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Antimicrobial Activity of Lactic Acid Bacteria from Bamboo Shoot Pickles Fermented at 15 °C

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Lactic Acid Bacteria (LAB) produces natural antimicrobial compounds that can inhibit and prevent the growth of spoilage bacteria. LAB can be isolated from fermented food such as pickles, which ferment at cool temperature. The objectives of this research were to isolate and to obtain LAB from yellow Betung bamboo (*Dendrocalamus asper*) shoots pickles that has antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. It was made by submerging yellow bamboo shoots in 2.5% brine solution and keeping them in sealed container, then fermenting them at cool temperature (15 °C) for 10 days. LAB was isolated using MRS agar and identified based on their morphological, physiological and biochemical characteristics. The result showed that LAB isolates identified as *Lactobacilli* and had antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. All *Lactobacilli* (21 isolates) isolated from fermentation at 15 °C were homofementative.

Key words: antimicrobial activity, bamboo shoots pickles, fermentation temperature, lactic acid bacteria

Bakteri asam laktat (BAL) menghasilkan senyawa antimikroba sehingga dapat menghambat dan mencegah pertumbuhan bakteri pembusuk. BAL dapat diisolasi dari makanan fermentasi seperti acar yang difermentasi suhu dingin. Tujuan penelitian ini adalah mengisolasi dan mendapatkan isolat BAL dari acar rebung yang mempunyai aktivitas penghambatan terhadap *Escherichia coli* dan *Staphylococcus aureus*. Rebung difermentasi dalam larutan garam 2.5% dalam wadah tertutup pada suhu 15 °C selama 10 hari. BAL yang berhasil diiolasi diidentifikasi sebagai *Lactobacillus* yang homofermentatif dan mempunyai aktivitas antimikroba terhadap *Escherichia coli* dan *Staphylococcus aureus.*

Kata kunci: Acar rebung, Aktivitas antimikroba, bakteri asam laktat, suhu fermentasi

Utilization of bacteria beneficial to human health in the food world is now more advanced. Basically the bacteria can be divided into two groups, the beneficial bacteria and harmful bacteria. Lactic acid bacteria (LAB) belong to the beneficial bacteria for producing lactic acid. LAB has made a great contribution in the development of fermented foods, particularly in foods that contain probiotics (Lindayani and Hartayanie 2013). LAB includes microorganisms of the genera *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Pediococcus*.

The lactic acid produced from the metabolic activity of LAB causes the condition of raw materials (food) becomes acidic. This is particularly advantageous, given the low pH condition that inhibits the damage caused by other microbes in almost all food (food spoilage microbes). According to Josephsen and Jespersen in Hui *et al*. (2004), LAB produces metabolites that function as antimicrobial compounds including organic acids (lactic acid and acetic acid), bacteriocins, hydrogen peroxide, diacetyl, and CO_2.

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Bamboo shoots are the young shoots of bamboo plants that appear at the bottom surface of the clump. The young shoots of bamboo can be consumed, thus classified as vegetable. Bamboo shoots that can be consumed are usually derived from species *Dendrocalamus asper* (yellow Betung)*, Gigantochloa verticillata*, *Dendrocalamus litiforus* (yellow bamboo), and *Bambusa aldhami* (green bamboo) (Andoko 2003). Raw bamboo shoots have water content of about 90% net weight (Rai 2007).

Bamboo shoots fermentation process takes place spontaneously. The fermentation is done by adding brine. Brine prevents the liquid contained in bamboo shoots drawn out through the process of osmosis and also serves to inhibit the growth of pathogenic bacteria, so only the lactic acid bacteria are expected to grow. The final result of fermentation process is often called pickled bamboo shoots.

The aim of this study is to isolate and identify the lactic acid bacteria of pickled yellow Betung bamboo *(D. asper)* shoots fermented at colder temperatures (15 °C) and 2.5% salt content, and to investigate the antimicrobial activity of the lactic acid bacteria.

MATERIALS AND METHODS

The Pickling of Bamboo Shoots. Two hundred fifty grams yellow Betung bamboo shoots was washed, cut and put in a jar, then 2.5% (w / v) saline solution was added and then the jar was sealed and kept in the incubator. The fermentation process lasted for 10 d at a cold temperature (15 °C) (Rahayu 2003 modified).

Isolation and Purification of Lactic Acid Bacteria. Twenty five mL liquid from pickled bamboo shoots was diluted with 225 mL sterile 0.1% peptone water (10^{-1}× concentration). The dilution process was conducted serially until the liquid was diluted down to 10^{-7}× the original concentration. 0.1 ml sample was taken from each dilution and inoculated into MRS agar medium containing 1% $CaCO_3$ by spreading and incubated at 37 °C for 48 h. Colonies that formed clear zone was further purified using the same methods and media (Yuliana and Dizon 2011).

Morphological Identification. Lactic acid bacterial isolates were identified by microscopic observation based on the shape of the bacteria using Gram staining and spore staining (Sneath *et al.* 1984).

Motility Test. Isolates were taken aseptically using a needle and inoculated by puncturing into motility medium (MRS with 0.7% agar). Incubation was performed at 37 °C for 48 h. Non-motile isolates would have grown only around the puncture, while motile isolates would have spread farther (Rahayu and Margino 1997).

Catalase Test. One loop of isolate was smeared onto an object glass that had been cleaned with alcohol. Then, drops of 3% H_2O_2 solution were added. The formation of gas bubbles indicated that isolates were catalase positive and vice versa (Gawad *et al*. 2010).

Gas Production Test. Fifty µL lactic acid bacteria inoculum was used to inoculate 5 mL MRS broth with 6.5% and 18% NaCl concentrations. Each medium that had been inoculated with lactic acid bacteria was subsequently incubated at 37 °C for 48 h. Observations of growth were done by measuring the absorbance value at 700 nm wavelength at 24 and 48 h (Rahayu and Margino 1997).

Growth at various pH (4.4 and 9.6). Fifty µL lactic acid bacteria culture was inoculated into 5 mL MRS broth media pH 4.4 and pH 9.6 and then incubated at 37 °C for 48 h. Observation of growth were done by measuring the absorbance value at 700 nm wavelength at 24 and 48 h (Rahayu and Margino 1997).

Growth at various temperatures (10 °C, 45 °C, and 50 °C). 50 µL lactic acid bacterial culture was used to inoculate 5 mL MRS *broth* media. Then, each media that had been inoculated was incubated at either 10 °C, 45 °C or 50 °C for 48 h. Observations of growth were done by measuring the absorbance value at 700 nm wavelength, 24 and 48 h after inoculation (Rahayu and Margino 1997).

Growth on Different NaCl Levels (6.5% and 18%). 50 µL lactic acid bacterial culture was used to inoculate 5 mL MRS broth media with different NaCl concentrations (6.5% and 18%). Each medium that had been inoculated with lactic acid bacteria was subsequently incubated at 37 °C for 48 h. Observations of growth were done by measuring the absorbance value at 700 nm wavelength, 24 and 48 h after inoculation (Rahayu and Margino, 1997).

Antimicrobial Activity. Measurement of antibacterial activity was conducted using wells (well assay). Lactic acid bacterial culture was diluted in 0.85% NaCl solution with turbidity level adjusted according to McFarland standard number 5 (Iniguez-Palomares *et al.*2007). One hundred µL of the diluted culture was taken and added to 10 mL liquid MRS medium (Khunajakr *et al.* 2008) for further 24 h incubation at 37 °C. Pathogenic bacteria was grown for 24 h then diluted in 0.85% NaCl solution with turbidity level adjusted to McFarland standard number 3 (Iniguez-Palomares *et al*. 2007). Ten mL of the diluted pathogenic bacterial culture was added to 10 mL of NA medium and poured into a petri dish and let to solidity. Then, a hole with 5 mm diameter was made and filled with 50 mL of lactic acid bacterial culture that had been grown in MRS broth media. Subsequently, the petri dish was stored at 4 °C for 3 h to make sure the bacteria had diffused into the media. After 3 h, the petri dish was moved to 37 °C and further incubated for 24 h. Clear zone formed was measured using a caliper.

RESULTS

Isolation and Identification of Lactic Acid Bacteria. Pickled yellow betung bamboo (*Dendrocalamus asper*) shoots, was used as source of microbial isolation using MRS containing 1% $CaCO_3$ and 10 ppm Na-Azide as selective medium. Na-Azide serves to inhibit aerobic microbial growth by binding to the free O_2. Pure culture was obtained by separating the colonies from one another. Twenty one isolates (Fig 1), which gave clear zone, were found.

All 21 isolates were subsequently identified based on their morphological and physiological characters.

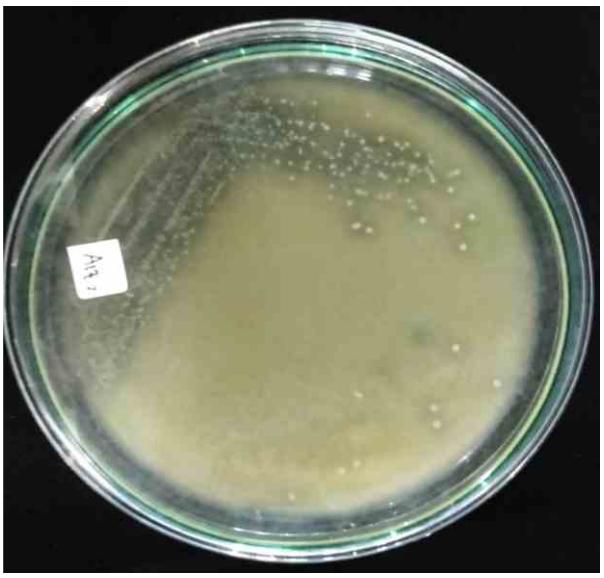


Fig 1 Colonies of lactic acid bacteria form a clear zone (isolate A17).

The identification was done by testing the morphological character by gram and spore staining, catalase test, motility test and gas production (Table 1).

All isolates were identified more specifically to determine the genus of the lactic acid bacteria. The identification was done by testing the ability to grow at different temperatures (10 °C, 45 °C, and 50 °C), different concentrations of NaCl (6.5% and 18%), and at different pH (4.4 and 9.6). It was known that all isolates could grow at 6.5% NaCl, pH 4.4 and 45 °C. Most isolates can grow at 10 °C and a small percentage of isolates can grow at 50 °C. However, none of these isolates could survive at pH 9.6 and 18% NaCl. These results show that all the tested isolates belong to the genus *Lactobacillus* (Table 2).

Antimicrobial Activity of Lactic Acid Bacteria. Lactic acid bacteria isolates were tested for antimicrobial activity. Antimicrobial activity test was conducted using agar diffusion method and using two bacterial pathogens, *Escherichia coli* strain ATCC 252 922 (Gram-negative) and *Staphylococcus aureus* strain ATCC 252 923 (Gram-positive) (Fig 2).

Isolate A12 had the highest antimicrobial capability against *S. aureus* with inhibition zone diameter of 11.07 mm, while isolate A20 had the highest antimicrobial capability against *Escherichia coli* with inhibition zone diameter of 10.93 mm (Fig 3). These results indicate that both isolates have very strong antimicrobial ability.

Some isolates (A1, A13, A19, and A23) showed no inhibition against *S.aureus* dan *E.coli*. The isolates which had antimicrobial activity showed larger clear zone against *S. aureus* than *E. coli* except A20, A24, and A31 (Table 3).

DISCUSSION

In this research, shoots of yellow Betung bamboo (*D. asper*) was used for fermentation. The fermentation process was carried out for 9 d at 15 °C and was completed when the pH decreased to below 4.5, because lactic acid bacteria will stop growing at lower pH (Rehm and Reed 1996). Fermentation performed at low temperatures takes longer than the fermentation process in general. The lower temperature can lead to inhibition of the growth of lactic acid bacteria, so that limited amount of lactic acid was produced due to slower fermentation process. Clear zone around the colony arised due to the reaction between the acid produced by bacteria with $CaCO_3$ in media resulting soluble Ca-lactate in the media and seen as a clear zone.

Table 1 showed that all isolates have characteristics similar to the common characteristics of lactic acid bacteria. They were gram-positive, rod-shaped or cocci, catalase negative, non-spore-forming, non-motile and produced acids (mainly lactic acid and acetate) (Ghiasi 2011; Aly *et al*. 2006). All isolates were identified as *Lacotobacillus* (Table 2). *Lactobacillus* growth characteristics include capability to grow at suitable temperatures 10 °C-50 °C, 6.5% NaCl and pH 4.4, but unable to grow at 18% NaCl and pH 9.6 (Rahayu and Margino 1997). This result is quite different from the research conducted by the Tamang and Sarkar (1996) in Choudhury *et al*, (2012), whostated that *Lactobacillus* were not the only LAB found in bamboo shoots fermented at room

Table 1 Identification of lactic acid bacteria in pickled betung bamboo shoots (*Dendrocalamus asper*)

Isolates	Clear zone	Gram staining	Spore Staining	Bacteria Form	Catalase	Motility	Gas production	Result
A1	+	+	-	b	-	-	+	B
A2	+	+	-	b	-	-	+	B
A3	+	+	-	b	-	-	+	B
A11	+	+	-	b	-	-	+	B
A12	+	+	-	b	-	-	+	B
A13	+	+	-	b	-	-	+	B
A15	+	+	-	b	-	-	+	B
A16	+	+	-	b	-	-	+	B
A17	+	+	-	b	-	-	+	B
A18	+	+	-	b	-	-	+	B
A19	+	+	-	b	-	-	+	B
420	+	+	-	b	-	-	+	B
A23	+	+	-	b	-	-	+	B
A24	+	+	-	b	-	-	+	B
A27	+	+	-	b	-	-	+	B
A31	+	+	-	b	-	-	+	B
A32	+	+	-	b	-	-	+	B
A33	+	+	-	b	-	-	+	B
A34	+	+	-	b	-	-	+	B
A43	+	+	-	b	-	-	+	B
A44	+	+	-	b	-	-	+	B

The bacterial form of basil: "-" = negative / no form: "+" = Positive / form: "B" = the lactic acid bacteria.

temperature, but also *Pediococcus* and *Leuconostoc*. This indicates that the fermentation temperature has an influence on the growth of LAB.

E. coli strain ATCC 252 922 (Gram-negative) and *S. aureus* strain ATCC 252 923 (Gram-positive) were used for antimicrobial test because both bacteria were the most common pathogens attacking human. *S. aureus* is a gram-positive that lives as a saprophyte in the membrane channels of the human body, the surface of the skin, sweat glands, and the intestinal tract, while *E. coli* is a gram-negative commonly found in the human colon as normal flora. All isolates have antimicrobial activity or inhibitory ability against pathogens as indicated by the appearance of clear zone around the holes was filled with isolate (Fig 2).

This is due to the differences in the composition and structure of the cell wall of Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E.coli*). Cell wall structure of Gram-positive bacteria is more simple, single-layered with a low lipid content (1-4%), to facilitate the bioactive material transport into the cell. On the other hand, Gram negative bacteria have

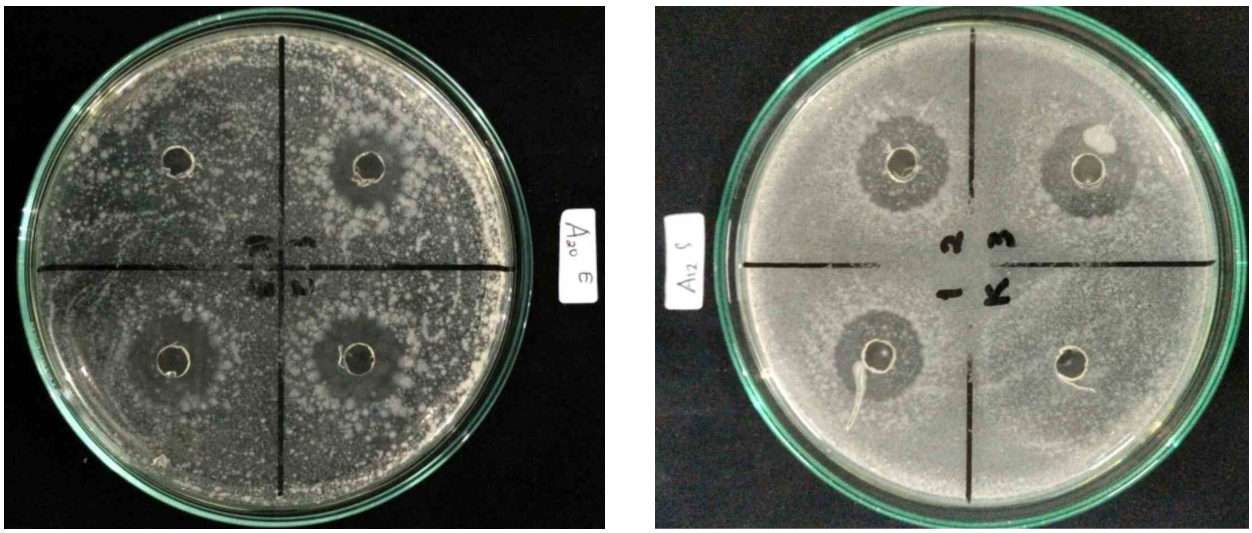
Table 2 Results identification of lactic acid bacteria growth bacteria based capabilities at various ph, temperature, and

Isolates	pH		Temperature (°C)			NaCl (%)		Genus
	4.4	9.6	10	45	50	6.5	18	
A1	+	-	-	+	+	+	-	*Lactobacillus*
A2	+	-	+	+	-	+	-	*Lactobacillus*
A3	+	-	+	+	-	+	-	*Lactobacillus*
A11	+	-	+	-	-	+	-	*Lactobacillus*
A12	+	-	+	+	-	+	-	*Lactobacillus*
A13	+	-	+	+	-	+	-	*Lactobacillus*
A15	+	-	-	+	+	+	-	*Lactobacillus*
A16	+	-	+	+	-	+	-	*Lactobacillus*
A17	+	-	+	+	-	+	-	*Lactobacillus*
A18	+	-	-	+	+	+	-	*Lactobacillus*
A19	+	-	+	+	-	+	-	*Lactobacillus*
420	+	-	+	+	-	+	-	*Lactobacillus*
A23	+	-	+	+	-	+	-	*Lactobacillus*
A24	+	-	+	+	-	+	-	*Lactobacillus*
A27	+	-	+	+	-	+	-	*Lactobacillus*
A31	+	-	+	+	-	+	-	*Lactobacillus*
A32	+	-	+	+	-	+	-	*Lactobacillus*
A33	+	-	+	+	-	+	-	*Lactobacillus*
A34	+	-	+	+	-	+	-	*Lactobacillus*
A43	+	-	+	+	-	+	-	*La ctobacillus*
A44	+	-	+	+	-	+	-	*Lactobacillus*

+ = isolates grow
- = isolates did not grow

more complex structure, consisting of a three-layered outer layer lipoprotein, lipopolysaccharide middle layer which acts as a barrier to the entry of antibacterial compounds, and peptidoglycan inner layer with a high lipid content (11-12%) (McKane and Kandel 1996).

The ability of lactic acid bacteria in inhibiting the growth of pathogenic bacteria was due to the antimicrobial components produced by lactic acid bacteria, one of which is lactic acid. The lactic acid caused a decrease in pH so that the growth of Gram-positive (+) and Gram negative (-) bacteria which cannot stand low pH would be inhibited. Although gram-negative bacteria have lipopolysaccharide layer, the lactic acid can still inhibit it . This is because lactic acid is a water-soluble molecule that can permeate into the outer membrane. Lipopolysaccharide layer was damaged by lactic acid so that other antimicrobials such as diacetyl, bacteriocins and hydrogen peroxide can enter the cells (Alokomi *et al*. 2000 in Afriani 2012). From this study, it can be concluded that twenty



A B

Fig 2 The test results on the antimicrobial activity of isolate A20 pathogenic bacteria *Escherichia coli* (a); A12 on *Staphylococcus aureus* (b). k: control (medium without the addition of isolates; 1: isolates test (I repeat); 2: isolates test (replications II); 3: isolates test (replications III).

Table 3 Antimicrobial activity

Isolates code	Diameter clear zone (mm)	
	S.aureus	*E.coli*
A1	0.00	0.00
A2	9.77	9.03
A3	6.67	10.5
A11	10.67	10.53
A12	11.07	7.97
A13	0.00	0.00
A15	9.00	8.20
A16	10.40	9.17
A17	0.00	0.00
A18	8.47	7.90
A19	0.00	0.00
A20	9.70	10.93
A23	0.00	0.00
A24	7.23	8.70
A27	7.60	7.27
A31	6.20	9.57
A32	10.27	7.47
A33	6.90	0.00
A34	14.53	9.30
A43	10.70	9.67
A44	427	400

one isolates of lactic acid bacteria found in pickled bamboo shoots fermented at 15 °C were identified as *Lactobacillus*. Seventeen isolates had antimicrobial activity that can inhibit the growth of pathogenic bacteria *E. coli* and *S. aureus*. Isolate A34 has the highest antimicrobial activity against *S. aureus* with inhibition zone diameter 14.53 mm and isolate A20 had the highest antimicrobial ability against *E. coli* with inhibition zone diameter 10.93 mm

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