Enumeration and characterization of bacteria from civet gastrointestinal tract

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Abstract. Civet coffee is one of expensive coffee in the world. The demand for civet coffee is increasing but unfortunately the production is very limited. This condition triggers frequent counterfeiting of civet coffee. Artificial civet coffee production is possible by imitating fermentation in the digestive tract of civet animals. However, up to now, information on the microbiological system of the civet digestive tract is still very limited. This study aims to determine the bacterial population from the mongoose digestive tract and to determine its potential in breaking down protein and pectin. The research was carried out by luwak surgery, isolation and enumeration of civet digestive tract bacteria and their characterization. Analysis of civet digestive tract bacteria is carried out by collecting fluid from the three parts of the civet digestive tract, namely the stomach, small intestine and large intestine. The results showed that the population of lactic acid bacteria (LAB) was lower than non-LAB in each part of the civet digestive tract. There were 11 pure isolates with various morphological and biochemical characteristics. Four potential isolates were obtained which had the ability to break down protein as well as pectin.

1. Introduction
Civet coffee is known as one of expensive coffees in the world. This coffee is produced from coffee cherries that have been eaten by civet (Paradoxurus hermaphroditus) where in the process of digestion the coffee beans are not digested so that they are released again during defecation. These coffee beans are then cleaned of civet droppings, washed and processed as in regular coffee production. In particular, this coffee is one of the best coffees in the world market because of its uniqueness and delicious taste [1], [2].

Along with the increasing popularity of civet coffee, the demand for civet coffee is also increasing. However, because this type of coffee is very dependent on the existence and biological system of the civet, its production is becoming very limited and is considered as a rare item which causes its high price. This is what causes civet coffee to be the target of counterfeiting [3]. Artificial civet coffee production is possible by imitating fermentation in the digestive tract of civet animals. The fermentation process of civet coffee is assumed to be similar to the wet coffee processing method (through the fermentation process) which is responsible for the formation of coffee aroma and flavor [3], [4].

Microorganisms and enzymes found in the civet gastrointestinal tract might play a role in the fermentation of civet coffee. However, until now, the biochemistry of civet coffee fermentation is not fully known. The digestive tract of the mongoose, like the digestive tract of other mammals, is a
complex ecosystem. Animal gastrointestinal microorganisms are related to the function of the digestive tract in maintaining the homeostasis of the immune system, absorption of nutrients and metabolites of host energy [5]-[7], fermentation of food and other metabolites [8], [9].

The coffee fruit eaten by the civet as a whole contains the fruit skin (pulp), mucilage layer (mucilage) and coffee beans but only the beans are removed intact. The main components found in the fruit skin and mucus layer are degraded by microorganisms in the fermentation of civet coffee and the enzymes it produces. Silva [10] states that the pectin and protein content in the fruit skin and mucus layer of coffee is quite high, reaching 42.3% and 18.9%. It is suspected that the bacteria that play a role in the fermentation of civet coffee have the ability to degrade pectin and protein. Pectinolytic and proteolytic bacteria are known to be able to decompose sugars, each of which can utilize pectin or protein as a substrate [11]-[13]. The decomposition of pectin will produce a number of simple sugar compounds [14], [15], while the results of protein decomposition are a number of peptides which are claimed as precursors to form aroma and flavor compounds [16]. Therefore, this study aims to determine the bacterial population from the mongoose digestive tract and to determine its potential in breaking down protein and pectin.

2. Materials and methods

2.1 Civet surgery
Analysis of civet gastrointestinal tract bacteria is carried out by collecting fluid from the three parts of the civet gastrointestinal tract, namely the stomach, small intestine and large intestine. The fluid collection was obtained through civet surgery which was carried out by the veterinary surgery team at the Fakultas Kedokteran Hewan Universitas Syiah Kuala Clinical Surgery and Radiology Laboratory [17]. Before surgery, the civet was fasted for 8 hours and the premedication drug atropine sulfate was injected subcutaneously at a dose of 0.02 mg / kg BW. After ten minutes, general anesthesia ketamine 10 mg / kg BW and xilazine 1 mg / kg BW were given intramuscularly. Furthermore, an incision is made to remove the digestive tract organs.

2.2 Isolation and enumeration of bacteria from the civet gastrointestinal tract
The fluid collection from each part of the gastrointestinal tract that has been incubated is taken 1 ml and performed serial dilutions. The results of the selected dilutions were cultured as much as 1 ml into each petri dish with different media, namely MRSA media for LAB and NA for non-LAB (two plates each) using the pour plate method and incubated at 37°C for 48 hours (MRSA dishes) and 24 hours (NA plate). Bacterial count was performed by counting the colonies of each plate expressed in CFU / ml. The data that can be used are those with a count between 25-250 colonies per plate [18]. Furthermore, macroscopic observations were made on the morphology of the growing colony (shape, color, elevation, margin, and size), while the cell shape was analyzed microscopically. Single and distinct colonies are separated and purified by streaking method.

2.3 Characterization of bacteria from the civet gastrointestinal tract
The pure isolates were then characterized by several biochemical tests. The tests carried out included the Gram stain test, catalase [19] and the ability to break down protein [12] and pectin [20].

3. Results and discussion

3.1 Civet gastrointestinal bacteria enumeration
LAB and non-LAB enumeration was carried out on three parts of the gastrointestinal tract, namely the stomach, small intestine and large intestine. The two types of bacteria were grouped based on differences in the cultivation media used, namely MRS and NA media. The results of bacterial enumeration in the civet digestive tract are presented in Figure 1.
Based on Figure 1, it can be seen that the total count of LAB and non-LAB dishes increases from the upper gastrointestinal tract (stomach and small intestine) to the lower part (large intestine). The total count of LAB plates in the stomach, small intestine and large intestine was 7.1 log CFU/ml, respectively; 8.4 log CFU/ml and 8.6 log CFU/ml were lower than non-LABs, namely 7.4 log CFU/ml; 8.5 log CFU/ml and 9.1 log CFU/ml. Results of enumeration of civet digestive tract bacteria by Suhandono et al. (2016) in the three sections is 6.1 log CFU/ml; 9.1 log CFU/ml and 9.5 log CFU/ml for LAB and 7.0 log CFU/ml; 9.3 log CFU/ml and 9.1 log CFU/ml for non-LAB. The LAB count in each part of the digestion was higher than non-LAB except in the large intestine.

In this study, it can be seen that the lowest total bacteria (LAB and non-LAB) were obtained in the stomach and the highest in the large intestine. The number of microorganisms in the upper digestive tract (stomach, small intestine) is lower, compared to the lower gastrointestinal tract, namely the large intestine [21], [22], the lowest bacterial population is found in the stomach [23], [24]. The low population of microorganisms in the stomach is thought to be related to physiological conditions and a very acidic environment. The stomach has a very low pH (2-3) so that microorganisms that grow are limited. In addition, the toxicity of bile salts and the relatively fast movement of the digesta are challenges for the growth of microorganisms [25]-[27].

Bacterial counts in the gastrointestinal tract can show varying results. The population of bacteria in the digestive tract of animals and humans reaches approximately 14 log CFU/ml [26]. The composition of the gastrointestinal microflora is influenced by many factors such as location of collection, analysis technique, age, diet, genetics, geography and a number of other factors [9], [23], [26].

There have been many attempts to estimate the number of bacterial species in the gastrointestinal tract but the results are considered controversial. It is known that most bacteria in the gastrointestinal tract cannot be cultured so that most of the estimated data do not fully describe the composition of the microflora of the gastrointestinal tract [23]. The bacteria that were isolated in this study were those that could grow on NA and MRS isolation media so that the bacteria obtained in this study were limited to only bacteria that could grow well on these media.

3.2 Characterization of civet gastrointestinal tract bacteria
A total of 11 pure isolates from the isolated civet gastrointestinal tract were selected for further characterization. The selection was made by observing the morphological characteristics of the colonies that grew on each petri dish on both MRS and NA media. Single and distinct colonies were isolated and purified by streaking method. All pure isolates have been characterized morphologically and biochemically with the results as shown in Table 1.
Table 1. Morphological characteristics of colony and cell of bacteria isolates from the civet gastrointestinal tract.

| Isolate | Colony Colour | Margin | elevation | size | Cell (form) |
|---------|---------------|--------|-----------|------|-------------|
| ICMM1 4007 | circular milky white | Entire raised | small | basil |
| ICMM2 4008 | circular milky white | Entire raised | moderate | coccus |
| ICMM3 4009 | circular milky white | Entire raised | small | basil |
| ICMM4 4010 | circular milky white | entire raised | small | coccus |
| ICMM5 4011 | circular milky white | entire raised | moderate | basil |
| ICMM6 4012 | circular milky white | entire raised | pinpoint | coccus |
| ICMM7 4013 | circular milky white | entire raised | pinpoint | coccus |
| ICMM8 2779 | circular yellowish white | undulate raised | moderate | cocobasil |
| ICMM9 2780 | circular yellowish white | entire raised | small | basil |
| ICMM10 2781 | circular light yellow | entire convex | small | basil |
| ICMM11 2782 | circular yellowish white | entire convex | small | basil |

Bacterial colony morphology can vary between strains due to differential gene expression [28]. Each colony has a distinctive size, shape, edge, texture, opacity and color [29]. Bacterial colonies grow from a single cell and are made up of millions of cells. The appearance of different colonies growing on the same media is assumed to come from different bacterial species. However, due to the large number of bacterial species that have the same colony morphology, this condition is not always true. The parameters observed in colonies can only be relied on for the initial identification of bacterial species [29], [30]. Further identification was carried out by biochemical tests which included Gram staining, catalase, the ability to break down protein and pectin. The test results can be seen in Table 2.

Table 2. Biochemical characteristics of bacteria from the civet gastrointestinal tract.

| Kode Isolat | Gram staining | Catalase | Proteolytic | Pectinolytic |
|------------|---------------|----------|-------------|--------------|
| ICMM1 4007 | +             | +        | +           | +            |
| ICMM2 4008 | +             | -        | +           | +            |
| ICMM3 4009 | +             | +        | -           | +            |
| ICMM4 4010 | +             | -        | +           | +            |
| ICMM5 4011 | +             | +        | +           | +            |
| ICMM6 4012 | +             | -        | +           | -            |
| ICMM7 4013 | +             | -        | -           | +            |
| ICMM8 2779 | -             | +        | -           | +            |
| ICMM9 2780 | -             | +        | +           | -            |
| ICMM10 2781| -             | +        | -           | +            |
| ICMM11 2782| -             | +        | +           | -            |

*: + = available, - = none

Based on Table 2, it can be seen that the biochemical and physiological characteristics of the isolates varied. The results of the Gram test showed that 7 isolates had Gram positive characteristics and 4 Gram negative isolates. Gram stain is the first step in bacterial classification. The cell wall of gram-positive bacteria has a thick web-like cell wall consisting of peptidoglycan (50% -90% of the cell wall), a complex collection of glycol-polymers and proteins. The thick peptidoglycan layer of Gram positive allows these organisms to maintain a crystal violet-iodine complex and stain cells purple. Another major component of the cell wall of gram-positive bacteria presents in the peptidoglycan layer is lipoteichoic acid. This component acts as a regulator of autolytic wall enzymes and also has antigenic properties that stimulate specific immune responses when released from the cell wall after cell death [31], [32].

In the catalase test, 4 isolates showed positive catalase results and 7 catalase isolates were negative. The catalase test is used as a biochemical test to classify bacteria into 2 groups, namely positive catalase
and negative catalase. Catalase is an enzyme produced by microorganisms that live in oxygenated environments to degrade oxygen metabolites, hydrogen peroxide [33, 34]. Anaerobic bacteria generally lack the enzyme catalase. Catalase negative bacteria live in anaerobic conditions and no hydrogen peroxide is formed during metabolism. All LAB grows anaerobically but unlike most anaerobic bacteria, LAB can grow well in the presence of oxygen (aerotolerant anaerobes). LAB that grows in aerobic conditions also shows negative catalase, namely using peroxidase enzymes to destroy hydrogen peroxide [34], [35].

Another biochemical characteristic analyzed is the ability to break down pectin and protein. The enzyme activity shown by each bacterial isolate varied. Some have only pectinolytic activity but do not show proteolytic activity or vice versa. There were also four isolates that had pectinolytic and proteolytic activity, namely isolates ICMM1, ICMM2, ICMM4 and ICMM5. Having specific enzyme activity is one of the requirements needed to become a starter. The ability of microorganisms to break down protein and pectin is important to know as an indicator that isolates can serve as potential starters in coffee fermentation.

Coffee contains a number of protein and pectin. Protein degradation produces peptides and some free amino acids [16], while pectin hydrolysis produces some simple sugars [36], [37]. The results of the degradation of these two components can affect the taste of coffee when roasted. Roasting causes major changes in the chemical composition of coffee such as protein, amino acids, reducing sugars, sucrose, trigonelin, chlorogenic acid, decreased water content and the formation of melanoidin, many of which are caused by the Maillard reaction [38]. Chemical reactions during roasting are very important in the formation of coffee aroma because they are responsible for producing a number of compounds that have an impact on coffee aroma such as pyrazine, pyrurol, thiol, furanone, pyridine, and thiophene [39]. In the next stage, it is necessary to confirm the ability of the four isolates as potential starters in fermenting coffee to produce artificial civet coffee.

4. Conclusion
The population of lactic acid bacteria (LAB) in the gastrointestinal tract was generally lower than that of non-LAB. LAB and non-LAB populations tend to increase from the upper gastrointestinal tract (stomach and small intestine) to the lower (large intestine). The bacteria isolated from the civet gastrointestinal tract have various morphological and biochemical characteristics. A total of four isolates were confirmed as LAB and the rest were non-LAB. Four isolates of civet bacteria have the ability to degrade protein and pectin at once. Further tests are needed to determine the potential of the four isolates in producing artificial civet coffee.

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