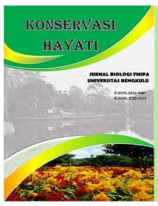

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Characterization of Lactic Acid Bacteria (LAB) from Tempeh Probiotic Drink with Combination of Dates and Skim Milk

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ABSTRACT

Tempeh can be processed into a probiotic drink because it contains Lactic Acid Bacteria (LAB), which are beneficial for the digestive system. A combination of dates and skim milk as prebiotics is required by LAB as a substrate to produce energy and cellular components, and to increase the population to produce sufficient amounts of acid. This study aims to describe the characteristics of LAB present in tempeh probiotic drinks with a combination of dates and skim milk. Isolation was performed using the total plate count (TPC) method with the spread plate technique. Selected isolates were identified macroscopically, microscopically, biochemically, and physiologically. The results showed that the average amount of LAB viable counts was 9.6 x 10^6 CFU/mL with 8 suspected isolates as LAB. Microscopic observation obtained all 8 isolates were Gram-positive with two round shapes and six-rod shapes. The catalase test results showed all 8 isolates were negative due to the absence of bubbles. The triple sugar iron agar (TSIA) test showed a yellow color, indicating the capability to ferment glucose, lactose, and sucrose. All selected isolates non-motile and positive MR and negative SCA testing. Some of the isolates showed tolerance to salts and acids based on physiological testing. The six most promising LAB isolates showed important characteristics of LAB that should be evaluated when selecting probiotic candidates, namely TEa-4130, TEb-4230, TEb-4130, TEb-3150, TEb-3250, and TEb-4250 isolates.

Keywords: *Characterization, Identification, LAB, Probiotic, Tempeh*

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INTRODUCTION

Tempeh, as a traditional fermented product, can be processed into alternative probiotic drinks besides milk. Tempeh is a potential source of probiotics because of its oligosaccharide content that can be metabolized by microorganisms such as Lactic Acid Bacteria (LAB). *Lactobacillus* sp., *Bifidobacterium* spp., and *Saccharomyces* spp. are included in LAB (Islam, 2016). LAB is generally recognized as Generally Recognized As Safe (GRAS) and can produce useful metabolites or by-products such as peptides, antimicrobials, ethanol, organic acids, fatty acids, and carbon dioxide (Gallego & Salminen, 2016; Marco *et al*., 2017; Macori & Cotter, 2018). LAB produces various enzymes such as

amylase, peptidase, proteinase, dehydrogenase, decarboxylase, and β–β-glucosidase to increase the food's nutritional value and digestibility (Li *et al*., 2021).

Tempeh probiotic drink inoculated with *L. bulgaricus* and *Streptococcus thermophilus* is useful as an inexpensive food supplement for menopausal women (Purwadaria *et al*., 2016). Probiotics are nonpathogenic microorganisms that induce beneficial effects in balancing the intestinal microflora of the host and are able to survive in gastric acidity (Haghshenas *et al*., 2016; Rizal *et al*., 2016). Łoniewski *et al.* (2022) stated that the use of probiotics has increased and is not limited to prescription only when used together with antibiotics.

Dates contain essential broad-effect nutraceuticals such as antimutagenic, antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, gastroprotective, anticancer, and immune-stimulating activities (Ahmed *et al*., 2016). Besides, dates contain minerals, vitamins, phenolic compounds, and sugars as a source of dietary fiber that can be used as prebiotics for intestinal microflora, especially probiotics (Al-Thubiani & Khan, 2017; Nadeem *et al*., 2019). Prebiotics are nondigestible foods that positively affect the host by selectively activating the growth of microorganisms in the intestinal flora (Işlek *et al*., 2014). Prebiotics are food for probiotics. The combination of prebiotics and probiotics showed antiviral activity in children with acute Rotavirus (RV) gastroenteritis. Diarrhea duration was shortened when *B. lactis* B94 and inulin were taken orally (Işlek *et al*., 2014). Moreover, fermented milk of *B. breve* C50, *S. thermophilus* 065, and a prebiotics combination prevented RV-induced diarrhea in lactating rats (Rigo-Adrover *et al*., 2019). Currently, the main functional ingredients used in the food industry are probiotics and prebiotics, which provide many health benefits (Balthazar *et al*., 2017).

The availability of nutrition for microorganisms becomes an important factor in probiotic drink formulation (Nainggolan *et al*., 2021). The probiotic drink is one of the food processing diversifications using Tempeh, dates, and skim milk. LAB activities can be enhanced by the combination of dates and skim milk with a certain concentration to produce sufficient amounts of acid. LAB also produces bacteriocin, which is considered a preservative and antimicrobials in food or beverages (Perez *et al*., 2014).

Rahayu *et al.* (2021) showed that a tempeh juice drink with ginger was successfully formulated. However, this study did not provide any information about the capabilities and characteristics of LAB as a probiotic drink. Therefore, to produce a drink with probiotic properties, this study examined the characterization of LAB from Tempeh probiotic drinks with a combination of dates and skim milk. This research also explored LAB with potential probiotic characteristics.

METHODS

Preparation of Sample

The 10 g of tempeh was cut into cubes, 7 dates were separated from the seeds and added to 250 mL of water with 5 g of skim milk, then mashed using a blender.

Isolation and Quantification of LAB

Isolation and quantification of LAB were performed using the Total Plate Count (TPC) method on de Man Rogosa and Sharpe Agar (MRSA) selective media with 5% calcium carbonate ($CaCO_3$). A total of 10 mL of tempeh juice sample was taken and placed into 90 mL of 0.9 % (w/v) physiological solution (NaCl) and then homogenized. Serial dilutions up to 10^{-6} were made using the spread plate technique on Petri dishes and then incubated at 37ºC for 48 hours. The colonies were counted using a colony counter with 30-300 colonies (Rizal *et al*., 2016). The results were expressed as colony forming unit/millimeter (CFU/mL)

by multiplying the average number of colonies by the reciprocal of the dilution factor (Mubin & Zubaidah, 2016).

Macroscopic Morphological Identification and Purification of LAB Isolates

Colonies with clear zones in MRSA and distinct morphologies were isolated. Presumptive colonies were purified by inoculating into the same media three times to obtain pure isolates. Morphological identification, macroscopically and microscopically, was carried out using Lactic Acid Bacteria Biodiversity and Taxonomy (Holzapfel & Wood, 2014). The selected isolates were evaluated based on biochemistry and physiology, such as the catalase test, hydrogen sulfide production test, test for determining the type of halophilic or halotolerant, and acid resistance test.

Microscopic Morphological Identification using Gram Staining

One loop of LAB isolate was spread on an object glass. Moreover, it was fixed by heating over a Bunsen burner, then dripped with crystal violet dye for 1 minute, later rinsed with distilled water and dried. After that, it was dripped with Lugol for 1 minute, then rinsed again with distilled water and subsequently with ethanol (acetone alcohol) for 10-30 seconds. After washing briefly, it was stained with safranin solution for 30 seconds, then washed with distilled water and dried. The slides were examined under a microscope with 100 times magnification using immersion oil.

Biochemical Testing

Catalase Test

One loop of 24-hour-old LAB isolate was aseptically taken from MRSA and transferred to object glass, and then 1-3 drops of 3% H_2O_2 were dripped (Sastry *et al*., 2016). A positive result was indicated by the formation of bubbles.

Triple Sugar Iron Agar Test (TSIA)

One loop of LAB isolate was streaked on TSIA media by piercing the loop up to a third of the bottom of the tube. Later, it was removed and streaked in a zig-zag manner on the surface then incubated for 120 hours at 37°C (Sastry *et al*., 2016).

Motility Test

One loop of LAB isolates from the culture stock is inserted into the upright Sulphide Indole Motility (SIM) medium and incubated for 24 hours at 37°C. The growth of bacteria around the puncture (not spreading) is only in the form of a line, indicating a negative test result, so the bacteria are non-motile. The growth of bacteria that spreads in the media shows a positive test result, meaning the bacteria are motile (Leboffe & Pierce, 2010).

Methyl Red (MR) Test

One loop of bacterial isolates was inoculated into MR media and incubated for 24 hours at 29ºC. After incubation for 24 hours, 3-4 drops of 1% methyl red indicator were added to the medium. A positive test is marked by a change in the color of the medium to red, meaning acid is formed and a negative test is marked by no color change in the medium (Ginting *et al*., 2021).

Simon Citrate (SCA) Test

One loop of LAB isolate was streaked onto a slanted agar medium for the citrate test and incubated at 37°C for 24 hours. A positive test is marked by the color of the medium

changing to blue and a negative test is marked by no color change in the medium (Ginting *et al*., 2021)

Physiological Testing

Halophilic or Halotolerant Typing Test

Isolate was grown on MRSA media + 1% $CaCO_3$, 7 % NaCl, and MRSA media + 1% $CaCO_3$ without NaCl. Then incubated at 37°C for 24-48 hours (Listiyo *et al*., 2017).

Acid Resistance Test

The amount of 1 mL LAB isolate was inoculated on MRSB-HCl media and incubated at 37°C for 48-72 hours. Bacterial growth in low acidity media (pH 2,5–3) showed a positive result (Rahmah *et al*., 2021).

Data Analysis

The data was presented in a qualitative descriptive including type of LAB isolate from Tempeh with a combination of dates and skim milk, macroscopic and microscopic characteristics, as well as biochemical and physiological testing in the form of figures and tables.

RESULT AND DISCUSSION

Isolation and Quantification of LAB

Tempeh probiotic drink, made with a combination of dates and skim milk is reported to be the potential alternative raw material for probiotic drinks besides milk. The results showed that the average amount of LAB viable counts was 9.6 x 10^6 CFU/mL which met the standards requirement for probiotic bacteria. Food and beverages containing probiotics should have a minimum of 10^6 cfu/mL active LAB when consumed (FAO/WHO, 2002). There is no SNI published regarding probiotic drinks from tempeh. The existing SNI is a flavored fermented milk drink whose total LAB contains at least 1.0x10^6 CFU / mL (Sari & Catarina, 2020). The results of the study Khotimah & Kusnadi (2014) showed that in the treatment the ratio of dates: water (1:5, 1:6, and 1:7) had a significant effect (a=0.05) on total LAB and total acid. The treatment of skim milk concentration had a significant effect (a=0.05) on total BAL, total acid, and reduction in total sugar.

The growth of LAB was also influenced by the sugar content in dates and skim milk as a carbon source. Dates contain amino acids and produce reducing sugars such as glucose (71.2 %) and fructose (81.6 %) (Assirey, 2015). Skim milk contains lactose as a high source of carbon that will be converted into lactic acid and protein as the source of nitrogen (Sintasari *et al*., 2014). According to Nainggolan *et al*. (2021), there was a significant difference in the total LAB at 2% skim milk compared to 0% and 10% of skim milk, but not a significant difference between 4%, 6%, and 8% of skim milk. Fadro *et al.* (2015) conducted a similar study on the addition of skim milk to corn milk probiotic drinks and found that the amount of skim milk added enhanced total LAB. This is influenced by the content in skim milk in the form of lactose and protein which can be utilized by LAB as an energy source for growth.

The growth of LAB is strongly influenced by the composition of the growth media and environmental factors. If sucrose and lactose are available in a fermentation medium, LAB will tend to utilize lactose then sucrose because of the existence of lactase then sucrase.

Macroscopic Morphological Identification and Purification of LAB Isolates

After 48 hours of incubation, presumptive LAB colonies were round, with convex elevation and white color. The morphological characteristics of the potential

probiotic bacteria obtained showed similarities with the research conducted by Aýun *et al.* (2023), which described small to medium round LAB colonies with flat edges and convex elevation. Isolation and purification were examined based on clear zones on MRSA media with 5% $CaCO_3$. Calcium carbonate has buffer activity and serves as an initial selection for lactic acid-producing bacteria. LAB that produces lactic acid will react with $CaCO_3$, resulting in the appearance of clear zones around the colonies after 2-3 days (Figure 1). According to Meryandini *et al.* (2020), the clear zone around the LAB colony indicates the presence of a water-soluble calcium-lactic compound. The eight LAB isolates with different macroscopic characteristics were subjected to physiological testing. Isolates with clear zones around the colonies were streaked on MRSA and incubated for 48-72 hours at 37°C. The eight LABs produce a clear zone and form uniform colonies after the quadrant streak process is carried out because of the purification process. According to Hamidah *et al.* (2019) the isolates that have similar cell morphology indicate that the bacteria belong to the same genera. Pure isolates were stored in Nutrient Broth (NB) with glycerol. Isolate LAB was then used as the selected isolate for the next stage.

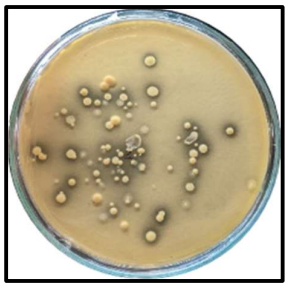

Figure 1. LAB isolates with clear zones around the colonies' growth on MRSA media with 5% $CaCO_3$

Microscopic Morphological Identification using Gram Staining

LAB isolates with clear zones were stained to examine color and cell shape based on cell wall structure differences. Purple color indicates Gram-positive bacteria while red color indicates Gram-negative bacteria. All 8 isolates showed a positive result in dark purple color. The results of microscopic identification obtained 8 LAB isolates with the characteristics presented in Table 1. The eight isolates had characteristics as LAB that were Gram-positive with two round (cocci) and six rod-shaped (bacilli).

Table 1. Gram-staining identification results

Isolate codes	The Microscopic Characteristic		
	Color	Cell shape	Gram
TEa-4130	purple	rod	positive
TEb-4230	purple	rod	positive
TEb-4130	purple	rod	positive
TEb-4340	purple	round	positive
TEb-3150	purple	rod	positive
TEb-3250	purple	rod	positive
TEb-4150	purple	round	positive
TEb-4250	purple	rod	positive

Morphology of LAB isolates from tempeh with a combination of dates and skim milk was observed microscopically in the form of round and rod-shaped cells (Figure 2). All 8 isolates were Gram-positive bacteria because they bound to a crystal violet color, making them appear purple. Microscopic observation carried out Pisol *et al.* (2013) showed

that isolate LAB from Indonesian soybean tempeh was Gram-positive and had the form of a cocci and bacilli. This characteristic can be used to determine the genus of LAB bacteria.

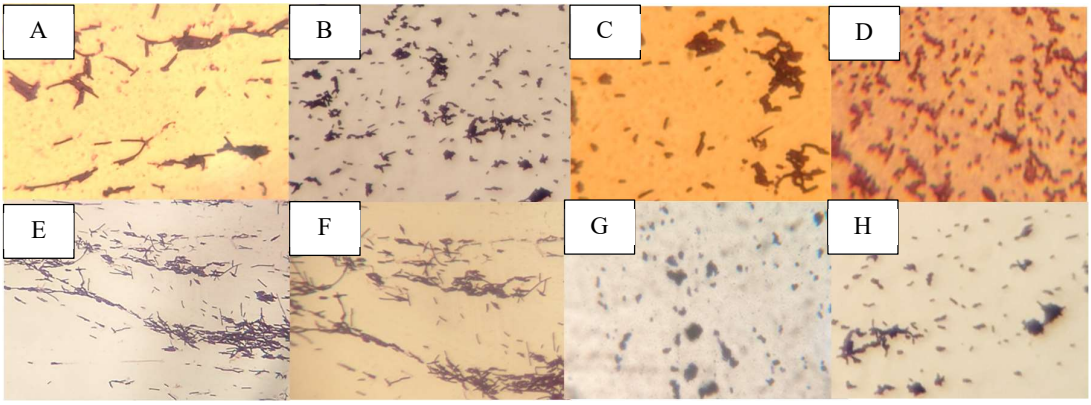

Figure 2. Gram staining results by various cell shapes with 100x magnification; (A, B, C, E, F, and H) = round (cocci) cell shape, meanwhile (D and G) = rod (bacilli) cell shape

LAB characteristic variations are normal, but their Gram-positive nature is absolute (Surono, 2004). The dominant cell shape in this research is rod-shaped. It is known that LAB in the form of rods are classified as *Lactobacillus, Lactococcus*, *Streptococcus*, *Leuconostoc*, *Enterococcus*, and *Pediococcus* (Rahmah *et al*., 2021). During the acidification process of tempeh, the species *Lactobacillus agilis* can also be found (Barus *et al*., 2021).

The cell wall of Gram-positive bacteria is mostly composed of 95% peptidoglycan and the rest is teichoic acid, allowing it to form complex bonds with the main dye, crystal violet (Ismail *et al*., 2017). On the other hand, the cell wall of Gram-negative bacteria is composed of lipid-protein, lipopolysaccharide, and a small amount of peptidoglycan (5-10%). The lipopolysaccharide layer in Gram-negative bacteria strengthens the rigidity of the cell wall through intermolecular cationic cross-linking (Sastry *et al*., 2016). The addition of ethanol to the cell can increase cell wall porosity by dissolving the lipids in the outer membrane, causing the purple complex to be released and the cells to become colorless. Additionally, the cells will become red due to the counterstain, safranin staining them.

Biochemical Testing

After observing macroscopically and microscopically, 8 pure cultures were obtained, and then identification of biochemical characteristics was carried out based on the formation of clear zones as initial characteristics of probiotics. The catalase test results showed that all isolates did not produce the catalase enzyme (negative catalase), which was indicated by the absence of bubbles (Figure 3). LAB does not produce the catalase enzyme, which converts hydrogen peroxide into water and oxygen. Some bacteria require oxygen to form hydrogen peroxide, which is a toxic by-product of aerobic metabolism. Meanwhile, LAB does not require oxygen, because LAB is an anaerobic bacteria (Sastry *et al*., 2016). Following Ismail *et al.* (2017), LAB was reported to have negative catalase.

Figure 3. Catalase test of LAB isolates showed the absence of bubbles

TSIA test is aimed to determine the nature of H_2S production and carbohydrate fermentation. H_2S production is indicated by the formation of a black color on the base of the media (Brown & Smith, 2015). Meanwhile, carbohydrate fermentation occurs when the slant (perpendicular) part of the media is red and the butt (slope) is yellow, indicating that the bacteria can ferment glucose. If both the slant and butt media are yellow, then the bacteria can ferment glucose, lactose, and sucrose (Ismail *et al*., 2017). Figure 4 shows that TSIA test results indicate that all 8 isolates were yellow in the slant and butt, indicating that the bacteria were able to ferment glucose, lactose, and sucrose. No black color was formed at the base of the TSIA media. According to Wikandari *et al.* (2012), the LAB of the genus *Pediococcus* are bacteria that are capable of fermenting sugar without producing gas.

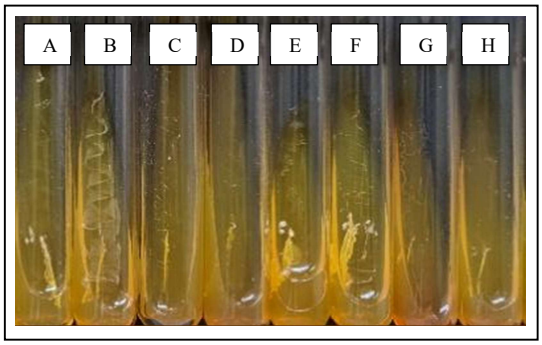

Figure 4. TSIA test results on 8 LAB isolates showed yellow color in the slant and butt media; (A) TEa-4130, (B) TEb-4230, (C) TEb-4130, (D) TEb-4340, (E) TEb-3150, (F) TEb-3250, (G) TEb-4150, (H) TEb-4250

Table 2 shows the results of the motility test, MR test, and SCA test. The motility test is carried out to determine the movement of bacteria. The results of the motility test showed that the eight isolates were non-motile, which was characterized by a white spread at the inoculation puncture site, which means the bacteria did not have a flagellum. To test the presence of acids, methyl red reagent added to the culture medium will be red (positive), which indicates that the microorganism is a producer of mixed acids (Fallo & Sine, 2016). The formation of organic acids from sugar fermentation activity which is able to lower the pH is a characteristic of LAB. The results of the SCA test on the LAB isolates showed negative results, which means that there was no change in the color of the media from green to blue. All results showed that the isolates examined could not use citrate as an energy source. LAB are only able to use sugar for limited growth in environments that contain sufficient sugar

Table 2. Biochemical testing identification results

Test Observation	LAB Isolate Codes							
	TEa-4130	**TEb-4230**	**TEb-4130**	**TEb-4340**	**TEb-3150**	**TEb-3250**	**TEb-4150**	**TEb-4250**
Motility test	non-motile	non-motile	non-motile	non-motile	non-motile	non-motile	non-motile	non-motile
Methyl Red	+	+	+	+	+	+	+	+
SCA	-	-	-	-	-	-	-	-

Physiological Testing

Physiological testing was carried out after biochemical testing. All selected isolates were characterized as shown in Table 3. Probiotic candidate microbes should be able to survive in extreme conditions such as in the digestive tract from the mouth to the intestines. A survival test for LAB was conducted on MRSA+ 1% $CaCO_3$ media containing 7% salt (NaCl) and media without salt. The results showed that 6 isolates grew on media with salt, namely TEa-4130, TEb-4230, TEb-4130, TEb-3150, TEb-3250, and TEb-4250. These results indicate that these 6 isolates are halophilic and can only grow in a high-salt environment. Meanwhile, TEb-4340 and TEb-4150 were unable to grow on media with salt. According to Sunaryanto & Marwoto, (2013), bile salts can penetrate and react on the lipophilic side of the cytoplasmic membrane to break the cell membrane causing bactericidal effects on commensal microorganisms in the human body. The acidity resistance test showed precipitation on the 6 isolates, namely TEa-4130, TEb-4230, TEb-4130, TEb-3150, TEb-3250, and TEb-4250. According to Oluwajoba *et al.* (2013), acid-tolerant microbes are potential probiotics as they can survive at 0.3% bile salt concentration and pH 2. This is due to the conditions in the human digestive tract containing 0.3% bile salts and pH 2-3.

Table 3. Comparison of physiological characteristics of 8 LAB isolates

Test Observation	LAB Isolate Codes							
	TEa-4130	**TEb-4230**	**TEb-4130**	**TEb-4340**	**TEb-3150**	**TEb-3250**	**TEb-4150**	**TEb-4250**
$CaCO_3$								
- MRSA+ 1% $CaCO_3$, 7% NaCl	+	+	+	-	+	+	-	+
- MRSA + 1% $CaCO_3$	+	+	+	+	+	+	+	+
HCl								
- pH 2,5	+	+	+	-	+	+	-	+
- pH 3	+	+	+	-	+	+	-	+

(+): positive (growth)
(-): negative (no growth)

CONCLUSION

Tempeh probiotic drink with a combination of dates and skim milk is reported to be a potential alternative raw material for probiotic drinks besides milk. Eight suspected isolates as LAB had characteristics of rounded colonies, convex elevations, and white in color with clear zones around the colonies. Microscopic observation revealed that all eight isolates were Gram-positive, with two round shapes and six rod-shaped. Catalase test results showed that all eight isolates were negative due to the absence of bubbles. The TSIA test showed a yellow color, indicating the capability to ferment glucose, lactose, and sucrose. All selected isolates non-motile and positive MR and negative SCA testing. Some of the isolates showed tolerance to salts and acids based on physiological testing. The six most promising LAB isolates exhibited important characteristics of LAB that should be evaluated when selecting probiotic candidates, namely TEa-4130, TEb-4230, TEb-4130, TEb-3150, TEb-3250, and TEb-4250 isolates.

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