sup>
A.22.1	7.67 <sup>figh</sup>	5.75 <sup>c</sup>	5.65 <sup>abc</sup>
A.22.2	3.79 <sup>a</sup>	3.67 <sup>a</sup>	4.90 <sup>ab</sup>
A.22.3	4.97 <sup>bc</sup>	7.61 <sup>ef</sup>	7.48 <sup>de</sup>
A.22.4	7.41 <sup>efg</sup>	5.05 <sup>c</sup>	4.91 <sup>ab</sup>
A.23.2	7.11 <sup>ef</sup>	6.91 <sup>e</sup>	4.64 <sup>a</sup>
A.23.4	7.11 <sup>ef</sup>	6.07 <sup>e</sup>	7.65 <sup>de</sup>

Values in the same columns followed by different letters (a-f) are significantly different ( $p < 0.05$ )

Differences in the sensitivity of pathogenic bacteria to LAB antibacterial activity were caused by the differences in strains, cell wall structure of pathogens and the different antimicrobial substances produced by LAB isolates<sup>42</sup>. Gram-negative bacteria contain cell wall structures that are much thinner than those of Gram-positive bacteria, such that antibacterial compounds accumulate more easily and damage the cell membrane. According to Jawetz *et al.*<sup>43</sup>, differences in cell wall structure determine the penetration, binding and antibacterial activity of the compounds. Inhibition of Gram-negative bacteria is due to antibacterial compounds, including organic acids such as lactic acid. Lactic acid can disrupt the permeability of Gram-negative bacteria by damaging their outer membrane.

#### Antibacterial activity of lactic acid bacteria cell-free supernatant:

Table 3 indicates that the cell-free supernatant isolated from okara also retains antibacterial activity. The greatest antibacterial activity was found against *Listeria monocytogenes* EP01. The diameter of the clear zone that formed around the wells was 7.14 mm, whereas the diameters of the clear zones inhibiting *Escherichia coli* 0157 and *Staphylococcus aureus* ATCC 25923 growth were 6.23 and 6.66 mm, respectively. Inhibition of the growth of gram-negative bacteria such as *Escherichia coli* 0157, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* EP01 is primarily due to antibacterial compounds, including organic acids such as LAB-produced lactic acid. Lactic acid can disrupt the permeability of gram-negative bacteria by damaging the bacterial outer membrane. According to Alakomi *et al.*<sup>44</sup>, lactic acid is a

water soluble molecule that can penetrate the periplasm of gram-negative bacteria through the outer membrane. Lipopolysaccharide (LPS), located on the membrane surface, can be marred by lactic acid. Damage to LPS on the exterior of the cell can allow other antimicrobial compounds such as diacetyl, hydrogen peroxide and bacteriocins to invade the cytoplasmic membrane and disrupt intracellular activity, resulting in cell death.

The diameters of the inhibitory zones generated by LAB isolates were greater than those of the cell-free supernatants. Figure 1-3 show a comparison of the antimicrobial activity of LAB isolates and of the cell-free supernatant against *Escherichia coli* 0157, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* EP01, respectively. These figures show that all LAB isolates displayed greater antimicrobial activity than that of the cell-free supernatants against each pathogenic bacterial species. This finding is likely due to the antimicrobial activity of the LAB isolates, which still produce primary and secondary metabolites, such as organic acids, bacteriocin, diacetyl, bactericidal proteins and hydrogen peroxide<sup>45</sup>, whereas cell-free supernatants only contain secondary metabolites of LAB, such as bacteriocin and hydrogen peroxide. As defined previously by Schillinger and Lucke<sup>46</sup>, the antimicrobial activities of the LAB in this study were considered positive when the LAB had a clear zone diameter of 0.5 mm or larger.

**Survival rates of LAB isolates under conditions of low pH and bile salt exposure:** For LAB to be successful probiotics, they must be able to tolerate gut conditions<sup>47</sup>. To reach the

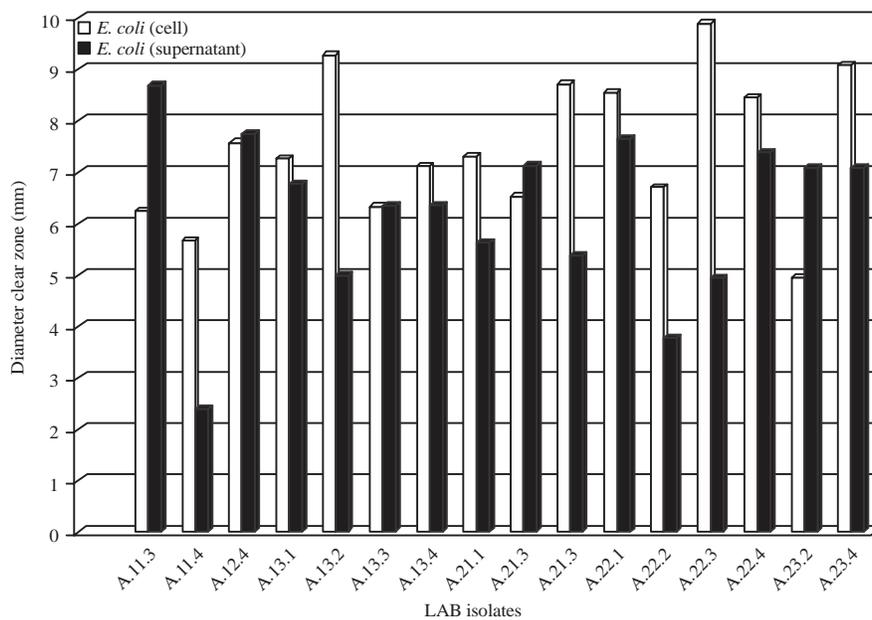


Fig. 1: Comparison of the antimicrobial activity of LAB isolates and CFS against *Escherichia coli* 0157

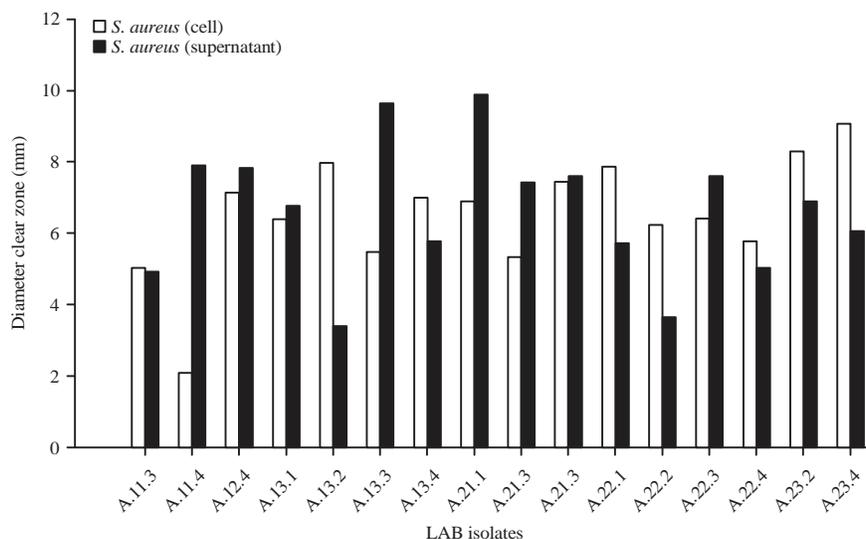


Fig. 2: Comparison of the antimicrobial activity of LAB isolates and CFS against *Staphylococcus aureus* ATCC 25923

intestine, LAB must be able to live in very extreme conditions found in the stomach (low pH 2-3); the time from initial consumption to passage through the stomach was estimated to be 90 min. These conditions can lead to a significant decrease ( $p < 0.05$ ) in LAB viability due to low pH. Table 4 shows that not all isolated LAB can survive at pH 2.5 and 3 for 90 min. Isolates A21.1 and A21.3 displayed very low tolerance to acidic conditions (pH 2.5 and 3) and two isolates (A22.4 and A23.2) displayed relatively high resistance at pH 3, with a survival rate of more than 90%. Food is known to stay in the stomach for an average of 3 h<sup>48</sup>.

Bile salt tolerance is also required for probiotics to survive in the small intestine<sup>49</sup>. The survival rate of LAB isolates on 0.5% oxgall as shown in Table 4, ranged from 77-139.80% after incubation for 5 h. Isolate A21.3 demonstrated the highest survival rate compared with other isolate survival rates. Three isolates showed survival rates of more than 50% (A23.4, A13.2 and A23.2) and three other isolates had very low survival rates, at less than 10%. This decrease in the survival rate of LAB is likely caused by changes in the cell membrane permeability of

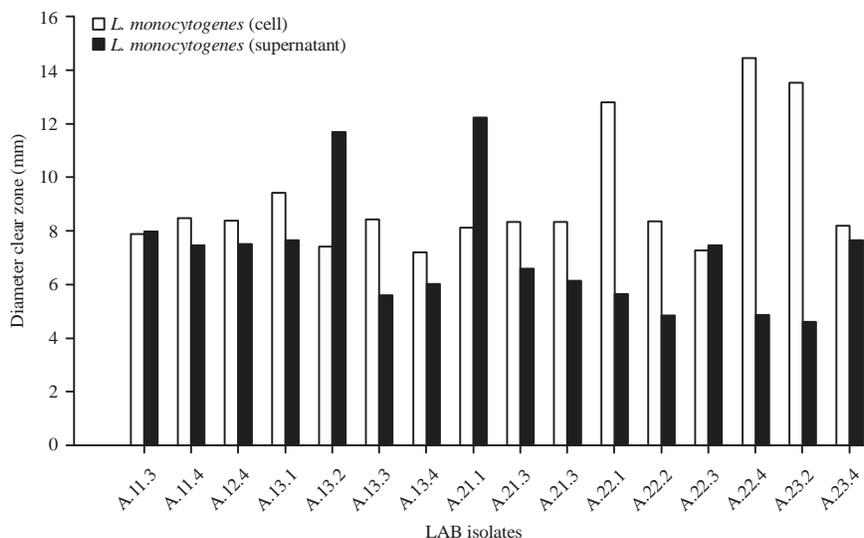


Fig. 3: Comparison of the antimicrobial activity of LAB isolates and CFS against *Listeria monocytogenes* EP01

Table 4: Survival rate of lactic acid bacteria isolates under conditions of low pH and bile salt exposure

Isolated strains	Survival rate (%) (90 min)		Survival rate (%) (5 h)
	pH 2.5	pH 3	Oxgall (0.5%)
A13.2	58.54 <sup>b</sup>	62.11 <sup>c</sup>	116.67 <sup>c</sup>
A21.1	4.07 <sup>a</sup>	7.59 <sup>b</sup>	124.10 <sup>c</sup>
A21.3	1.29 <sup>a</sup>	20.00 <sup>b</sup>	139.80 <sup>d</sup>
A22.4	59.71 <sup>b</sup>	90.53 <sup>d</sup>	77.00 <sup>a</sup>
A23.2	69.99 <sup>c</sup>	95.21 <sup>e</sup>	90.00 <sup>b</sup>
A23.4	62.50 <sup>b</sup>	89.9 <sup>d</sup>	134.00 <sup>d</sup>

Values in the same columns followed by different letters (a-e) are significantly different (p<0.05)

Table 5: Identification of isolated LAB using the standard API 50 CHL kit

Isolated LAB	Significant LAB taxa	Accuracy (%)	Remarks
A13.2	<i>Lactobacillus pentosus</i>	99.9	Excellent identification
A21.1	<i>Lactobacillus pentosus</i>	86.1	Good identification
A21.3	<i>Lactobacillus plantarum</i> L.	99.9	Excellent identification
A22.4	<i>Lactobacillus pentosus</i>	99.4	Very good identification
A23.2	<i>Lactobacillus pentosus</i>	99.4	Very good identification
A23.4	<i>Lactobacillus plantarum</i> L.	91.7	Good identification

Gram-positive bacteria, resulting in leakage of intracellular material due to cell lysis, resulting in cell death.

**Identification of candidate probiotics using the standard API 50 CHL kit:**

The LAB isolates were selected based on their antibacterial activity and their survival rates under conditions of low pH and 0.5% bile salt. Isolates were then identified by their carbohydrate fermentation patterns using an API 50 CHL kit (Table 5). The API 50 CHL kit results showed that the LAB isolated from okara consisted of *Lactobacillus pentosus* for isolates A13.2, A21.1, A22.4 and A23.2 and *Lactobacillus plantarum* for isolates A1.3 and A23.4. According to Delgado *et al.*<sup>50</sup> the species *Lactobacillus pentosus*, isolated from plants, produces high bacteriocin

titers. Probiotics with properties of *Lactobacillus plantarum* have been used in food fermentation<sup>51,52</sup>. These species are both used in food fermentation because they have antimicrobial activity against pathogenic bacteria and are able to tolerate low pH and bile acid exposure<sup>53,54</sup>. Lactic acid bacteria contribute greatly to the environment and have health benefits<sup>55</sup>. Another study found that *Lactobacillus pentosus* and *Lactobacillus plantarum* isolated from fermented olives has probiotic potential<sup>56</sup>.

The identified LAB isolated from okara displayed various probiotic properties. These LAB showed antimicrobial activities against pathogenic bacteria (*Escherichia coli* 0157, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* EP01) and displayed survival rates of more

than 50% under conditions of low pH and bile salt exposure. These findings require further studies, especially with regard to the molecular identification of LAB, identification of their antimicrobial compounds and their applications in food as probiotics and food biopreservatives.

### CONCLUSION

The antimicrobial activity of LAB isolated from okara varied among individual isolates and the antibacterial activity of the LAB isolates was higher than that of the cell-free supernatant. Based on the antimicrobial activity and probiotic properties of the LAB isolated from okara, these isolates belong to the species *Lactobacillus pentosus* and *Lactobacillus plantarum*.

### SIGNIFICANCE STATEMENTS

This study isolated LAB from okara and identified them as the species *Lactobacillus pentosus* and *Lactobacillus plantarum*. This study will help the researchers to uncover the benefits of indigenous LAB isolated from okara, that was a waste product of soymilk production. Using these LAB for producing functional food products from soybean and other foodstuffs and for biopreserving foods will be proven new explorations.

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