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Full Length Article

Identification of Lactic Acid Bacteria from Luwak (*Paradoxurus hermaphroditus*) Gastrointestinal Tract

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Abstract

Luwak is a mammal that is widely cultivated to produce luwak coffee. Luwak coffee is a rare and one of the world's most expensive coffees from Indonesia. Numerous enzymes and microorganisms, many of which are lactic acid bacteria, contribute to the production of the coffee aroma. This research aims to identify lactic acid bacteria from luwak digestion accurately down to the species level. The parameters tested in this study were Gram staining, bacterial resistance to acid, bacterial resistance to bile salts, and genotypic identification of isolates using the 16S rRNA gene sequencing method. The results found that US1, FS3, FS4 and FS6 were identified as *Leuconostoc pseudomesenteroides*, US2 and US3 were identified as *Lactobacillus plantarum*, FS2 was identified as *Weissella cibaria* and FS5 was identified as *Staphylococcus haemolyticus*. All of these isolates were included in Gram-positive based on the results of Gram staining. However, among the 8 isolates obtained, one isolate, namely FS5 (*S. haemolyticus*), was not a lactic acid bacterium, but a pathogenic bacterium. This information can be used for the development of luwak origin bacteria for *in vitro* coffee fermentation purposes. © 2021 Friends Science Publishers

Keywords: Lactic acid bacteria, Luwak, Gastrointestinal tract, 16S rRNA

Introduction

Asian palm civet (*Paradoxurus hermaphroditus*), known as the "luwak" in Indonesia, is a mammal belonging to the Viverridae family. This animal has a long tail, short and small legs (Rahardjo 2012; Maha *et al*. 2018) and has glands that can release a pandan aroma (Rahardjo 2012). Luwak is a nocturnal animal that is active at night searching for food (Rahardjo 2012; Nijman *et al*. 2014; Winaya *et al*. 2020). This species is an omnivore that generally eats fruits such as banana, papaya, or coffee but sometimes consumes insects, small vertebrates, or reptiles (Rahardjo 2012; Fitri *et al*. 2019; Winaya *et al*. 2020). Basically, luwak is a wild animal that lives in the forest. However, in Indonesia, these animals are widely cultivated to produce luwak coffee (Schmidt-Burbach *et al*. 2014; Winaya *et al*. 2020).

Luwak coffee is a rare and one of the world's most expensive coffees from Indonesia. The fermentation processes in the gastrointestinal tract of luwak give a unique aroma and an excellent taste to the coffee, which is preferred by coffee consumers worldwide (Fitri *et al*. 2019; Winaya *et al*. 2020). Various enzymes and microorganisms, which are lactic acid bacteria, play a role in the coffee aroma formation (Fitri *et al*. 2019).

The high consumer interest in luwak coffee has increased the curiosity of researchers to explore the bacteria found in the digestive tract of luwak. Efforts to isolate and identify these bacteria are starting to be carried out. However, the identification carried out was only limited to phenotypic identification (Fauzi 2008).

Identification of lactic acid bacteria can be carried out based on the phenotypic and genotypic properties. Phenotypic identification is based on the colony morphological observations, microscopic observations (Gram stain), physiological, metabolic (biochemical), or chemotaxonomic tests. Meanwhile, genotypic identification is carried out using molecular methods, such as the Polymerase Chain Reactions (Ammor *et al*. 2005; Donelli *et al*. 2013). The phenotypic identification method has many weaknesses, including identification that takes a long time, and the level of accuracy is low due to its subjective interpretation (Donelli *et al*. 2013).

This study aims to accurately classify lactic acid bacteria isolated from luwak digestion down to the species level. The 16S rRNA gene sequencing method was used to identify the bacteria in this study.

Materials and Methods

Materials

Lactic acid bacteria samples used were isolated from luwak digestion (colon and feces) from previous research, which were coded with US1, US2, US3, FS2, FS3, FS4, FS5 and FS6 (Tawali *et al*. 2019).

Gram staining

Gram staining was done by dripped a solution of crystal violet dye, iodine, 95% ethanol, and safranin sequentially on each sample.

Acidity resistance test

The resistance of microbial isolates to gastric acid in the digestive tract with low pH was carried out by inoculated each sample into MRS Broth + HCl 0.1 N (set at pH 3) and incubated for 2 × 24 h at 37ºC. A positive result was indicated by the occurrence of bacterial growth in a medium that has low acidity. The result was negative if there was no microbial growth in the medium.

Bile salt resistance test

MRS Broth was supplemented with 5% synthetic bile salt (ox bile). Each sample was inoculated into MRSB-salt medium and incubated for 2 x 24 h at 37ºC. Positive results were demonstrated by the presence of sediment at the tube's bottom and an improvement in the media's clarity relative to before incubation.

DNA extraction

Genomic DNA extraction was performed according to the protocol included in the TIANamp Genomic DNA Kit.

DNA amplification using PCR

This procedure was performed on extracted DNA isolates. To amplify the 16S rRNA sequence, primers 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGTGTGTACAAGGC-3') were used. The PCR master mix composition used was as follows: GoTaq MasterMix 16 μL, 2 μL forward primer, 2 μL reverse primer, 4 μL H_2O and 2 μL DNA gene. The PCR machine used the following conditions: 5-min pre-denaturation at 95ºC, 1-min denaturation at 95ºC, 1-min annealing at 57ºC, 1-min extension at 72ºC, 10-min post-extension at 72ºC, and kept at 4ºC until 30 cycles were completed.

Visualization of PCR results by electrophoresis

1.5% agarose gel was prepared in Erlenmeyer by dissolving 1.5 g of agarose powder in 100 mL of TAE Buffer, heated for 2 min until homogeneous, and then 8 μL of Ethidium Bromide was added. Following that, the gel liquid was cooled to room temperature. After allowing the gel liquid to cool slightly, it was poured into a gel electrophoresis mold using a gel comb with a comb count of 24 wells. 5 μL of each amplification product was put into a 1.5% agarose gel well submerged in a tank containing TAE buffer. Additionally, 50 min of electrophoresis at a constant voltage of 100 volts was performed. After 50 min the electrophoresis was terminated and the gel was extracted for UV light observation.

DNA sequencing

PCR products from samples that showed positive electrophoresis results were continued to the DNA sequencing. DNA samples were carried out by 1st Base through PT Genetika Indonesia. The DNA sequencing process was carried out using the Sanger dideoxy method. The sequencing results were analyzed by performing a BLAST search against the NCBI database at www.ncbi.nlm.nih.gov using the nucleotide sequences from the 16S rRNA gene sequencing results.

Results

Morphology characterization of isolates

Gram staining results performed on isolates can be used to determine the morphological characteristics of bacteria in their shape and Gram characteristics. The results of Gram staining of luwak digestive isolates can be seen in Table 1 and Fig. 1. Based on Table 1, it can be seen that three isolates obtained from luwak gastrointestinal, namely US2, US3, and FS2, had bacilli form, while five isolates, namely US1, FS3, FS4, FS5, and FS6, had cocci form. All isolates obtained were purple under the microscope (Fig. 1).

Resistance of isolates from acid and bile salt

The results of the resistance assay of luwak digestive isolates to acidity and bile salts (Table 1) showed that only one isolate, namely FS5, was unable to grow on acid or high concentration of bile salt conditions. In contrast, the other seven isolates, namely US1, US2, US3, FS2, FS3, FS4 and FS6, were able to grow on these conditions.

Molecular identification of isolates from luwak gastrointestinal

Molecular identification of isolates in this study was carried out using the 16S rRNA gene sequence technique.

Table 1: Resistance to pH, bile salt and morphological observation results of luwak digestive lactic acid bacteria

Isolates	Cell Morphology		pH 3 Resistance	Bile Salt Resistance
	Shape	Gram		
US1	Cocci	Positive	+++	+++
US2	Bacilli	Positive	+++	+++
US3	Bacilli	Positive	+++	+++
FS2	Bacilli	Positive	++	++
FS3	Cocci	Positive	++	++
FS4	Cocci	Positive	++	++
FS5	Cocci	Positive	-	-
FS6	Cocci	Positive	+++	+++

The pH and bile salt resistance were evaluated as growth (+) and non-growth (-)

Table 2: BLAST result of isolates from luwak gastrointestinal

Sample Code	Homologous Species with GenBank	Query Coverage	Identities	Accession
US1	*Leuconostoc pseudomesenteroides* strain MG5216	99%	99,18%	MN368204.1
US2	*Lactobacillus plantarum* strain CAU:227	99%	99,67%	MF369879.1
US3	*Lactobacillus plantarum* strain IMAU20905	99%	98,71%	MK369825.1
FS2	*Weissella cibaria* strain MG5327	98%	99,15%	MN368586.1
FS3	*Leuconostoc pseudomesenteroides* strain L12001	99%	98,92%	KT952379.1
FS4	*Leuconostoc pseudomesenteroides* strain: CF102	99%	98,12%	AB854189.1
FS5	*Staphylococcus haemolyticus* strain WS1-3	99%	98,15%	MN448416.1
FS6	*Leuconostoc pseudomesenteroides* strain: Ni1324	99%	99,58%	AB598984.1

16S rRNA gene sequences were performed after amplification of the DNA isolate by PCR. The visualization results in Fig. 2 show that all amplified isolates DNA had a size of <1500 bp. The bands formed are single, thick, and bright bands. That condition showed the success of the PCR process carriesd out on isolate. Based on the analysis results using BLAST (Table 2 and Fig. 3), it was known that sample US2 and US3 were homolog with *Lactobacillus plantarum*, sample FS2 was homolog with *Weisella cibaria*, sample US1, FS3, FS4 and FS6 were homolog with *Leuconostoc pseudomesenteroides*, while FS5 was homolog with *Staphylococcus haemolyticus.*

Discussion

Bacteria, especially lactic acid bacteria, are commonly found and are closely related to all processes that take place in the digestive system of monogastric animals (Kraatz 2011). Various methods can be used to identify lactic acid bacteria. The identification method using the 16S rRNA gene sequence is an identification method that is widely used to identify lactic acid bacteria more effectively. For example, to identify lactic acid bacteria from chicken digestion (Lee *et al*. 2008), native Aceh duck digestion, longan fruit (Hidayat *et al*. 2019) and buffalo milk (Melia *et al*. 2018).

Morphological observations and Gram staining are an initial screening to identify bacteria before the 16S rRNA gene sequence is performed. Gram staining is the most common method in the laboratory used to distinguish between Gram-positive and Gram-negative microorganisms (Becerra *et al.* 2016). Gram-positive bacteria have thick

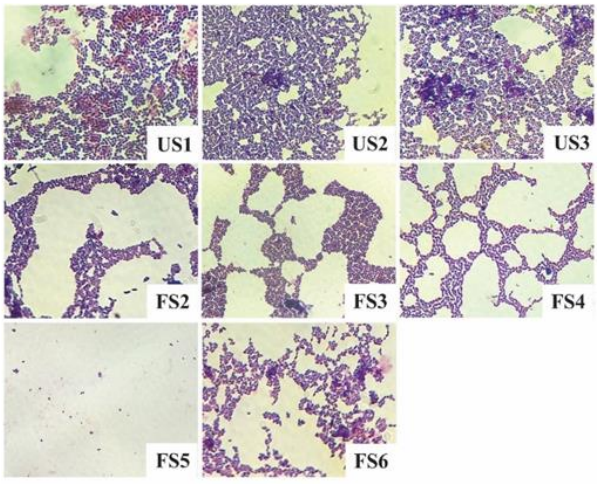

Fig. 1: Microscopic gram stain results of isolates from luwak gastrointestinal tract

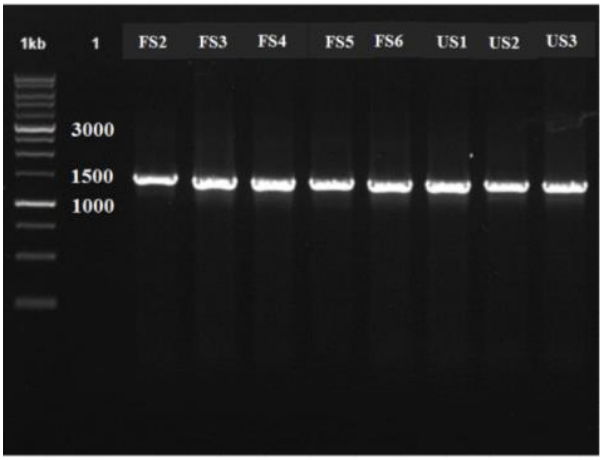

Fig. 2: Electrophoresis visualization of the amplification results of US1, US2, US3, FS2, FS3, FS4, FS5, dan FS6

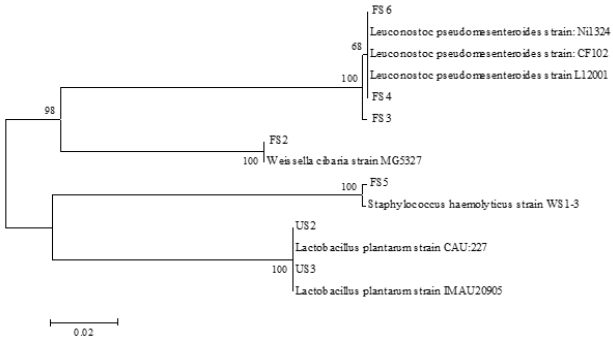

Fig. 3: Phylogenetic tree of LAB isolates from luwak gastrointestinal tract

peptidoglycan walls so that they can maintain the color of crystal violet after decolorization, while Gram-negative bacteria have thin peptidoglycan walls, so they are unable to maintain color after washing with alcohol (Yazdankhah *et al*. 2001; Bailey 2020).

The purple color visualized under the microscope indicates that all of the isolates obtained were Gram-positive bacteria. Ismail *et al*. (2018) stated that lactic acid bacteria are included in the Gram-positive bacteria group. Gram-positive bacteria show purple or blue color under a microscope as a result of Gram staining. Thus, based on Gram staining, the eight isolates obtained from luwak gastrointestinal tract could be categorized as lactic acid bacteria as an initial screening.

Isolates analyses based on their morphology are not sufficient to identify a bacterial species. Other tests are needed, for example, from the ability of bacteria to survive certain conditions. The resistance of bacteria to high acidity and bile salts could be used to test further to identify isolated bacteria. Lactic acid bacteria could be classified as probiotics (Zielińska and Kolohyn-Krajewska 2018). Probiotics are live microorganisms that can benefit the body, one of which is by improving the health of the human gut. Probiotics must survive in any conditions in the digestive tract to be able to carry out their functions (Jensen *et al*. 2012; Zielińska and Kolohyn-Krajewska 2018). Based on the result, it was shown that the eight isolates obtained from luwak digestion suspected that US1, US2, US3, FS2, FS3, FS4 and FS6 belong to the lactic acid bacteria group. At the same time, FS5 was not included in the lactic acid bacteria group.

Molecular identification is the most precise way used to identify lactic acid bacteria down to the species level. The isolates identification in this study was carried out using the 16S rRNA gene sequence technique. The first step to identify isolates by 16S rRNA gene sequence was DNA extraction. DNA extraction aims to obtain DNA from an organism by separating it from the cell membrane and other cellular components (Gupta 2019). DNA extraction consists of three main stages: cell wall destruction (lysis), separation of DNA from other components, and DNA purification (Corkill and Rapley 2008). The DNA obtained will be used as a template in the amplification stage with PCR. The purified PCR products are then determined by sequencing the nucleotide sequence (Rinanda 2011).

DNA sequencing was done by Single Pass DNA Sequencing. Sequencing data were analyzed using nucleotide blasts at ncbi.nlm.niv.gov. Analysis of blast results provides information about organisms or bacteria that have homologous DNA sequences to the sample. The information can be used to identify the species of the isolates.

Based on the results of analysis using BLAST (Table 2 and Fig. 3), it was known that eight isolates obtained from luwak digestion were identified into four species, namely *L. plantarum*, *L. pseudomesenteroides*, *Weissella cibaria*, and *S. haemolyticus* with query coverage reaching 98–99% and identities ranged from 98.12–99.67%. Query coverage is the percentage of nucleotide length that is in line with the database contained in BLAST, while identity is the highest value of the percentage of identity or the match between query sequences and aligned database sequences (Newell *et al*. 2013). *S. haemolyticus* is a type of bacteria that is included in the coagulase-negative staphylococci group. These bacteria are normal bacteria that live on human skin and flora but can be pathogenic and cause urinary tract infections and other diseases (Suhartono *et al*. 2019). So that from eight isolates, only seven isolates from luwak digestion were identified as lactic acid bacteria, namely US1, US2, US3, FS2, FS3, FS4 and FS6.

Conclusion

Lactic acid and non-lactic acid bacteria can be found in the luwak gastrointestinal. The 16S rRNA sequence method using universal primers 63f and 1387r not only can identify the lactic acid bacteria but also pathogenic bacteria such as *S. haemolyticus*. *L. pseudomesenteroides*, *L. plantarum* and *W. cibaria* are types of lactic acid bacteria found in the digestive tract of luwak. This information can be used for the development of civet origin bacteria for *in vitro* fermentation purposes.

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Author Contributions

FF, ABT, AL, and ZD designed the research flow. FF and ZD performed the research and wrote the manuscript. ABT and AL edited the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a reasonable request

Ethics Approval

Ethical approval is not applicable in this study.

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