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Preliminary Selection of Lactic Acid Bacteria Isolated from Cocoa Beans *(Theobroma cacao)* as Probiotics Candidates

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ABSTRACT

Cocoa beans are covered in mucilage which is rich in carbohydrates, and they are a good medium for the growth of lactic acid bacteria (LAB). These bacteria can be used as probiotics that are beneficial to human health. Furthermore, LAB improves the quality of cocoa beans during fermentation. Therefore, this study aims to isolate and identify LAB in spontaneously fermented cocoa beans which are potential probiotics candidates. The isolation was selectively conducted using an MRS broth medium supplemented with 1% $CaCO_3$. In addition, the potential of LAB as probiotics was selected based on acid resistance, bile salt resistance, antimicrobial activity, and sensitivity test to antibiotics. The identity of LAB was confirmed based on the API 50 CHL test. The results showed that three LAB isolates of strains SK1B1, SK2T2, and SK3B4 had survival tolerance at pH conditions in gastric acid (pH 2.5, pH 3, and pH 4), were to survive on bile salts (0.3%, 0.5% and 1%) and has an antibacterial ability. Furthermore, the LAB isolates were identified as a species of Lactobacillus plantarum (ID 99.9%).

SELEKSI AWAL BAKTERI ASAM LAKTAT DARI BIJI KAKAO (Theobroma cacao) SEBAGAI KANDIDAT PROBIOTIK

ABSTRAK: *Biji kakao diselimuti oleh lendir yang kaya akan kandungan karbohidrat. Oleh karenanya biji kakao merupakan media yang baik untuk pertumbuhan bakteri asam laktat. Bakteri asam laktat (BAL) memiliki potensi sebagai probiotik yang sangat bermanfaat bagi kesehatan manusia. Pada fermentasi biji kakao, BAL memiliki kontribusi penting terhadap peningkatan kualitas biji kakao. Penelitian ini bertujuan untuk isolasi dan identifikasi BAL yang tumbuh pada biji kakao terfermentasi spontan yang berpotensi sebagai kandidat probiotik. Isolasi dilakukan secara selektif menggunakan medium MRS agar yang dilengkapi dengan 1% $CaCO_3$. Potensi BAL sebagai probiotik diseleksi berdasarkan uji ketahanan asam, uji ketahanan terhadap garam empedu, uji aktivitas antimikrobia, dan uji sensitivitas terhadap antibiotik. Identitas BAL dikonfirmasi berdasarkan uji API 50 CHL. Hasil penelitian menunjukkan tiga isolat BAL yaitu strain SK1B1, SK2T2 dan SK3B4 memiliki toleransi hidup pada kondisi pH pada asam lambung (pH 2,5, pH 3 dan pH 4), mampu bertahan pada garam empedu (0,3%, 0,5 % dan 1%) serta memiliki*

kemampuan antibakteri. Tiga isolat BAL tersebut diidentifikasi sebagai spesies Lactobacillus plantarum (ID 99,9%).

INTRODUCTION

Cocoa is a plantation commodity that contributes to the Indonesian economy, making its production very important (Manalu, 2019). This condition opens up opportunities to improve the quality of cocoa beans which is still relatively low through the fermentation process (Meryandini et al., 2019). The application of lactic acid bacteria (LAB) for cocoa beans fermentation improves the quality of products, especially to ensure delicacy, shelf life, and safety for consumption (Fahrurrozi et al., 2019).

Cocoa beans (*Theobroma cacao* L.) are the raw material for chocolate production. The fermentation of cocoa pulp by microorganisms is important for developing chocolate flavor precursors (Ho et al., 2015). The process is complex and in the first, second, and third phase, it involves the succession of anaerobic growth of various yeast species, LAB and acetic acid bacteria, and possibly *Bacillus* species, other bacteria, and filamentous fungi (Vuyst et al., 2010). During fermentation, microbes dissolve the pulp around the beans and produce various metabolic end products (such as alcohol and organic acids). This will diffuse into the beans causing death and changes that induce a series of biochemical reactions and produce chemicals that pioneer the taste, aroma, and color of chocolate (Ardhana & Fleet, 2003).

Lactobacillus plantarum and *Lactobacillus fermentum* are the most dominant LAB species found in cocoa beans fermentation (Ho et al., 2015). Fahrurrozi et al., (2019) identified the species *Pediococcus acidilactici, L. plantarum subsp. plantarum*, and *Lactobacillus pentosus* isolated from fermented cocoa beans. Furthermore, the characterization of *L. plantarum* and *L. fermentum* isolates from Brazilian cocoa fermentation showed significant results for the development of new probiotic cultures (Melo et al., 2017). *Lactobacillus fermentum* obtained from natural cocoa beans fermentation plays a role in reducing fungal contamination (Winata, 2019) and reducing mycotoxin content (Ariyanti & Suprapti, 2018; BAHARUDIN et al., 2020; Fitriyana et al., 2016).

Therefore, it is necessary to develop LAB strains from local resources to be used as probiotics or starter cultures. It is still relevant to Improve the quality of cocoa beans. Kusuma tested cocoa beans as a source of antioxidants (Kusuma et al., 2013) and Kayaputi tested the phytochemical components as preservatives in cocoa beans (Kayaputri et al., 2014). Furthermore, Hendradi (Hendradi et al., 2013) examined cocoa beans' SPF content for cream preparations.

Efforts to develop LAB strains from local resources to be used as probiotics or starter cultures to improve the quality of cocoa beans are still relevant. Therefore, this study aims to identify LAB isolates that have the potential as probiotic candidates to improve the quality of cocoa beans in giving a distinctive chocolate character.

METHOD

The samples of cocoa beans were obtained from farmer groups Margo Dadi, Hargo Mulyo and Sido Muncul in the Gunung Kidul area, Yogyakarta. The cocoa pods taken were of the Lindak da criollo variety, and each cocoa bean was measured in length and diameter. It was divided into three parts, namely top, middle, and bottom, and about 25 g of LAB isolation and its mucus (pulp) was taken.

This study phase was divided into three parts, and the first was the isolation of LAB and its morphological and physiological identification. The second was the screening and selection of potential LAB candidates as probiotics through acid resistance testing and the ability to grow in bile salts as well as antimicrobial activity. The third was the

identification of selected LAB isolates based on the API 50 CHL test.

a. Isolation and Selection of Bacterial Isolates from Cocoa Beans

The isolation of LAB was conducted by growing 25 g of beans and pulp samples in 225 ml of MRS (Oxoid) broth, then incubated at 37°C for 48 hours. Furthermore, the isolation method started from bacterial isolates cultured in an MRS broth medium enriched with $CaCO_3$ by pour plate, then incubated at 37°C for 48 hours (Fitriyana et al., 2015).

The selected colonies with bright zones were purified to obtain single colonies. Meanwhile, the colonies were taken through a loop and streaked on MRS broth using the quadrant method. The Petridish was incubated for 24 hours at 37°C, and the single colonies obtained were re-cultured in MRS broth since they were slanted as pure isolates (Amaliah et al., 2018). Furthermore, the LAB characterization was conducted based on the method of Amaliah et al. (2018). It was modified with morphological characterization, Gram staining, and biochemical tests consisting of catalase, motility, and gas production test, as well as the ability to grow at temperatures of 10°C and 45°C.

b. LAB Isolate Screening as Probiotic Candidate and Antibacterial Producer

Probiotic candidates were selected based on the bacteria resistance to acid and bile salt conditions as well as antimicrobial activity (Shi et al., 2012).

Test for Resistance of Lactic Acid Bacteria to Acid

The LAB isolates tested were grown in 3 ml of MRS broth medium with a pH of 2.5; 3; and 4 for 48 hours. Meanwhile, the cells were then harvested by centrifugation at 13,500 rpm for 15 minutes, and the pellets were washed with phosphate-buffered saline (PBS, Merck) twice and dissolved in 100 µl of PBS solution. The formed suspension was grown in 5 ml of MRS broth at 37°C for 4 hours, and the LAB growth was confirmed in MRS agar using the streak plate method (Shi et al., 2012).

Test for Resistance of Lactic Acid Bacteria to Bile Salts

The LAB isolates were grown in 3 mL MRS broth with bile salt concentrations of 0.3% each; 0.5%; and 1.0% for 24 hours at 37°C. Furthermore, the LAB cultures were then grown in MRS agar on a streak plate for 24 hours at 37°C (Shi et al., 2012).

Antibacterial Activity Test

The antibacterial activity test was conducted using a cell-free culture supernatant (CFCS) solution prepared by growing LAB isolate in MRS broth medium at 37°C for 18 hours. Meanwhile, the cells were harvested by centrifugation at 10,000 rpm for 10 min, and the supernatant was neutralized at pH 6.5 using 1 N NaOH solution and stored in a refrigerator (Siddiqi et al., 2018).

The antibacterial activity test was conducted based on the well-diffusion method, and the indicator bacteria used were *Staphylococcus aureus* ATCC 25923, *Salmonella typhi* NCTC 786 (PT Biofarma), and *Salmonella typhi* BPE 122.4CCA (Amarantini et al., 2019). Furthermore, the bacteria were swabbed using a sterile cotton swab on the surface of the Mueller Hinton Agar (MHA, Himmedia) medium and an 8 mm well was made. In each well, 150 µl of CFCS solution was added, incubated at a cold temperature to allow faster diffusion of the solution into the medium. After incubated at 37°C for 24 hours, the diameter of the clear zone formed around the well was measured (Siddiqi et al., 2018; Fernandez et al., 2013; Klayraung & Okonogi, 2009). Therefore, the zone of inhibition less than 10 mm was categorized as weak, 10-15 mm was categorized as moderate, and greater than 15 mm was categorized as strong (Alsaid et al., 2010).

c. Identification of LAB isolates using the API 50 CHL kit

The LAB isolates were grown in MRS broth for 48 hours, and the cells were harvested and inoculated in 10 ml of API 50 CH medium. Meanwhile, the turbidity was standardized using a 0.5 McFarland solution, and the cell suspensions were inoculated into wells on API CHL 50 strips. Each well was added with paraffin oil to provide anaerobic conditions and incubated at 37°C. After 48 hours, the ability of the isolates to ferment various types of sugar was observed, and the identity was known after the data was processed using *Apiweb*™ software (Biomerieux, 2009) (Utama et al., 2018).

RESULTS AND DISCUSSION

a. Isolation and selection of lactic acid bacteria from cocoa beans

The LAB growing on an MRS broth medium containing 1% $CaCO_3$ was shown by the presence of a clear zone around the colonies. Furthermore, 22 isolates were obtained from these colonies with morphological characteristics of short rod-shaped cells, Gram-positive, non-motile, catalase-negative, homofermentative, and can grow at temperatures of 10°C and 45°C (Table 1). The LAB colonies were seen as transparent yellow or cream, growing in groups or single with neat edges (Figure 1). Also, the morphological characteristics of the LAB isolate cells were following the group isolated from similar studies conducted by (Amarantini et al., 2019; Amaliah et al., 2018; Bennani et al., 2017; Fitriyana et al., 2016).

b. Screening of Lactic Acid Bacterial Isolates as Probiotic Candidates

The results of LAB isolate screening based on survival in acidic conditions showed that 11 out of 22 LAB survived at pH 2.5. Furthermore, the isolates that grew at pH 3.0 and pH 4.0 were more than those at pH 2.5, and about 18 of the 22 survived on 1.0% bile salts (Table 2). Meanwhile, about 10 of the 11 isolates growing at pH 2.5 were known to be able to grow on 1% bile salts. Therefore, the 10 LAB isolates were potential probiotic candidates (Shewale et al., 2014). The LAB should be tolerant of extreme conditions in the human digestive tract such as acidic pH and bile salts as a probiotic candidate (Makinen et al., 2012). Also, they should counteract the pH of the gastric acid fluid in the human body, which is around pH 3.0 with a digestion time of 1-3 hours (Qian et al., 2018).

Table 1. Characteristics of LAB isolated from Cocoa Pulp and Beans

Isolate	Cell		Homo/ Hetero-Ferment	Motility	Catalase	Temperature (°C)	
	Shape	Gram				10	45
SK1A1	Rod	+	Homo	-	-	+	+
SK1T1	Rod	+	Homo	-	-	+	+
SK1T2	Rod	+	Homo	-	-	+	+
SK1B1	Rod	+	Homo	-	-	+	+
SK2A1	Rod	+	Homo	-	-	+	+
SK2A2	Rod	+	Homo	-	-	+	+
SK2T1	Rod	+	Homo	-	-	+	+
SK2T2	Rod	+	Homo	-	-	+	+
SK2T3	Rod	+	Homo	-	-	+	+
SK2B1	Rod	+	Homo	-	-	+	+
SK2B2	Rod	+	Homo	-	-	+	+
SK3B1	Rod	+	Homo	-	-	+	+
SK3B2	Rod	+	Homo	-	-	+	+
SK3B3	Rod	+	Homo	-	-	+	+
SK3B4	Rod	+	Homo	-	-	+	+
SK3B5	Rod	+	Homo	-	-	+	+
SK3B6	Rod	+	Homo	-	-	+	+
SK3B7	Rod	+	Homo	-	-	+	+
SK3T1	Rod	+	Homo	-	-	+	+
SK3T2	Rod	+	Homo	-	-	+	+
SK3T3	Rod	+	Homo	-	-	+	+
SK3T4	Rod	+	Homo	-	-	+	+

Description: + = growth; - = no growth to the tested character

The antimicrobial activity results showed that not all LAB isolates had the ability to inhibit pathogenic bacteria. Table 2 showed that only 4 of the 22 had inhibition against the test indicator bacteria in the moderate inhibitory category (Alsaid et al., 2010). Furthermore, the LAB isolates were SK1B1, SK2T2, SK3B3, and SK3T3. BAL K1B1 isolate only inhibited Gram-negative indicator bacteria (*S. typhi* BPE 122.4CCA). The SK3T3 isolate was known to inhibit

Gram-positive indicator bacteria (*S. aureus* ATCC 25923).

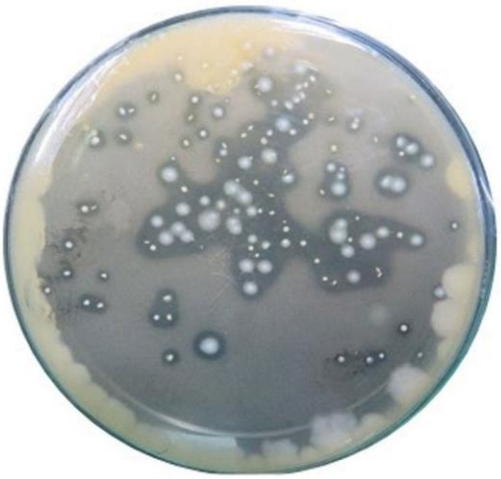

Figure 1. Characteristics of LAB colonies on MRS broth $CaCO_3$ media. LAB colonies isolated from cocoa pulp and beans.

Table 2. Test Results of LAB Isolates as Probiotic Candidates

Isolate	pH			Bile salts (%)			Inhibition Category		
	2.5	3	4	0,3	0,5	1	*S. typhi* BPE 122.4 CCA	*S. typhi* NCTC 786	*S. aureus* ATCC 25923
SK1A1	+	+	+	+	+	+	w	w	w
SK1T1	+	+	+	+	+	-	w	w	w
SK1T2	+	+	+	+	+	+	w	w	w
SK1B1	+	+	+	+	+	+	m	w	w
SK2A1	-	+	+	+	+	+	w	w	w
SK2A2	+	+	-	+	+	+	w	w	w
SK2T1	-	+	+	+	+	+	w	w	w
SK2T2	+	+	+	+	+	+	m	m	m
SK2T3	-	-	+	+	+	+	w	w	w
SK2B1	-	-	+	+	+	+	w	w	w
SK2B2	-	-	+	+	+	+	w	w	w
SK3B1	+	+	+	+	+	+	w	w	w
SK3B2	+	+	+	+	+	+	w	w	w
SK3B3	-	+	+	+	-	+	w	w	w
SK3B4	+	+	+	+	+	+	w	m	m
SK3B5	-	-	+	+	-	-	w	w	w
SK3B6	+	+	+	+	+	+	w	w	w
SK3B7	+	+	+	+	+	+	w	w	w
SK3T1	-	+	+	+	+	+	w	w	w
SK3T2	-	+	+	+	-	-	w	w	w
SK3T3	-	+	+	+	+	+	w	w	m
SK3T4	-	+	+	+	+	-	w	w	w

Description: + = growth; -= no growth; w= weak; m= moderate

Two other LAB isolates (SK2T2 and SK3B4) inhibited *S. aureus* ATCC 25923 and *S. typhi* BPE 122.4CCA. Most of the isolates in acidic pH conditions and bile salts were known to have no inhibitory power against pathogenic bacteria (Table 2). The LAB isolates from fermented cocoa beans had an inhibitory effect on *Escherichia coli, Bacillus subtilis*, and *S. aureus* (Fahrurrozi *et al*., 2019). Furthermore, it had inhibitory activity on *Salmonella sp., S. aureus,* and *E. coli* (Sari *et al*., 2018).

c. Identification of LAB Isolates using the API 50CHL kit

Based on the screening of LAB isolates that have potential as probiotics, three were selected, namely strains SK1B1, SK2T2, and SK3B4, and were identified using the API 50CHL kit. The three isolates had the same ability to ferment glucose, fructose, lactose, maltose, galactose, sucrose, mannose, ribose, sorbitol, mannitol, cellobiose, melibiose, trehalose, melezitose, raffinose, N-acetyl-glucosamine, amygdalin, arbutin, arbutin. , salicin, β-gentiobiose, and D-turanose (Table 3). In addition, the LAB isolates SK1B1 and SK2T2 were identified as *Lactobacillus plantarum 1* (99.9%).

The LAB strain SK3B4 fermented L-arabinose and 22 types of sugars such as SK1B1 and SK2T2 (Table 3). Meanwhile, SK3B4 was identified as *L. plantarum* 1 species (99,9%). Fahrurrozi *et al*., (2019) found that the species *L. plantarum subsp. plantarum, L. pentosus,* and *Pediococcus acidilactici* are lactic acid bacteria in cocoa beans. Similarly, Ayertey et al., (2017) found *Lactococcus sp.* and *Lactobacillus sp.* in cocoa beans. Based on the identification of the *L. plantarum* species, the results of this preliminary study showed the potential application of indigenous lactic acid bacteria to be developed as probiotics to produce better quality chocolate.

Table 3. Fermentation Test Results for Various Carbon Sources using the API 50 CHL kit

No	Type of carbon sources	Isolates		
		SK1B1	SK2T2	SK3B4
0	*Control*	-	-	-
1	*Glycerol*	-	-	-
2	*Erythritol*	-	-	-
3	*D-Arabinose*	-	-	-
4	*L-Arabinose*	-	-	+
5	*Ribose*	+	+	+
6	*D-Xylose*	-	-	-
7	*L-Xylose*	-	-	-
8	*Adonitol*	-	-	-
9	*B Methyl-D-Xyloside*	-	-	-
10	*Galactose*	+	+	+
11	*Glucose*	+	+	+
12	*Fructose*	+	+	+
13	*Mannose*	+	+	+
14	*Sorbose*	-	-	-
15	*Rhamnose*	-	-	-
16	*Dulcitol*	-	-	-
17	*Inositol*	-	-	-
18	*Mannitol*	+	+	+
19	*Sorbitol*	+	+	+
20	*Methyl-D-Mannoside*	-	-	-
21	*Methyl-D-Glucoside*	-	-	-
22	*N-Acetyl-Glucosamine*	+	+	+
23	*Amygdalin*	+	+	+
24	*Arbutin*	+	+	+
25	*Esculin*	+	+	+
26	*Salicin*	+	+	+
27	*Cellobiose*	+	+	+
28	*Maltose*	+	+	+
29	*Lactose*	+	+	+
30	*Melibiose*	+	+	+
31	*Sucrose*	+	+	+
32	*Trehalose*	+	+	+
33	*Inulin*	-	-	-
34	*Melezitose*	+	+	+
35	*Raffinose*	+	+	+
36	*Starch*	-	-	-
37	*Glycogen*	-	-	-
38	*Xylitol*	-	-	-
39	*B Gentiobiose*	+	+	+
40	*D-Turanose*	+	+	+
41	*D-Lyxose*	-	-	-
42	*D-Tagatose*	-	-	-
43	*D-Fucose*	-	-	-
44	*L-Fucose*	-	-	-
45	*D-Arabitol*	-	-	-
46	*L-Arabitol*	-	-	-
47	*Gluconate*	-	-	-
48	*2-Keto-Gluconate*	-	-	-
49	*Keto-Gluconate*	-	-	-

CONCLUSIONS AND SUGGESTIONS

Three LAB isolates of K1B1, K2T2, and K3B4 from cocoa beans grew at gastric pH conditions with bile salt resistance up to 1% and had antimicrobial activity. Furthermore, the LAB isolates were identified as *L. plantarum* (ID 99.9%), and the results of the initial screening showed that they can act as probiotics. Also, they improve the quality of cocoa beans during the fermentation process.

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