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Probiotic candidates of lactic acid bacteria from fermented food cincalok and tempoyak from Kalimantan, Indonesia

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Abstract. *Murwani R, Anggraeni R, Ambariyanto A. 2024. Probiotic candidates of lactic acid bacteria from fermented food cincalok and tempoyak from Kalimantan, Indonesia. Biodiversitas 25: 2705-2712.* Lactic acid bacteria (LAB) belong to beneficial bacteria with the potential as probiotics to prevent pathogenic bacterial infections, improving digestive enzyme activity and the immune system. The objective of this research was to determine the probiotic potential of five lactic acid bacterial species isolated from fermented foods and to evaluate their antibacterial activity against pathogenic *Bacillus subtilis, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae,* and *Staphylococcus nepalensis*. The gastric simulation test results showed a significant decrease in all five LAB isolates from 10^9 to 10^8 CFU/mL ($P<0.05$). Subsequent intestinal simulation decreased the number of *Levilactobacillus brevis* and *Lactiplantibacillus plantarum* numbers from 10^8 to 10^7 CFU/mL ($P<0.05$), which remains high. All five bacterial strains were found negative for the DNase test. In the hemolytic test, only *Enterococcus faecalis* and *Pisciglobus halotolerans* lysed red blood cells but not *Tetragenococcus halophilus*, *L. brevis*, and *L. plantarum*. In conclusion, *T. halophilus, L. brevis*, and *L. plantarum* were promising probiotic candidates. Further in vivo studies are necessary to test the probiotic effect of these three LAB strains.

Keywords: Antimicrobial, bile salt, cincalok, DNase, hemolytic activity, lactic acid bacteria, probiotics, tempoyak

INTRODUCTION

Lactic Acid Bacteria (LAB) are Gram-positive, aerotolerant, homo- or heterofermentative bacteria found in soil, water, digestive tract, human urogenital tract, animals, and food (plants, meat, milk, and vegetables), as well as in fermented food products. Homofermentative LAB produces two moles of lactic acid from one mol of glucose, while heterofermentative produces CO_2, acetic acid, ethanol, and lactate, besides lactic acid (Villalobos et al. 2020). LAB are found in numerous fermented foods (Rhee et al. 2011; Sukmarini et al. 2014; Sulistiani et al. 2014; Angmo et al. 2016; Afiati et al. 2018; Harnentis et al. 2019; Nurhikmayani et al. 2019) and add taste, texture, and aroma (Souza et al. 2023). The dominant LAB strain in fermented foods is *Lactiplantibacillus plantarum* (Rahayu et al. 2015; Rahayu 2019). Previous studies found *L. plantarum, Pediococcus pentosaceus,* and *Streptococcus thermophilus* in mandai cempedak (Rahayu 2003; Juwana et al. 2020). Meanwhile, *L. plantarum*, *Streptococcus* sp., *Pediococcus acidilactici*, *Weissella paramesenteroides*, *Enterococcus gallinarum*, and *Enterococcus faecalis* were found in tempoyak (Rahayu 2003; Yuliana and Dizon 2011; Pato and Surono 2013). *Lactobacillus confusus*, *Staphylococcus piscifermentans*, *Piscibacillus halophilus*, *Pediococcus dextrinicus*, *Lactobacillus rhamnosus*, *Staphylococcus saprophyticus*, *Pediococcus acidilacti*, and *Levilactobacillus brevis* were found in cincalok (Hajar and Hamid 2013; Elegado et al. 2016; Khairina et al. 2016; Pribadhi et al. 2021). *Tetragenococcus halophilus* from cincalok and *L. brevis* from tempoyak are new isolates not isolated from the two fermented foods (Murwani et al. 2024).

Some LAB strains from various fermented foods are probiotics (Adesulu-Dahunsi et al. 2018; Choi et al. 2018; Zielinska and Krajewska 2018; Amelia et al. 2020; Ayivi et al. 2020; Jung et al. 2020; Motey et al. 2021; Pratama et al. 2021; Suwannaphan 2021). Probiotics can produce bacteriocins and hydrogen peroxide, which can kill and inhibit pathogens, prevent dysbiosis (Noori et al. 2023), increase enzyme activity, maintain serum glucose levels (Khalili et al. 2019), reduce cholesterol assimilation (Vasiee et al. 2020), improve intestinal health (Pereira et al. 2018; Jha et al. 2022), act as an immunomodulator and anti-inflammatory (Gueimonde et al. 2013; Plaza-Diaz et al. 2019), and improve immune system (Dicks and Botes 2010).

Screening tests for potential probiotics can be conducted using in-vitro and in-vivo tests. The main in vitro screening to determine the probiotic's potential includes testing for resistance to gastric acid (low pH of 1-3) and the small intestine environment where food is digested enzymatically (pancreatin) in the presence of bile salts with a pH close to neutral (Pinto et al. 2020). The gastric and intestinal tolerance tests can be carried out separately or continuously. In the separate method, lab exposure to gastric simulation was carried out separately

from intestinal simulation. In the continuous method, exposure to low pH was followed by the small intestine environment with higher pH (±8.0). The continuous method also mimics more of the in vivo system. The safety of probiotics included blood hemolytic and DNase activity tests (Riceto et al. 2015; Bujnakova et al. 2017; Mangia et al. 2019; Tarrah et al. 2019; Dwivedi et al. 2022). The objective of this research was to determine the probiotic potential of five lactic acid bacterial species isolated from fermented foods and to evaluate their antibacterial activity against pathogenic *Bacillus subtilis, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae,* and *Staphylococcus nepalensis*. The study is expected to provide the probiotic candidates from the five lab strains for further study in-vivo.

MATERIALS AND METHODS

Collection of LAB isolates

The five LAB isolates used were collected from the bacterial collection of the Natural Products Laboratory, UPT Laboratorium Terpadu, Universitas Diponegoro, Indonesia. They were obtained from fermented foods, cincalok (*T. halophilus, E. faecalis,* and *P. halotolerant*), and tempoyak (*L. brevis* and *L. plantarum*). The isolates were grown in de Man Rogosa Sharpe Agar (MRSA from HiMedia India) supplemented with 1% $CaCO_3$.

Preparation of LAB cultures

20 mL of Man Rogosa Sharpe Broth (MRSB, HiMedia India) was used to inoculate each isolate. The LAB was grown in MRSB, incubated aerobically at 37°C, and shaken at 200 rpm for 24 hours (Bose et al. 2023). The 24-hour LAB cultures were used for subsequent tests.

Gastrointestinal simulation test

Gastrointestinal simulation test was done according to the method of Ayyash et al. (2021), Włodarczyk et al. (2021), Gupta et al. (2023) with modifications. Gastric juice was made by preparing 0.2% NaCl in double distilled water, and pH was adjusted by adding concentrated HCl until pH 2 was reached. Pepsin (Sigma, USA) 3% was prepared by dissolving it in gastric juice (). Next, 8 mL of gastric juice and 1 mL of 3% pepsin were mixed and sterilized with a 0.2 µm filter. Finally, one mL of LAB from 24 hours of grown broth culture was added to make a total of 10 mL gastric simulation volume. The gastric simulation juice was incubated in a shaker at 37°C at 100 rpm for 3.5 hours (Gupta et al. 2023). After 3.5 hours, one mL of the simulated gastric mix was taken, serially diluted using physiological salt (NaCl 0.9%), and inoculated in MRSA to calculate LAB viability (CFU). Another one mL of the gastric simulation mix was used for intestinal simulation.

The intestinal simulation was performed according to the method of Lee et al. (2014), Hyacinta et al. (2015), and Shuhadha et al. (2017) with modifications. Intestinal condition was examined by preparing a sodium bicarbonate ($NaHCO_3$) buffer pH 8 containing 0.2% NaCl in double-distilled water. The pH 8 was adjusted using 0.5 M NaOH. Oxgall 3% (Sigma, USA) was prepared in the $NaHCO_3$ buffer. Pancreatin (Sigma, USA) 1% was also prepared in the same buffer. Next, 7 mL of buffer, 1 mL of Oxgall, and 1 mL of pancreatin were mixed and sterilized using a 0.2 µm filter. Finally, one mL of the previous gastric simulated mix was added to make a total volume of 10 mL of intestinal simulation. The intestinal simulation mix containing 0.3% Oxgall and 0.1% pancreatin was run in a shaker at 37°C at 100 rpm for 4 hours. After 4 hours, one mL of the simulated intestinal mix was taken, diluted using physiological salt (NaCl 0.9%), and inoculated in MRSA to calculate LAB viability (CFU). The gastrointestinal simulation tolerance test is summarized in Figure 1.

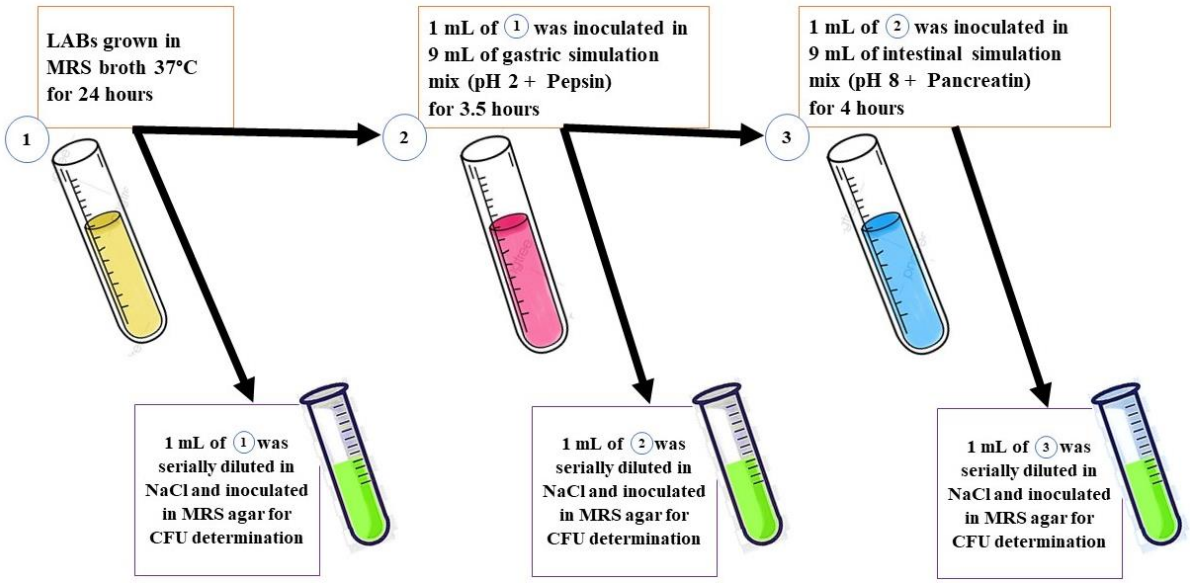

Figure 1. Steps of gastrointestinal simulation tolerance test

Calculation of the number of colonies (colony forming unit/CFU)

1 mL of the sample was transferred into 9 mL of physiological salt solution (NaCl 0.9%) until the first dilution was obtained (10^{-1}). The dilution was continued until it reached 10^{-6}. 50 µL of each dilution was transferred into MRSA and incubated at 37°C until colony growth was visible. The number of colonies was calculated following the formula of Shori et al. (2022):

N (CFU) = Number of colonies x number of dilutions/Total dilution volume.

Antimicrobial activity

The pathogenic bacteria *B. subtilis* and *E. coli* were obtained from the Food and Nutrition Culture Collection (FNCC) at the Centre for Food and Nutrition Studies, Universitas Gajah Mada, Yogyakarta, Indonesia. *B. cereus*, *K. pneumoniae,* and *S. nepalensis* were collected from the collection of pathogenic microbes at the Natural Products Laboratory, Universitas Diponegoro, Semarang. The agar plug method was used to test the antimicrobial activity against each pathogen. All pathogenic microbes were grown on Mueller Hinton Agar (MHA, HiMedia India) for 24 h at 37°C. LAB isolates grown in MRSA were collected with a cork-borer (6 mm) and placed on top of a pathogenic microbe agar culture, ensuring the LAB colonies were in direct contact with pathogen colonies. The tests were done for 3-5 days. A clear zone around the LAB colony indicates the presence of antimicrobial activity, and the diameter of inhibition zones was measured using calipers (Murwani et al. 2021).

DNase test

DNase agar media (HiMedia, India) was added with methyl blue dye. The LAB were inoculated on the media and incubated at 37°C for 5×24 hours. A clear zone formation around the inoculant indicates the DNase activity (Hickey et al. 2013; Franz et al. 2015). The DNase test was done in five replicates.

Hemolytic test

Sheep blood agar media (HiMedia, India) was prepared by adding 10% sheep blood (HiMedia, India). The LAB were inoculated on the media and incubated at 37°C for 5×24 hours. The formation of a clear zone around the inoculant indicates that LAB was β-hemolysin positive, and a slightly clear dark zone indicates ƛ-hemolysin (Fernández-Pacheco et al. 2021). The hemolytic test was done in five replicates.

Data analysis

The CFU data were analyzed using analysis of variance (ANOVA) to determine the effect of in vitro gastrointestinal digestive simulation on the number of LAB isolates. Duncan's test was carried out at a 5% significant level when a significant effect was found. The antibacterial, DNase, and hemolytic tests were analyzed descriptively.

RESULTS AND DISCUSSION

Results showed that five bacterial isolates grew well on MRS Agar media at pH 6.5. It was also observed that their number differed for each type of LAB grown for 24 h ($p<0.05$). After gastric simulation (gastric condition pH 2 in the presence of pepsin), a significant decrease was noted in the number of all LAB tested from 10^9 to 10^8 CFU/mL ($P<0.05$). Subsequently, in the intestinal simulation test (intestinal juice in the presence of bile salt and pancreatic enzyme), the number of *L. brevis* and *L. plantarum* decreased from 10^8 to 10^7 CFU/mL ($P<0.05$). Even though the number of BAL decreased tenfold, namely from 10^8 to 10^7 CFU/mL, and was statistically significant, the number was still in the high category. However, the number of the other three strains remained the same (same superscript letter in Table 1).

Antimicrobial activity, DNase, and hemolysis tests

The results showed that all five isolates showed no antimicrobial activity against selected bacterial isolates. The DNase test of five bacterial strains was negative. However, the hemolysis test showed that *E. faecalis* and *P. halotolerans* lysed red blood cells, as indicated by a clear zone around the colonies (Table 2, Figures 2 and 3). Meanwhile, *T. halophilus, L. brevis,* and *L. plantarum* did not lyse red blood cells.

Table 1. Tolerance of lactic acid bacteria to gastrointestinal simulation test

LAB strains	Bacterial inoculum (CFU/mL)	Gastric acid tolerance (CFU/mL)	Intestinal digestion tolerance (CFU/mL)	SEM	*p*
	24 (h)	3.5 (h)	4 (h)		
Tetragenococcus halophilus	$0.20\pm0.02 \times 10^{9a(A)}$	$0.08\pm0.09 \times 10^{8b(A)}$	$0.09\pm0.02 \times 10^{7b(A)}$	0.02×10^9	0.00*
Enterococcus faecalis	$1.62\pm0.12 \times 10^{9a(C)}$	$0.11\pm0.01 \times 10^{8b(A)}$	$0.06\pm0.01 \times 10^{7b(A)}$	0.20×10^9	0.00*
Pisciglobus halotolerans	$0.15\pm0.04 \times 10^{9a(A)}$	$0.10\pm0.04 \times 10^{8b(A)}$	$0.02\pm0.00 \times 10^{7b(A)}$	0.02×10^9	0.00*
Levilactobacillus brevis	$1.03\pm0.15 \times 10^{9a(B)}$	$1.50\pm0.12 \times 10^{8b(B)}$	$1.24\pm0.00 \times 10^{7c(B)}$	0.12×10^9	0.00*
Lactiplantibacillus plantarum	$2.20\pm0.12 \times 10^{9a(D)}$	$2.80\pm0.06 \times 10^{8b(C)}$	$3.00\pm0.01 \times 10^{7c(C)}$	0.26×10^9	0.00*
SEM	0.16×10^9	0.02×10^9	0.00×10^9	-	-
p	0.00*	0.00*	0.00*	-	-

Notes: Each set of data is an average of five replicates. Small letters for rows and capital letters for columns. Different superscripts in the same row or column show significant differences ($p<0.05$)

Table 2. Antimicrobial activity, hemolysis, and DNase tests of five LAB strains

LAB strains	Antimicrobial activity	Tests	
		DNase	Hemolysis
Tetragenococcus halophilus	Negative	Negative	Negative
Enterococcus faecalis	Negative	Negative	Positive
Pisciglobus halotolerans	Negative	Negative	Positive
Levilactobacillus brevis	Negative	Negative	Negative
Lactiplantibacillus plantarum	Negative	Negative	Negative

Notes: Each data is an average of five replicates. Positive: lysed red blood cells, Negative: Not lysed

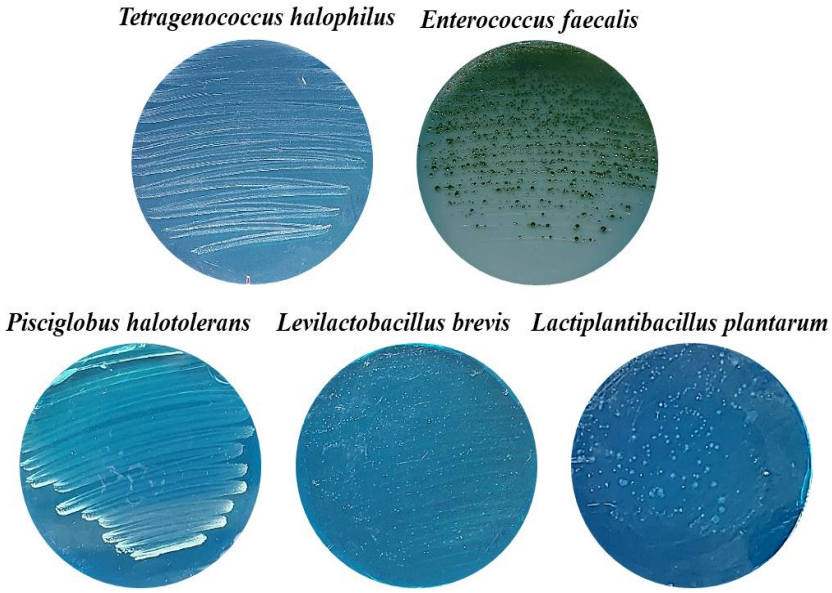

Figure 2. DNase test of five LAB isolates

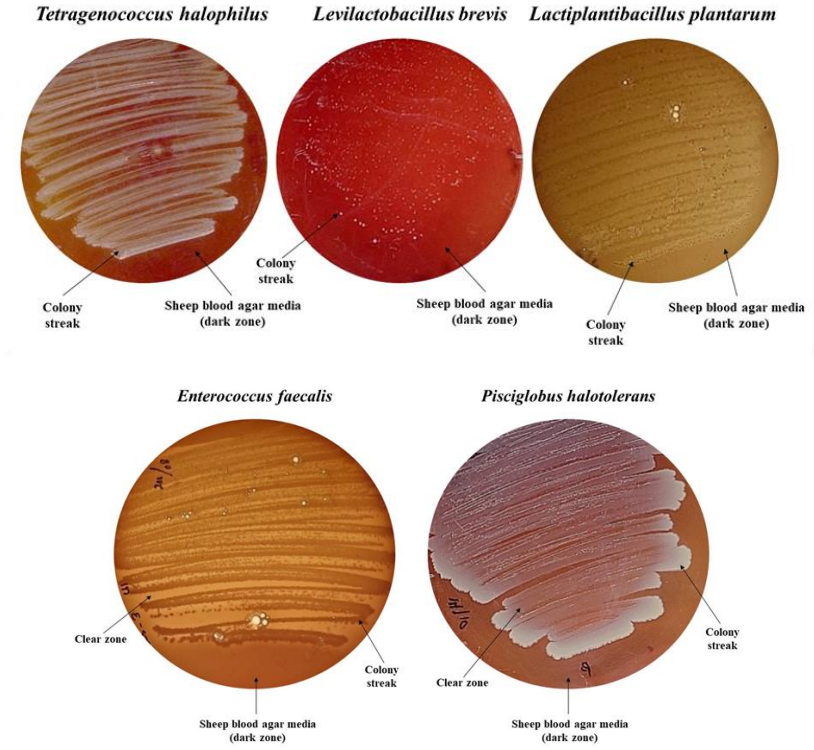

Figure 3. Hemolytic test of five LAB isolates

Discussion

The LAB isolates from cincalok and tempoyak from Kalimantan, Indonesia, were tested in vitro for their probiotic potential. After gastric simulation (pH 2 in the presence of pepsin), there was a significant decrease ($P<0.05$) in all LAB tested, with the remaining number still high (from 10^9 to 10^8 CFU/mL). These results aligned with previous research on the number of *L. plantarum* that experienced a decrease in gastric pH 2 (Khalil et al. 2018; Pinto et al. 2020; Saboori et al. 2022). LAB can grow optimally at a pH of 3.5-6.5 (Daba and Elkhateeb 2020; Śliżewska and Chlebicz-Wójcik 2020; Villalobos et al. 2020). Each LAB has a different ability to work and survive at different pH (Dianawati et al. 2016). LAB's low pH tolerance was due to a transport system of lactic acid and protons to the cell membrane (Singhvi et al. 2018; Mbye et al. 2020). However, highly acidic conditions of gastric pH change the cell's physiological condition, causing membrane and intracellular damage and even death (Guan and Liu 2020). Adding pepsin enzyme makes LAB more susceptible to proteolysis, leading to cell damage and a further decrease in number (Sharma and Yadlapati 2021; Li et al. 2022).

After exposure to intestinal simulation in the presence of bile salt and pancreatin enzyme, *L. brevis* and *L. plantarum* tested significantly decreased in number (from 10^8 to 10^7 CFU/mL; $P<0.05$). A decrease at the end of gastric and intestinal simulation appeared to depend on the initial inoculation number, which was prepared similarly in MRSB. However, their number significantly differed after 24 hours in MRSB and CFU determination ($p<0.05$). This difference could be due to the different growth rates of each LAB. The studies of *Lactobacillus acidophilus, Lactobacillus delbrueckii, Lactobacillus bulgarius, Lactobacillus plantarum, Weissella viridescens,* and *Latilactobacillus sakei* showed that each strain has a different growth time, speed, and exponential phases (Najim and Aryana 2013; Silva et al. 2018). Other studies also found similar results after intestinal simulation, where the *L. plantarum* number was reduced (Ahmad et al. 2018; Khalil et al. 2018; Pinto et al. 2020). This reduction was due to bile salt properties, which have an amphipathic nature that could damage cell membranes and walls, leading to bacterial death (Fonseca et al. 2019). Bile salt concentration can increase the ƛ-galactosidase enzyme activity that converts lactose into lactic acid (Saqib et al. 2017), leading to further cell membrane permeability. The intestinal simulation's pancreatin enzyme (with lipolytic and proteolytic activities) further damages the cell membrane and, hence, the LAB number.

All five LABs did not show antibacterial activity against pathogenic *B. subtilis, E. coli, B. cereus, K. pneumoniae,* and *S. nepalensis.* Some LAB produces antibacterial peptides such as bacteriocin (Harnentis et al. 2019; Kadyan and Pradan 2020) that can prevent pathogenic bacterial growth (Saranraj et al. 2013; Parasthi et al. 2020). *Lactobacillus* strains have a low ability to inhibit *K. pneumonia*, while *L. acidophilus* does not have antagonistic properties against pathogens (Shokryazdan et al. 2014). Some LABs are ineffective against gram-negative bacteria (Heredia-Castro et al. 2015). The probiotic mechanisms for health and food processing are not limited to their antibacterial activity. Studies in vivo showed that non-living inactivated probiotics (para probiotics) exert their health-beneficial properties by various mechanisms. For example, in cholesterol reduction (*Bifidobacterium longum*), recovery of intestinal injuries (*L. brevis*), modulation of inflammation (*L. rhamnosus*) (de Almada et al. 2016), anti-adhesion, and anti-colonization by *E. coli* (*L. plantarum*) (Gao et al. 2016; Ahn et al. 2018; Singh et al. 2018), splenic cytotoxic activity enhancement (*L. brevis*) (Sasaki et al. 2015), and stimulation of vertebrate host immunity (*Lactococcus lactis*) (Kimoto-Nira 2018). LAB in the whole system can affect the diversity and abundance of the whole microbiome (Murwani et al. 2024).

The DNase test aims to evaluate the ability of LAB to use DNA as a carbon source by the enzyme DNase, resulting in the breakdown of DNA into phospho-mononucleotides, marked by a clear zone around the colony (Huligere et al. 2023). *T. halophilus, E. faecalis, P. halotolerans, L. brevis, and L. plantarum* did not show clear zones around the colonies in the DNase test, providing evidence of their non-pathogenic nature. The test was carried out to determine the pathogenic potential of LAB and ensure that they are safe to use in vivo. In contrast, other studies suggest it could benefit LAB as probiotics in vivo and food processing (Alakomi et al. 2000; Zapaśnik et al. 2022; Dey et al. 2023). In vivo, probiotics can help the breakdown of extracellular DNA associated with biofilm produced by pathogenic bacteria (Vinderola et al. 2019). In food processing, DNase activity can help raw materials DNA degradation, improving the product's texture and flavor (Chaudhari et al. 2019). *T. halophilus, L. brevis*, and *L. plantarum* cannot hydrolyze blood, so they were potential probiotics. In contrast, *E. faecalis* and *P. halotolerans* had beta-hemolytic properties (lyse red blood cells), resulting in hemoglobin damage, characterized by a clear zone around the colony. Kim et al. (2022a) also found that *E. faecalis* lysed red blood cells. Bacteria that lyse blood are also considered pathogens (Savković et al. 2022). However, other research shows that hemolytic activity obtained from fermenting red and yellow peppers makes LAB strains produce the desired taste and texture (Di Cagno et al. 2009). Regarding health, LAB strains can inhibit *L. monocytogenes* growth in fresh cheese and act as a biocontrol agent in food products (Quattrini et al. 2018). However, all five isolates may be considered probiotic candidates in food processing. Previous LAB isolation studies found pathogenic bacteria from cincalok *S. saprophyticus* (Tsai et al. 2018) and from shrimp paste *Bacillus* sp., *Staphylococcus* sp., *Micrococcus varians, Corynebacteria* sp., *Enterococcus* (E) sp., *Pseudomonas* sp. (Nuñal et al. 2016). A previous study that examined the LAB isolates and microbiome of cincalok and tempoyak found *Staphylococcus carnosus, Corybacterium phoceense, Vagococcus vulneris,* and *Priestia filamentosus* (Murwani et al. 2024). Those studies showed that pathogenic bacteria are always present in food products, food systems, and the gastrointestinal tract, but their numbers do not cause ailments; this is due to other

types of microbes, especially LAB, that can suppress pathogenic bacteria (Nuñal et al. 2016; Tsai et al. 2018; Ayivi et al. 2020; Murwani et al. 2024).

LAB from cincalok and tempoyak samples that have been isolated and tested for their probiotic properties is *L. plantarum* (Ahmad et al. 2018; Khalil et al. 2018), while the other two (*T. halophilus* and *L. brevis)* have not been reported to have been tested for probiotic properties in vitro. *T. halophilus* has been tested as an isolate from soybean paste (Lim 2016), fermented soybean (Kim et al. 2022b), and Miso (Kumazawa et al. 2018). *Levilactobacillus brevis* that have been tested for probiotic properties are isolates from soybean paste (Lim 2016), dental plaque (Fang et al. 2018), Kimchi (Jang et al. 2019), chicken fecal and feed samples (Noohi et al. 2021), fermented fruit juice (Sathiyaseelan et al. 2022), and fermented *Carica papaya* L. (Sreepathi et al. 2023). Several other studies show that *E. faecalis* is considered a probiotic because it does not have hemolytic activity, is not resistant to antibiotics, and has high coaggregation and hydrophobicity scores. In conclusion, the probiotic candidates of LAB isolates from cincalok and tempoyak were *T. halophilus*, *L. brevis*, and *L. plantarum*. Further in vivo studies are needed for their probiotic application for animals and humans.

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