Characteristics of lactic acid bacteria and acetic acid bacteria isolated before and after fermentation of cacao beans from Pidie, Indonesia and detection of the flavour compounds

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Abstract. The objectives of this research were to characterise lactic acid bacteria (LAB) and acetic acid bacteria (AAB) which were isolated before and after fermentation of cacao beans, and to detect the flavor compounds in each of these steps. Samples were collected from a farmer in the Pidie District, Aceh Province, Indonesia who supplies the fermented cacao beans for a local chocolate factory located in Pidie Jaya District. The samples were collected before fermentation (F0) and after 5 days fermentation (F5) steps in duplicate. Five LAB and nine AAB were characterised for colony and cell morphology, catalase and oxidase tests, and growth resistance at low pH. The qualitative analysis of flavor compounds of the cacao beans were identified by Gas Chromatography Mass Spectrometry Solid Phase Microextraction (SPME-GCMS). The results showed that all isolates have a negative result on oxidase tests and only one AAB isolate could grow at pH 3. The reduction of phenethylamine during fermentation is desired because it gives off a fishy odor as a result of decarboxylation of amino acids by lactic acid bacteria. The detection of 3-octanyl acetate by its sweet floral fruity odor is expected. It is also interesting to note that a nutty taste and earthy aroma were detected indicating that trimethyl pyrazine and dimethyl pyrazine were present after fermentation even though they were not a major compound.

1. Introduction
Cocoa (Theobroma cacao L.) is a plantation crop that is widely cultivated in Indonesia and has become one of Indonesia's leading export commodities aside from coffee. In 2019, Indonesian cocoa production reached 200,000 tons [1]. Indonesia is the third largest producer of cocoa after Ivory Coast with a total production of 2,154,000 tons, and Ghana with a total production of 812,000 tons. According to the [2], the areas that produce the most cocoa in Indonesia include: South Sulawesi, West Sulawesi, Southeast Sulawesi, Central Sulawesi, Maluku, Papua, East Nusa Tenggara, East Java, Lampung, West Sumatra, North Sumatra, and Aceh. From the total number of areas which produce cocoa in Indonesia (1,658,421 ha) in 2017, more than 97% of Indonesia's cocoa plants came from...
smallholder plantations. In the Aceh Province, the total land used for cocoa plantations reached 101,203 Ha and produced around 27,129 tons of cocoa in 2014. The majority of these smallholder cocoa plantations can be found in Pidie, Pidie Jaya, Southwest Aceh, North Aceh, Bireun, East Aceh and Aceh Tenggara Districts.

According to data from the Forestry and Plantation Service, the largest cocoa production in the Aceh Province, Indonesia was in Pidie Jaya District, which is spread into eight sub-districts (Bandar Baru, Pante Raja, Trienggadeng, Meureudu, Meurah Dua, Ulim, Jangka Buya and Bandar Dua). The largest cocoa production from the eight sub-districts is in Bandar Baru. In addition, Pidie Jaya Regency also has a cocoa processing industry in Baroh Musa Village, Bandar Baru District. Processed products produced are chocolate bars, powder chocolate, various chocolate cakes, and chocolate drinks.

For food products, the current market demand for cocoa requires good quality cocoa beans which has the distinctive aroma and taste of chocolate [3]. Thus, important quality indicators of cocoa beans include chemical qualities such as the amount and type of volatile compounds (aroma), as well as physical qualities such as seed colour, acidity of seeds, and seed size [4]. However, the complex composition of the aroma of cocoa beans depends heavily on seed genotype, soil type, age of cocoa trees, postharvest treatment such as pulp precondition, fermentation and the roasting process [5].

The aroma component of cocoa beans consists of volatile compounds which are formed from peptide and carbohydrate remodelling during the fermentation process which involves microbes such as lactic acid bacteria, acetic acid bacteria, and yeast [6]. The three microbes are very instrumental in the formation of the volatile components of cocoa. According to [7], components 2-3-5-6-tetramethylpirazine are components of pyrazine which are very influential in providing the desired chocolate aroma in cocoa paste. Therefore, in this study, the focus of the research lies in the isolation of microbes that play an important role during the fermentation process of cocoa beans and identification of volatile components formed before and after fermentation.

2. Materials and Methods

2.1. Samples Collection

The raw material used was lindak (bulk) cacao beans. The beans were collected from a local farmer in Padang Tiji Sub-district, Pidie District, Aceh Province, Indonesia who supplies the fermented cocoa beans to a local chocolate factory in the Pidie Jaya district. Samples were collected from before fermentation (F0) and after 5 days of fermentation (F5) in duplicate. Samples were kept in clear bottles and covered with aluminium foil. The temperature during transport was maintained by using a controlled cooling box.

2.2. Characterisation of Isolates

Cacao bean samples were grinded, diluted in buffered peptone water, and grown on MRS agar (Oxoid) and YGC agar (7.5 g yeast extract, 37.5 g D-glucose, and 3.75 g CaCO₃ in 750 ml aquadest) by the pour plating method. Both agars containing samples were incubated at 30°C for 48 hours. All isolates with different physical characteristics (colony morphology) were isolated and further characterised for cell morphology, catalase, and oxidase tests. Then, isolates from each agar and each stage were examined for their growth resistances at various low pH levels (3, 3.5, and 4).

2.3. Identification of Flavor Compounds

The qualitative analysis of flavor compounds was identified by Gas Chromatography Mass Spectrometry Solid Phase Microextraction (GCMS-SPME). Volatile compounds from the cacao beans (2.0 g) were extracted using a solid phase microextraction technique (SPME). The system used was a direct system (split less mode) with an injector temperature of 240°C for 30 seconds. The detector was quadrupole with an ionization system at 70 eV and a temperature of 260°C. Compounds were identified by two criteria: the first was by comparing the mass spectrum of each mass spectrum
compound from the Wiley 275L library; and second by comparing the retention index with literature data.

3. Results and Discussions
3.1. Characterisation of Isolates
Before fermentation, two LAB isolates and four AAB isolates were isolated, while after fermentation, three LAB isolates and five AAB isolates were isolated. Characteristics of all isolates can be seen in Table 1 where all isolates have negative result on oxidase test. Interestingly from Table 2, only one isolate from YGC agar could grow at a low pH level (pH 3). In Malaysian spontaneous cocoa pulp fermentation, two dominant bacterial species were found, *Lactobacillus fermentum* and *Acetobacter pasteurianus* [8]. Cocoa beans from East Java, Indonesia, were dominated by the presence of *Bacillus pumilus* and *Bacillus licheniformis* in the later stages of fermentation [9]. According to [10][11], these lactic acid bacteria have a negative catalase and are tolerant of acids.

### Table 1. Characteristics of lactic acid bacteria and acetic acid bacteria

| Microbe’s Group | Stage | Isolate Code | Gram Staining | Cell | Catalase | Oxidase |
|-----------------|-------|--------------|---------------|------|----------|---------|
| **Lactic Acid Bacteria (LAB)** | Before Fermentation (F0) | MRS 26 | Negative | Rods | Positive | Negative |
| | | MRS 31 | Positive | Rods | Negative | Negative |
| | After Fermentation (F5) | MRS 102 | Positive | Coccus | Negative | Negative |
| | | MRS 105 | Negative | Rods | Negative | Negative |
| | | MRS 106 | Positive | Coccus | Positive | Negative |
| **Acetic Acid Bacteria (AAB)** | Before Fermentation (F0) | YGC 29 | Negative | Rods | Positive | Negative |
| | | YGC 30 | Negative | Rods | Positive | Negative |
| | | YGC 34 | Negative | Rods | Positive | Negative |
| | | YGC 35 | Negative | Rods | Positive | Negative |
| | After Fermentation (F5) | YGC 97 | Negative | Rods | Positive | Negative |
| | | YGC 98 | Negative | Rods | Positive | Negative |
| | | YGC 99 | Negative | Rods | Positive | Negative |
| | | YGC 100 | Negative | Rods | Positive | Negative |
| | | YGC 101 | Negative | Rods | Positive | Negative |

### Table 2. Low pH resistance of lactic acid bacteria and acetic acid bacteria

| Microbe's Group | Stage | Kode Isolat | pH 3 | pH 3.5 | pH 4 |
|-----------------|-------|-------------|------|--------|------|
| **Lactic Acid Bacteria (LAB)** | Before Fermentation (F0) | MRS 26 | + | ++ | ++ |
| | | MRS 31 | + | + | + |
| | After Fermentation (F5) | MRS 102 | + | ++ | ++ |
| | | MRS 105 | + | ++ | ++ |
| | | MRS 106 | + | ++ | ++ |
| **Acetic Acid Bacteria (AAB)** | Before Fermentation (F0) | YGC 29 | - | - | + |
| | | YGC 30 | - | - | + |
| | | YGC 34 | - | + | ++ |
| | | YGC 35 | + | + | + |
| | After Fermentation (F5) | YGC 97 | - | - | ++ |
| | | YGC 98 | - | + | + |
| | | YGC 99 | - | + | ++ |
| | | YGC 100 | - | + | ++ |
| | | YGC 101 | - | - | + |
3.2. Identification of Flavor Compounds

Figure 1 shows that propanal, ethanedioic acid, 2-heptanol, 2-nonanone, and phenethylamine were detected as the main components before fermentation, while from Figure 2, 2,3-butanediol, 2-heptanol, 3-octanyl acetate, 2-nonanone, 2-dodecanol, decanoic acid, cyclopentasiloxane, and phenol,2,4-bis(1,1-dimethyl) were detected as the main components after fermentation. [11] identified a total of 58 compounds grouped into six main chemical groups which were esters (20), alcohols (12) and acids (11) groups showed higher numbers of individual compounds than aldehydes and ketones (8), pyrazines (4) and other compounds (3). A similar number of compounds were also found by [12] in fermented and dried Criollo cocoa. They found 9 alcohols, 11 aldehydes and ketones, 4 esters, 6 acids, and 6 pyrazines.

![Figure 1. Chromatogram of cocoa beans before fermentation (F0).](image1)

![Figure 2. Chromatogram of cocoa beans after fermentation (F5).](image2)
It is interesting to note that the component of trimethylpyrazine which provides a nutty flavor and dimethylpyrazine that provides earthy aroma were detected after fermentation although they were not the main components. Therefore, improvement in the fermentation process is needed. Decreasing phenethylamine during fermentation is desirable because this component provides a fishy aroma as a result of decarboxylation of amino acids by lactic acid bacteria. Detection of 3-octane acetate is desired which provides Aceh's cocoa with a sweet floral fruity aroma. Cyclopentasiloxane compounds are typically the components used in the manufacture of hair and skin conditioning products. The presence of a higher alcohol content is thought to be produced by yeast during fermentation. High alcohol is likely to give the cocoa a fruity (as well as floral) aroma. According to [13], the aroma of fruit in cocoa is also closely related to its acidity which increases during fermentation.

4. Conclusions

Tetramethylpirazine is a component of pyrazine which is very influential in providing the distinctive aromas of chocolate. Interestingly, it was not found to be one of the main components produced in cocoa beans that have been fermented for 5 days. In conclusion, the processing of cocoa beans from the Pidie district, of Aceh could potentially be used to develop non-food processed products such as perfumes, cosmetics, and lotion.

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