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Diversity of Culturable Bacterial in Various Parts of Luwak's (*Paradoxurus hermaprodithus javanica*) Gastrointestinal Tract

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Luwak coffee is a highly-priced coffee produced exclusively by the palm civet or *luwak* (*Paradoxurus hermaphrodites* ssp.). The purpose of this study was to determine the diversity of culturable bacteria in the gastro intestinal tract of luwak. The bacterial isolates were phenotypically characterized by their morphology and molecularly by analysis of their 1 500 bp 16s rDNA sequence. The results showed that *Enterobacter cloacae* and *Lactobacillus brevis* were found all over luwak's digestive tract. *E. cloacae* was the most common species. The most diverse bacterial population was found in small intestine. Seven bacterial genera were successfully identified from the small intestine and colon, compared to only five genera found in the stomach.

Key words:16s rDNA, coffee, culturable bacteria, gastrointestinal tract, luwak

Kopi luwak merupakan kopi yang dihasilkan secara eksklusif oleh musang atau luwak (*Paradoxurus hermaphrodites* ssp.) Tujuan penelitian ini adalah untuk mengetahui keragaman bakteri yang dapat dikultur dari saluran pencernaan luwak. Fenotipe isolat bakteri diidentifikasi secara morofologi dan secara molekuler dengan menggunakan sepasang primer yang dapat mengamplifikasi sekuen 16s rDNA. Hasil penelitian menunjukkan bahwa *Enterobacter cloacae* dan *Lactobacillus brevis* merupakan bakteri-bakteri yang ditemukan pada seluruh saluran pencernaan luwak. *E. cloacae* teridentifikasi merupakan bakteri yang paling umum. Populasi bakteri yang paling beragam ditemukan dalam usus kecil. Tujuh genus bakteri lainnya berhasil diidentifikasi dari usus kecil dan usus besar, sedangkan lima genus ditemukan di dalam perut luwak.

Kata kunci : 16s rDNA, bakteri yang dapat dikultur, kopi, luwak, saluran pencernaan

Coffee is an important commodity and one of the most popular beverages in the world. Over 2.25 billion cups of coffee are consumed in the world every day (Ponte 2002). In 2012, Indonesia ranked third after Brazil and Vietnam in the global production of green coffee beans (USDA 2012). Five species of coffee that are commonly cultivated in the world include *Coffea arabica, C. canephora*, *C. robusta*, *C. liberica*, and *C. excelsa* (Doyle *et al*., 2001). In Indonesia *C. robusta* and *C. arabica* are the species mostly planted (Neilson, 2008). Due to its high market demand, coffee processing has developed to produce various flavors to satisfy the consumers. One type of coffee that is increasingly popular is *luwak* coffee (*Kopi Luwak*) which is produced exclusively by the Indonesian palm civet or *luwak* (*Paradoxurus hermaphrodites* ssp.). *Luwak* is a small mammal (Carnivora, Viverridae) living in the rain forests of Java and Sumatera, although its various subspecies are intensely distributed in South East Asia (Patou 2010). The distinctive taste of this coffee has been recognized since coffee plantation started in Indonesia during the Dutch colonial era. The luwak eats fruits, including coffee fruits or coffee cherries. Only the best and mature coffee cherries would be eaten by the luwak. This process will produce coffee with distinctive flavor. The price of luwak coffee is relatively high because only a small quantity of this coffee is produced (Marcone 2004).

Once ingested by luwak, coffee cherries will pass through its entire digestive system. This process removes the outer layers of the fruit. After digestion, the remaining coffee beans were collected from the forest floor, cleaned, roasted, and ground; with the same process as the harvested coffee beans. Thus the unique flavor of Kopi Luwak comes from the digestion process in luwak, which includes mechanical, biochemical, and fermentation processes (Marcone 2004). Microorganisms, especially bacteria, are closely associated with the digestive process in monogastric animals (Kraatz 2010). Several studies have been conducted to determine the types of bacteria involved in the fermentation process, for example by trying to isolate the bacteria from faeces of civet and the luwak's coffee beans (Fauzi 2014). Currently, there is no information about the bacterial diversity in parts of

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the civet's digestive organs. Civet coffee fermentation process is highly dependent on the animal. On the other hand, the existence of luwak is diminishing and the ability of civet in the process of fermentation is limited.

This condition can lead to decreased production of civet coffee. Therefore, it is necessary to find other alternatives that can replace the role of civet in the process of civet coffee fermentation. *In vitro* fermentation can be an option that may replace civet's role in the production of civet coffee. The research on bacterial diversity in the digestive tract of the mongoose is important as the basis in determining the types of bacteria involved in the *in vitro* fermentation process of civet coffee. The objective of this study is to investigate the diversity of culturable bacteria in different parts of luwak's gastrointestinal (GI) tract.

MATERIALS AND METHODS

Sampling, Isolation, and Enumeration of Bacteria. Adult civet (*P. hermaphrodites* javanica) specimen was obtained from natural forest in Purwakarta, West Java. The animal was then aseptically dissected to separate the GI tract into parts. The whole GI tract (stomach, intestine, and colon) with samples of digested food were separately removed under sterile conditions. Samples from each GI part were serially diluted in phosphate buffered saline (from 10^{-1} to 10^{-9}), plated onto LB (Luria Broth) and MRS (de Man Rogosa and Sharp) agar media followed by incubation at 37 °C for 36 h. Concentrations of culturable bacteria were estimated by counting the average CFU (colony forming units) per milliliter on each agar plate. Colonies with different morphologies were selected using four-ways streaking on the plates and stored in 50% (v/v) glycerol at -80 °C.

Phenotypic Identification. Phenotypic analysis of the isolates from luwak digestive system was conducted according to the Bergey's Manual of Systematic Bacteriology using Trypticase Soy Agar (TSA), Trypticase Soy Broth (TSB) and Nutrient Agar (NA). Media for identification used were broth contaning glucose, lactose, sucrose and manitol, MR-VP (Methyl Red-Voges Proskauer), SIM (sulfur-indole-motility), and Simmon's Citrate and Nutrient Gelatin media. The staining, morphology, and biochemical properties were determined as described by Cowan (Cowan 1974). The staining was performed using crystal violet, safranin, Lugol's iodine, Barritt's, and Kovac's reagents, red methyl, p-aminodimetilanilin oxalate, and 3% of H_2O_2.

DNA Extraction. DNA was extracted by boiling method (Queipo-Ortuño 2008). Overnight bacterial suspension was harvested by centrifugation at 5000 rpm for 10 min. The pellet was then washed 3 times using 1.5 mL acetone. Each preparation was centrifuged at 9000 g for 10 min at 4 °C in order to remove potential PCR inhibitors. The pellet was suspended in 200 µL TE buffer 10 mmol/l Tris-HCl (pH 8.0), 1 mmol/lEDTA, and the mixture was briefly mixed in a vortex mixer. The suspension was placed in boiling water bath for 1 min, subjected to 3 freeze-thaw cycles alternating between liquid nitrogen for 3 min and 100 °C for 2 min, and then centrifuged at 13000 rpm for 5 min. A 100 µL aliquot of the supernatant was transferred to a sterile tube and stored at -20 °C until PCR testing.

Amplification of 16S rDNA Gene Fragments. Bacterial 16S rDNA gene was amplified by PCR using universal primers specifically designed for bacterial DNA, 16S rDNA forward primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3'); reverse primer 1492 (5'-GGTTACCTTGTTACGACTT-3') from Baker *et al*. (2003) in 50 µL reactions each containing 25 µL Dream taq Green PCR Mix 2x (FERMENTAS), 4 µL Dimethyl sulfoxide (DMSO), 2.5 µL of each primer for final concentration 0.1 µM, and 2 µL DNA template. The PCR program was as followed: initial denaturation at 95 °C for 10 min, followed by 25 cycles, consisting of 95 °C for 30 s, 45 °C for 30 s and 72 °C for 2 min, and a final extension cycle 72 °C for 10 min (2720 thermal cycler Applied Biosystem). The amplified 16S rDNA gene from the isolate was eluted and purified from the gel slice using Gel Extraction Kit (GeneAid, USA). Purified PCR products were directly sequenced using both primers by automated DNA sequencing (Macrogen Inc., Seoul, Korea) to obtain the complete nucleotide sequences of the 16S rDNA gene.

Sequence Analysis and Taxonomic Classification. The 16S rDNA gene sequences were compared with the available sequences in GenBank using the BLASTN bioinformatics program from the National Center for Biotechnology Information (NCBI). Based on the maximum identity score, the first ten sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated using RDP-II database and the phylogenetic tree was constructed according to the Neighbour-Joining method (Saitao 1987), using MEGA 5.1 Molecular Evolutionary Genetics Analysis software version 5.1 (Tamura *et al*. 2011) for the inquiry of the evolutionary relationship. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history

of the taxa analyzed. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site.

RESULTS

The total plate counts of aerobic bacteria found in the samples of digested food isolated from the digestive track on LB and MRS agar (expressed in log(CFU mL^{-1}) were between 0 - 9 (Fig 1). The total plate count of bacteria isolated from the stomach were 6.1 non lactid acid bacteria and 7.06 lactic acid bacteria. The total bacterial number in the colon was higher compared to stomach and small intestine (9.58 log (CFU mL^{-1})). The largest population of lactic acid bacteria was found in the small intestine (9.36 log (CFU mL^{-1})). Morphological, physiological, and molecular analyses suggested that Enterobacteriaceae was the dominant culturable bacteria in the digestive tract of luwak. Other genera such as *Bacillus*, *Pseudomonas, Pantoea*, *Escherichia*, *Lactobacillus*, *Ochrobactrum*, and *Kocuria* were also observed in the cultures. The 16S rRNA gene sequence analysis was performed after PCR amplification in order to identify the isolates. The amplified products of 16S rDNA were presented as single bands of 1 500 bp. The purified (Purification Kit, GeneAid, USA) fractions were then quantified by 1 kb DNA mass ruler standard Fermentas (Fig 2).

Phylogenetic tree was constructed from the 25 isolates. The phylogenetic tree suggested that L2LAB1

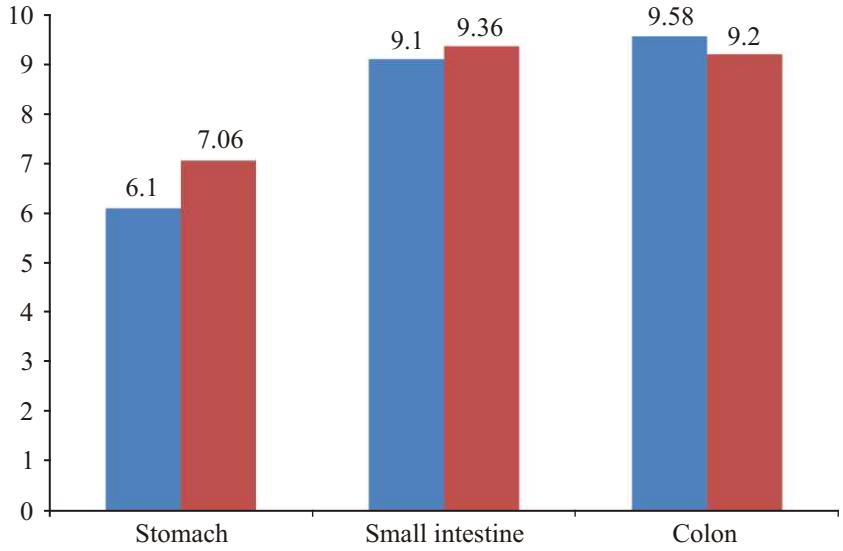

Fig 1 Number of bacteria in the digestive tract of luwak (*Paradoxurus hermaprodithus* var *javanica*). Non lactic acid bacteria (■) and lactic acid bacteria (■).

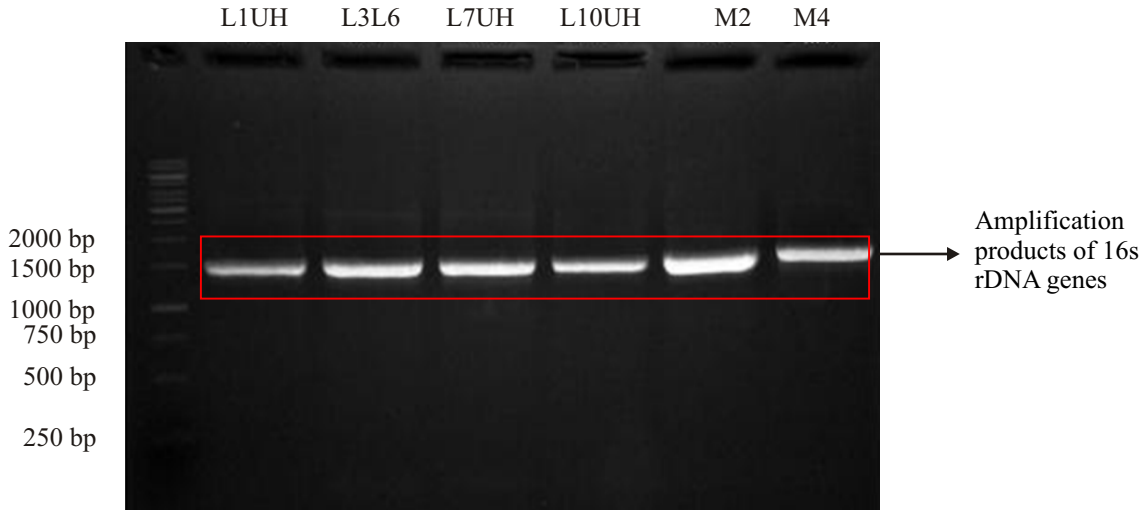

Fig 2 Agarose gel electrophoresis visualization of 16S rRNA PCR product of L1UH, L3L6, L4UH, L10UH, M2, and M4 isolates.

culture was closely related to *Bacillus subtilis*. The same analysis was also used to identify the other isolates. Bacterial identification and distribution in the digestive tract of luwak from BLAST analysis and phylogenetic analysis of the 16SrDNA gene are shown in Fig 3. In the stomach of luwak, we found *Bacillus methylotrophicus* strain PY5 (L2LAB1), *Enterobacter cloacae* JQ.993364.1 (L3L8), *Enterobacter* sp. (L3L6), *E. cloacae* (L9L1), *E. cloacae* JQ.993364.1 (L6L9), *Cedecea davisae* JQ396389.1 (L8L4), *Enterobacter* sp. 1054 (L7L9), *Enterobacter agglomerans* strain A123 (L7L8), *Enterobacter turicensis* (L5L9), *Bacillus* sp. (L10L4), and *Lactobacillus brevis* (M2). Meanwhile, in the small intestine, we found *Bacillus substilis* (UH3LAB3), *Escherichia coli* (UH3LAB1), *Enterobacter sp.* (L10UH), *E. cloacae* (L1UH), *E. cloacae* (L2UH), *Bacillus pumilus* (L3UH), *Pseudomonas aeruginosa* (L4UH), *E. cloacae* strain T137 (L5UH), *E. cloacae* (L8UH), *Kocuria palustris* (L9UH), *Ochrobactrum sp.*(UB3LAB1) and *Lactobacillus brevis* (M2). In the colon, we found *E. cloacae* (L1UH), *E. cloacae* (L2UH), *B. pumilus* (L3UH), *P. aeruginosa* (L4UH), *E. cloacae* strain T137 (L5UH), *E. cloacae* (L8UH), *K. palustris* (L9UH), *Ochrobactrum* sp. (UB3LAB1), *E. coli* (UH3LAB1), and *L. brevis* (M2) .

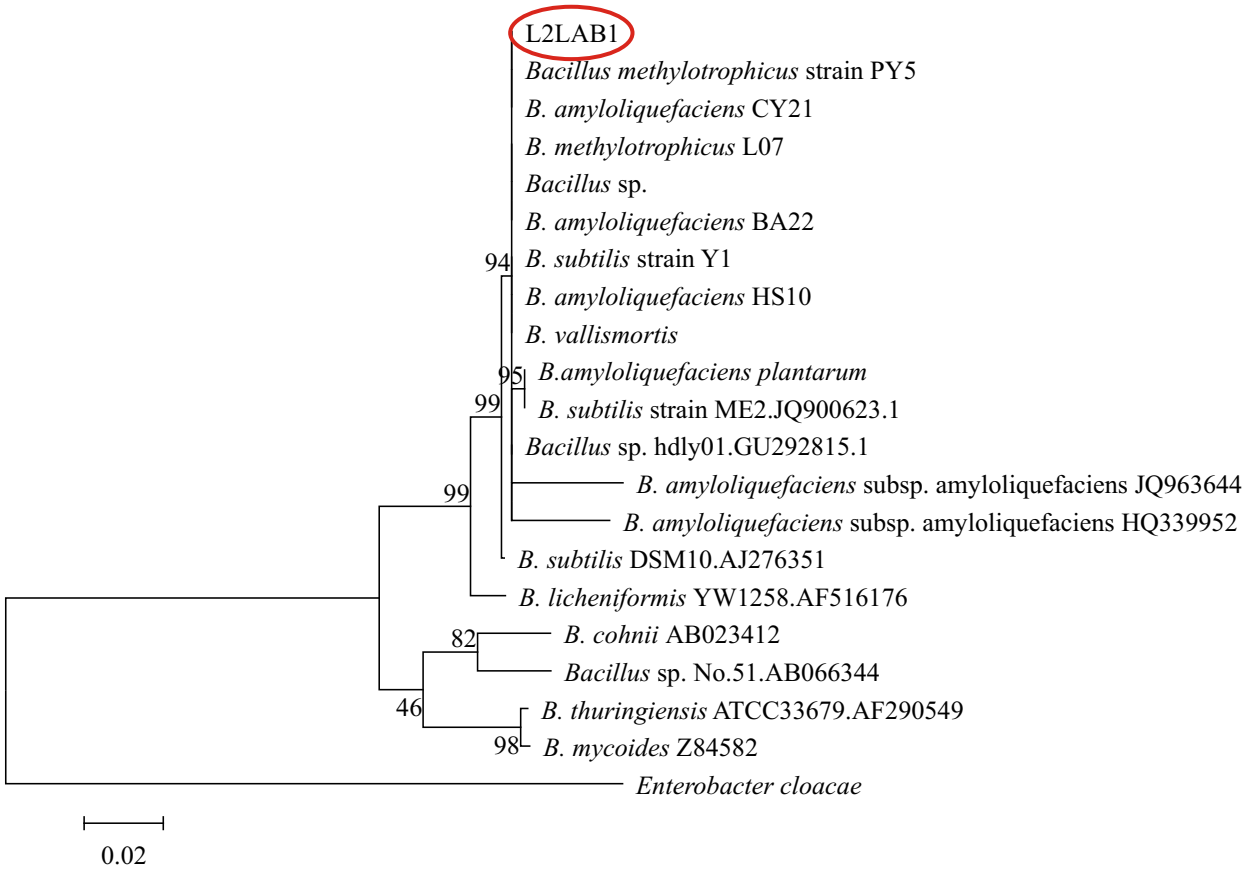

Fig 3 Phylogenetic tree by Neighbour Joining based on alignment 16S rDNA fragment L2LAB1isolate.

DISCUSSIONS

This study is the first recorded diversity study of the culturable bacteria from the digestive tract system of civet (*P. hermaphrodites* java). The total number of isolates and colony forming unit (CFU) presented in Fig 1 indicated that the number of bacteria increased gradually from the jejunum (small intestine) to the colon (large intestine) and were most numerous in the colon. Peristalsis and antimicrobial effects of stomach acid made the stomach and the proximal part of the small intestine habitable only to certain bacteria. According to Baron (1996), genera reportedly found at this location include *Lactobacillus*, *Enterococcus*, *Streptococcus*, and some acid-resistant Gram-positive bacteria. Most aerobic bacteria are in the jejunum, while the large intestine was dominated by aerobic and facultative anaerobic bacteria.

The 16S rDNA fragment analysis showed that *E. cloacae* and *L. brevis* were found all over the gastrointestinal system, indicating that they can survive

in that condition. Bevilacqua *et al*. (2009) reported that *E. cloacae* could be cultivated by fermentation at temperatures between 10-37 °C and pH between 4-5 and 8-10). Meanwhile, *L. brevis* was a lactic acid bacteria that carries out the reactions, that is the conversion of carbohydrate to lactic acid plus carbon dioxide and other organic acids, without needing oxygen.

Low bacterial diversity in the stomach might be attributed to the physiology and pH of the stomach. The low bacterial diversity in the stomach may result from acidic environment and extracellular enzymatic digestion (Pawar *et al*. 2012). The bacterial communities in the digestive tract of this animal may have important role in the digestion of the mucilage layer of coffee beans. We were assuming that the different digestive tract regions such as the esophagus, stomach, intestine, and colon are highly specialized compartments, and each could have distinct roles to play in the digestion. These functionally specialized tract regions may be unique microenvironments that harbor unusual bacterial communities. Research suggested that the relationship between gut flora and monogastric animal is not merely commensal (a non-harmful coexistence), but rather a mutualistic relationship (Sears 2005). The microorganisms may function as fermentation agent inducing the immune system, and competitor for harmful, pathogenic bacteria (Guarner and Malagelada 2003). The type of cultured bacteria is dependent on the techniques and media used (Pham and Kim 2012). In this research, the Lauria Broth and MRS were used as isolation media for bacteria from luwak's digested tract. Therefore, our finding was limited only for bacteria that grow on these media. To obtain larger bacterial diversity, metagenomic method or more diverse selections of media might be used in the future. David *et al*. (2005) found that extended incubation times were able to isolate many members of rarely group bacteria. It is possible to accommodate some of the bacteria that have long incubation periods.

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