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Phenotypic Identification of Lactic Acid Bacteria From Civet (*Paradoxorus hermaphroditus)*

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***Abstract*— Kopi luwak (civet coffee) is widely known as the most expensive coffee in the world. As quality is known, the popularity of kopi luwak also increases, which leads to the increasing demands for this coffee. Kopi luwak has high economic value due to its rarity, unique flavor. and a distinctive production process. An alternative for kopi luwak production is employing an in vitro fermentation to imitate the natural fermentation on the digestive system of civet. Fermentation is usually carried out by indigenous microflora, the existence of the microflora thought to have a role in civet coffee production. A type of bacteria suspected a role in the digestive tract is lactic acid bacteria (LAB). The study aims to identify LAB from the digestive tract of civet. The isolation of LAB was carried out by using MRS agar, and their characterization by phenotypic analysis. The results showed that the characteristic of LAB varied. Based on API analysis, the study successfully identified LAB, which are *Pediococcus pentosaceus, Lactococcus lactis* spp. *lactis, Lactobacillus fructivorans*, and *Lactobacillus brevis*. LAB with protease and pectinase activity might be used as a starter culture for the production of artificial kopi luwak. Hence, a further study related to their technical characteristics must be done to maintain kopi luwak availability.**

***Keywords*— kopi luwak; civet; LAB; phenotypic; fermentation.**

I. INTRODUCTION

Civet (*Paradoxurus Hermaphroditus*) is known as *Luwak* or *musang* in Indonesia. It is one of the species of small mammals that belong to South East Asia. Civet can be found throughout tropical East Asia, including Vietnam, Srilanka, India, Philippines, and Malaysia, where the little mammal inhabits tropical rain forests as well as coffee plantation and fruit yards. According to Smith [1], civet is a solitary animal (used to live alone), arboreal, and also shows nocturnal activity. They can live in a variety of different habitats, depending on the food supplies of the season. Kopi luwak or also popular as civet coffee is claimed as one of the most expensive coffee nowadays. People have been discussing this unique coffee because of its uncommon harvesting and distinctive origin. The process starts with civet picks and eats the coffee berries according to its preferences. Then the coffee berries pass through its digestive system. However, since the civet is unable to digest the seed (coffee bean), then it excretes among its feces. Local coffee farmers are eagerly waiting to collect it, which then goes under a long post-harvest process from cleaning, drying to roasting become drinkable [1]-[3].

As quality is known, the popularity of kopi luwak also increases, which leads to the increasing demands for this coffee. As stated by supply and demand law, the limited supply of kopi luwak influences its high price. Kopi luwak is roughly available 500-700 kg per year [1]. Not only the rarity supply, but its economic value is also attributed to its unique flavor and distinctive production process. The increasing demand for kopi luwak has led to civet abuse and malnutrition [4]. As a wild animal, civet is unlikely able to produce kopi luwak for commercial usage. Neither the caged production nor considers as a better option. Production of kopi luwak without civet (in vitro fermentation) is an alternative. It is suggested that digestive conditions of the

civet impart the unique flavor and aroma to kopi luwak. Fermentation takes place throughout the digestive tract of all animals, but the intensity of fermentation is according to numbers of the microbe and its population. According to Marcone [5], enzymatic process and bacteria found in the digestive tract of civet change the chemical composition of coffee beans and manifest its specific taste. The presence of different microbes in the digestive tract of civet will differ and characterize the fermentation process. Therefore, comprehensive knowledge about the microbiology of civet's digestive tract could facilitate the understanding and application of starter cultures for the in vitro kopi luwak fermentation process. Unfortunately, the microflora study of civet's digestive tract is still scarce.

One type of bacteria predicted has a role in the digestive tract is lactic acid bacteria (LAB). LAB are common bacteria found in the gastrointestinal and genitourinary tract of humans and animals [6]. In these environments, the LAB has an essential role in health-promoting functions such as immunomodulation, intestinal integrity, and pathogen resistance. LAB encompasses the heterogeneous group of microorganisms that have a common metabolic property to produce lactic acid as the main product at the end of carbohydrates fermentation. LAB members are nonpathogenic organisms, majority LAB strains have a positive impact on human health and are generally regarded as safe (GRAS). Many species of LAB have a role as the manufacture and preservation of fermented foods to control fermentations. Enzymatic activities of LAB contribute to the final sensory, nutritional, and rheological characteristics of fermented products [6][7].

The exploitation of LAB as a starter and probiotic cultures is very significant. Recently, studies related to LAB sources, the characteristics, and applications have boomed in. As to conclude, LAB is a promising option to control the fermentation process and to promote coffee quality [6]. According to Pereira et al. [8], the use of LAB in coffee processing can improve coffee quality, specially on-farm wet coffee processing. From a microbiological perspective, there is little information available regarding the microbial ecology of civet's digestive tract. The study of LAB characteristics from civet's digestive tract is a vital point since it is the basis in determining their capacity on coffee fermentation. The bacteria group is thought to have a role in kopi luwak fermentation. Thus, this research aims to characterize LAB species isolated from the digestive tract of civet.

II. Material and Method

A. Sample Preparation, Quantification and Isolation

Wild civet was obtained from the natural forest in Takengon, Central Aceh. The animal was then aseptically dissected by a veterinarian. The liquid sample about 10 ml was taken from 3 parts of the civet's digestive tract (stomach, intestine, and colon) then placed onto flasks with 90 ml of sterile peptone water, homogenized in a shaker and used for decimal serial dilution. Samples were diluted serially in sterile peptone water (10^{-1} to 10^{-9}), plated onto MRS (Merck) agar media followed by incubation under an anaerobic condition at 37^{o}C for 48 h. Quantification of LAB was estimated by counting the average CFU (colony-forming units) per milliliter on each agar plate. Single colonies with different morphologies (color, form, and size) were selected from each plate and purified by quadrant streaking on new agar plates. The pure isolates were preserved in 10% (v/v) glycerol and stored in a -20^{o}C freezer for further observation.

B. Phenotype Characteristics

Phenotype characteristics of isolates are conducted by examined morphological and biochemical properties. The LAB confirmation of the isolates was performed using the Gram staining and catalase test. Gram staining was conducted using conventional methods, while the catalase test was conducted by adding 3% of hydrogen peroxide. The presence of catalase was identified by bubbles production [9].

Enzymatic activity of isolates was also observed. The ability of isolates degrades the protein (protease activity), and pectin (pectinase activity) was detected by inoculated the isolates into an appropriate media and observed the clearance zone.

Qualitative analysis of protease activity of the isolates was performed by culturing of the isolates in medium containing 1% skim milk as sole carbon sources. After 24 h of incubation, the clear zone formed around the colonies measured [10]. Pectinase activity of isolates was conducted by culturing the isolates in medium containing 1% pectin as sole carbon source. After 24 h incubation, the zone of clearance was checked by adding 2% congo red as a detecting agent [11].

Carbohydrates fermentation pattern analyzed by API 50 CH. API 50 CH is a standardized system consisting of 50 biochemical tests for the study of carbohydrate metabolism by microorganisms. Ten (10) ml of pure water was poured into the incubation box with the strip placed in the incubation box. The bacterial culture from the petri dish was included in 2 ml of 0.85% sterile NaCl solution; then, it homogenized using vortex. The cell suspension was dripped slowly into other test tubes containing 5 ml of 0.85% NaCl sterile until the turbidity is the same as turbidity at No. 2 Mc Farland standard. Then it inserted into API 50 CHL media. After homogenization, the suspension put into microtube strips carefully so as not to exist air bubbles inside the microtube. The suspension from microtube dripped with 2 drops of mineral oil. The tray was then closed and incubated at 37º C for 48 hours. Each microtube was observed for changing color phenomena on 24 hours and 48 hours. Fermentation of carbohydrates in the medium was indicated by medium changing (bromcesol purple indicator contained in the medium) to yellow. Exception for esculin test (tube 25), the positive test showed by medium changing from purple to black. The results were recorded on the result sheet and analyzed with APIWeb software (http://apiweb. biomerieux.com) for then the species is determined [12].

III. Results and Discussion

A. Quantification and Isolation of LAB

The digestive tract of animals is colonized by a complex ecology of microorganisms, known as the intestinal microbiota, consisting mostly of bacteria that are classified as indigenous or transient. Lactid acid bacteria (LAB) is one of the common bacteria found in the digestive tract of

humans and animals [13,14]. The population number of LAB on civet's digestive tract is shown in Table 1.

TABLE I
ENUMERATION OF THE LAB IN DIFFERENT PARTS OF CIVET'S DIGESTIVE TRACT

No	Part of civet's digestive tract	Total LAB (cfu/ml)
1	Stomach	1.41×10^7
2	Small Intestine	2.51×10^8
3	Large Intestine (Colon)	3.71×10^8

The total LAB number was between $0.14 \times 10^7 - 3.71 \times 10^8$ cfu/ml. This result lower than those reported by Suhandono et al. [15] and Muzaifa et al. [16]. Table 1 indicated that the total number of LAB increased from the stomach to the small intestine and colon. The lowest total LAB number was found in stomach ($0,14 \times 10^8$ cfu/ml). The low number of LAB in the stomach is related to its environmental condition. The pH of the stomach is lower; hence, only the limited numbers of microbes can thrive in the region [17]. According to Tannock et al., [18], microbial numbers are restricted in the stomach area because of the pH of the stomach (as low as pH 2), the toxicity of bile salts, and the relatively swift flow of the digest. Bacteria along the digestive tract have several roles, many of which are beneficial for health (including vitamin production), ion absorption (Ca, Mg and Fe), protection against pathogens, histological development, enhancement of the immune system and fermentation of non-digestive foods and other metabolites [19].

A total of 4 different LAB were isolated from three parts of the civet's digestive tract. A summary of the morphology of the LAB isolates can be seen in Table II. As stated by Christopher and Bruno [20], bacteria colonies come from single cells that develop and multiply in numbers to million cells. Every colony has a determined appearance in the form of size, shape, edge, opacity, color, and texture. In the present study, colony characteristics of all isolates were relatively similar, except colony size. Therefore if colonies which grown on similar medium show a different appearance, it can be claimed as a different species of bacteria. But since many kinds of species reported to share a similar kind of colony morphology, the conclusion above shall not be considered. By doing so, observing the colony characteristics remains as reliable and preferable parameters for preliminary identification of bacterial species [20,21].

B. Phenotype Characteristics of LAB isolated from civet's digestive tract

The phenotype characteristics of LAB are shown in Table 3. All isolates showed as Gram-Positive Bacteria (GPB) and catalase-negative. The results confirm that all isolates are matched as lactic acid bacteria. Gram stain is known as a preliminary procedure to determine the initial characterization and also to classify the identified bacteria.

The cell wall of GPB has a thick layer, which consists of 50-90% peptidoglycan, a complex assemblage of glycopolymers and proteins. As the peptidoglycan forms a thick layer in GPB, it enables these bacterias to keep the crystal violet-iodine complex and then colors the cells as purple. Furthermore, GPB also has lipoteichoic acid, which is firmly placed in the peptidoglycan layer. This acid compound has antigenic properties and also acts as an enzyme regulator for the autolytic wall. Later after the cell death, the acid is released from the cell wall, and then its antigenic properties react as immune responses [22,23].

Catalase test used as one of the biochemical tests for grouping bacteria that divided bacteria into catalase-positive and catalase-negative bacteria (CNB) groups. Catalase is an enzyme that is produced by microorganisms that live in oxygenated environments to degrade the toxic form of oxygen metabolites, hydrogen peroxide [24.25]. Anaerobic bacteria generally lack the catalase enzyme. Many of the CNB live in anaerobic conditions, and the unable to for hydrogen peroxide during metabolism. All LAB grow anaerobically, but unlike most anaerobes, they grow in the presence of oxygen (anaerobic aerotolerant bacteria). LAB that grows in anaerobic conditions also classified as CNB. They use the peroxidase enzyme for destruction the of hydrogen peroxide [25,26].

The enzyme activity (protease and pectinase) varies between isolates. Having specific enzyme activity from an isolate is one of the requirements as a starter. The ability of microorganisms to break down proteins and pectins is an indicator that the isolates could be served as a potential starter in coffee fermentation. Coffee contains many proteins and pectins. Protein degradation yields peptides and some free amino acids [27], while pectin hydrolysis yields some simple sugars [28,29]. The results of the degradation of these two components can affect the taste of coffee during roasting. Roasting leads to profound changes in the chemical composition of coffee such as protein, amino acids, reducing sugars, sucrose, trigonelline, chlorogenic acid, water decreasing, and melanoidins formation, many of which are due to the Maillard reaction [30]. The reaction is the primary avenue for coffee aroma formation as it is responsible for the production of a comprehensive class of coffee aroma-impact compounds such as pyrazines, pyrroles, thiols, furanone, pyridines and thiophenes [31].

The result of carbohydrate fermentation by the API 50 CH system is presented in Table 4. The API 50 CH system consists of 50 microtubes used to study of carbohydrate family and its derivatives. The first tube is used as a negative control, which does not contain any active ingredient. A positive reaction was indicated by a color changed of media. Carbohydrates are the main energy source for bacterial growth. However, each bacterial species is capable of fermenting certain types of carbohydrates and not being able to ferment other carbohydrates. Therefore, carbohydrates fermentation tests can be used as a basis for the identification of LAB [32].

TABLE II
COLONY CHARACTERISTICS OF LAB FROM CIVET'S DIGESTIVE TRACT

Isolates	Source	Colony characteristics				
		Form	Colour	Margin	Elevation	Size
M1	Stomach	Circular	milky white	Entire	Raised	moderate
M2	Stomach	Circular	milky white	Entire	Raised	Small
M3	Stomach	Circular	light milky	Entire	Raised	Moderate
M4	Intestine	Circular	milky white	Entire	Raised	Small
M5	Intestine	Circular	milky white	Entire	Raised	Small
M6	Intestine	Circular	milky white	Entire	Raised	Moderate
M7	Colon	Circular	milky white	Entire	Raised	Small
M8	Colon	Circular	milky white	Entire	Raised	Pinpoint
M9	Colon	Circular	milky white	Entire	Raised	Pinpoint

TABLE III
COLONY CHARACTERISTICS OF LAB FROM CIVET'S DIGESTIVE TRACT

Isolates	Source	Cell form	Phenotype characteristics			
			Gram	Catalase	Protease	Pectinase
M1	Stomach	Bacilli	+	-	-	-
M2	Stomach	Bacilli	+	-	+	+
M3	Stomach	Bacilli	+	-	+	+
M4	Small intestine	Bacilli	+	-	+	+
M5	Small intestine	Coccus	+	-	+	+
M6	Small intestine	Bacilli	+	-	+	-
M7	Colon	Coccus	+	-	-	+
M8	Colon	Coccus	+	-	+	+
M9	Colon	Coccus	+	-	+	-

TABLE IV
CARBOHYDRATE FERMENTATION OF LAB FROM CIVET'S DIGESTIVE TRACT

Fermentation	Isolates								
	M1	M2	M3	M4	M5	M6	M7	M8	M9
0	-	-	-	-	-	-	-	-	-
Gliserol	-	-	-	-	-	-	-	-	-
Eryhtritol	-	-	-	-	-	-	-	-	-
D-arabinosa	-	-	-	-	-	-	-	-	-
L-arabinosa	+	+	-	+	+	+	+	-	-
D-ribose	+	+	-	+	+	+	+	+	+
D-xynose	+	+	-	+	+	+	+	-	-
L-xynose	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-
Methyl xylopyranoside	-	-	-	-	-	-	-	-	-
D-galactose	+	+	-	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+	+
D-fructose	+	+	-	+	+	+	+	+	+
D-mannose	+	+	-	+	+	+	+	+	+
L-sorbose	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-
Inocitol	-	-	-	-	-	-	-	-	-
D-mannitol	-	-	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	-	-	-
Metyl-αD mannopyranoside	-	-	-	-	-	-	-	-	-
Metyl-αD glucopyranoside	-	+	-	-	-	-	-	-	-
N-acetyl glucosamine	+	+	-	+	+	+	+	+	+
Amygdaline	+	-	-	+	+	+	+	+	+
Arbutine	+	-	-	+	+	+	+	+	+
Esculine	+	+	-	+	+	+	+	+	+
Salicine	+	+	-	+	+	+	+	+	+
D-cellobiose	+	+	-	+	+	+	+	+	+
D-maltose	+	+	-	+	+	+	+	+	+
D-lactose	+	+	-	+	+	+	+	+	+

Fermentation	Isolates								
	M1	M2	M3	M4	M5	M6	M7	M8	M9
D-melibiose	-	+	-	-	-	-	-	+	+
D-sacharose	-	+	-	+	-	-	-	-	+
D-tetralose	+	+	-	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-
D-melizitose	-	-	-	-	-	-	-	-	-
D-raffinose	-	+	-	-	-	-	-	-	-
Amidone	-	-	-	-	-	-	-	-	-
Glycogene	-	-	-	-	-	-	-	-	-
ZYNitol	-	-	-	-	-	-	-	-	-
Gentibiosa	+	+	-	+	+	+	+	+	+
D-turanose	-	+	-	-	-	-	-	-	-
D-lyxose	-	-	-	-	-	-	-	-	-
D-tagadose	+	-	-	+	+	+	+	-	-
D-fucose	-	-	-	-	-	-	-	-	-
L-fucose	-	-	-	-	-	-	-	-	-
D-arabitol	-	+	-	-	-	-	-	-	-
L-arabitol	+	-	-	-	-	-	-	-	-
Potasium gluconate	+	+	-	+	-	+	-	-	-
Pot-celogluconat	+	+	-	+	+	+	+	-	-
Pot-2 celogluconat	-	-	-	-	-	-	-	-	-

+ : Positive, - : negative after 48 h of incubation at 37^{o}C

TABLE V
IDENTIFICATION OF LAB BASED ON API 50 CH

No	Isolates	Identified LAB	Similarity
1	M1	*Lactobacillus brevis*	99.9%
2	M2	*Lactobacillus brevis*	97%
3	M3	*Lactobacillus fructivorans*	86%
4	M4	*Lactobacillus brevis*	99.9%
5	M5	*Pediococcus pentasaceus*	86%
6	M6	*Lactobacillus brevis*	99.5%
7	M7	*Pediococcus pentasaceus*	82%
8	M8	*Lactococcus lactis* spp. *lactis*	74.9%
9	M9	*Lactococcus lactis* spp. *lactis*	56.1%

Based on the result of carbohydrate fermentation, four species LAB were identified, i.e., *Lactobacillus brevis, Lactobacillus fructivorans, Pediococcus pentasaceus,* and *Lactococcus lactis* spp. *lactis. Lactobacillus brevis* strains (M1, M2, M4, and M6) have a similarity up to 97-99,9% while the remained strains have a similarity value 56,1-86% (Table 4). According to Walter [33], *Lactobacillus brevis* is one of *Lactobacillus* species commonly detected in human feces. Lactobacilli are still regarded as dominant microorganisms of the humans and animals gut [34-36].

IV. CONCLUSIONS

In conclusion, LAB successfully isolated from the digestive tract of civet. All LAB isolates have Gram-positive and catalase-negative. The total LAB number was between $0.14 \times 10^7 - 3.71 \times 10^8$ cfu/ml. The phenotypic characteristics of LAB isolates were varied. This study revealed the presence of a wide variety of LAB from civet's digestive tract, four species of LAB were identified by API 50 CH, i.e., *Lactobacillus Brevis, Lactobacillus fructivorans, Pediococcus pentasaceus* and *Lactococcus lactis* spp. *Lactis.* LAB with protease and pectinase activity might be used as a starter culture for the production of artificial kopi luwak. Hence, a further study related to their technical characteristics must be done to maintain kopi luwak availability.

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