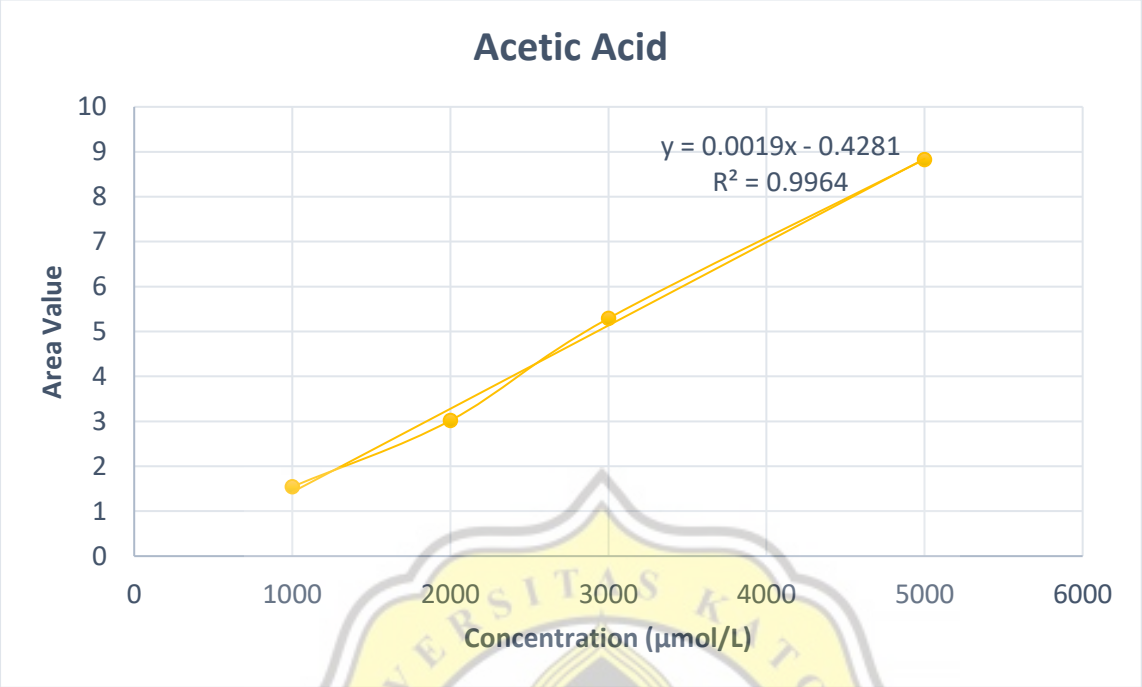
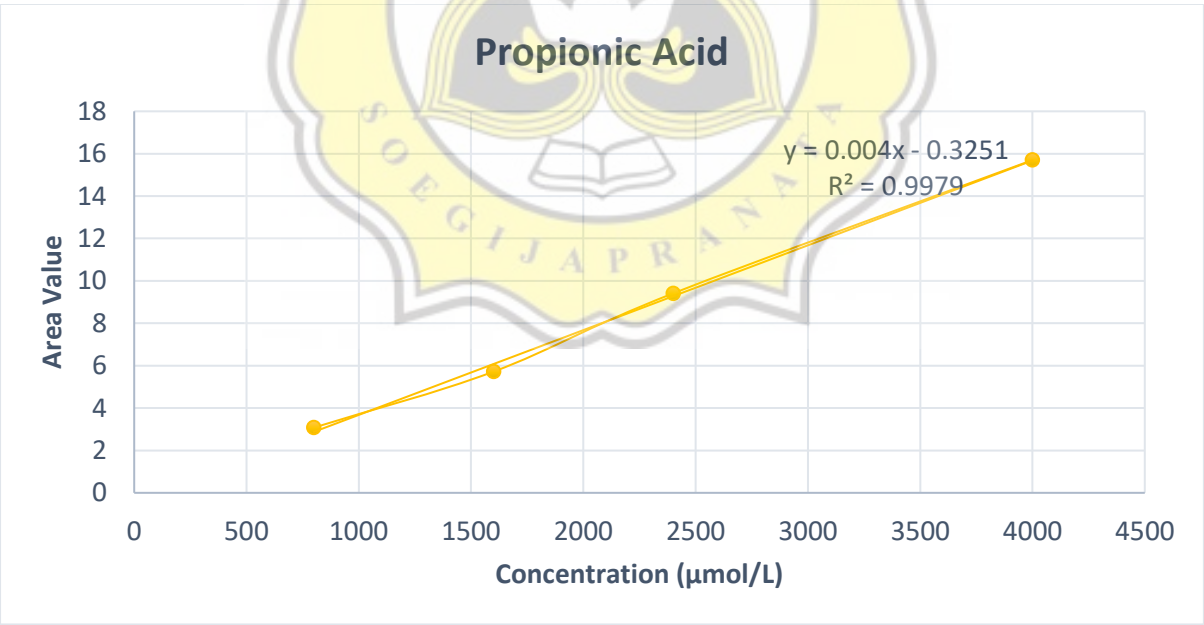
6. APPENDIX

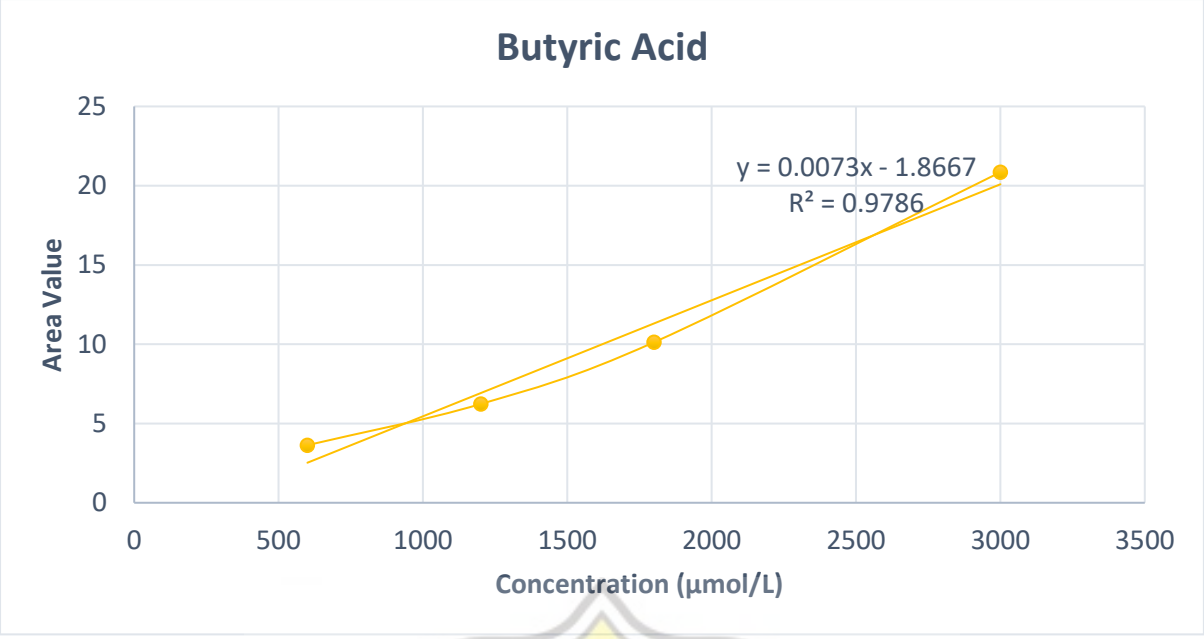
6.1. Standard curve of acetic, propionic and butyric acid



Additional Figure 1. Standard Curve of Acetic Acid



Additional Figure 2. Standard Curve of Propionic Acid

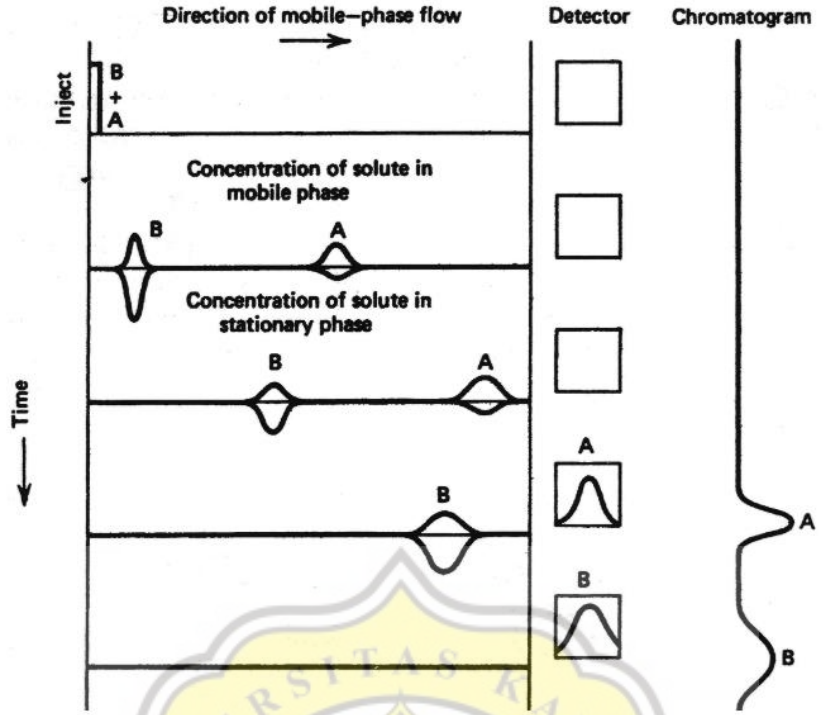


Additional Figure 3. Standard Curve of Butyric Acid

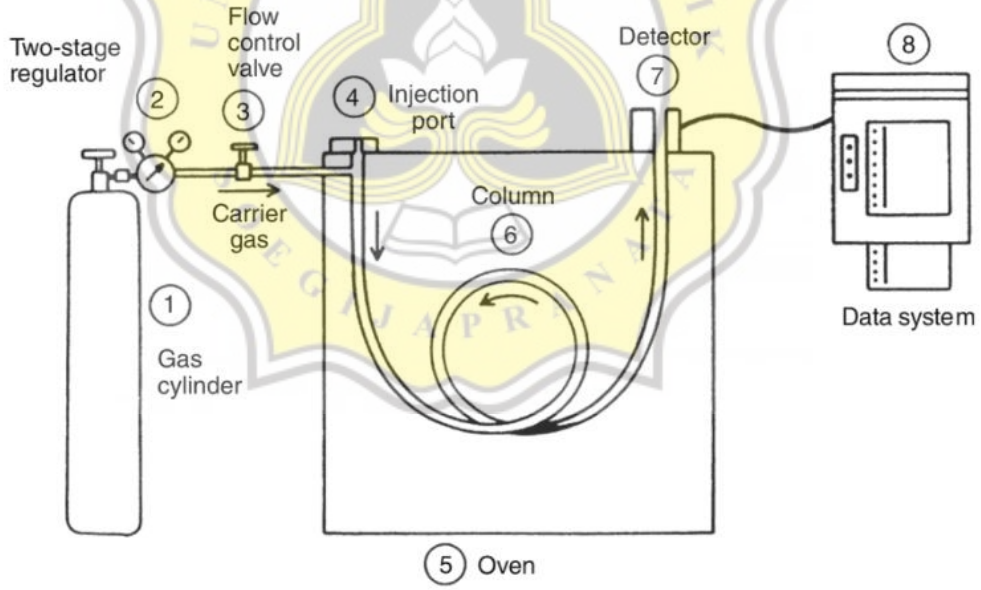
6.2. Measurement of Short Chain Fatty Acids by Gas Chromatography

6.2.1. Gas Chromatography

Gas chromatography is a separation technique that use the affinity of the sample carried by the mobile phase (gas phase), with the stationary phase (column) to separate each compound present in the sample. In the gas phase, the sample will be carried by the carrier gas into the chromatographic column, where it interacts with the stationary phase. Because each compound has a different affinity for the stationary phase and mobile phase, the compounds were separated. Each compound will then reach the detector and be analyzed its physicochemical properties, strengthen the signal, and transfer the data of each compound to the data system. Additional Figure 4 and 5 illustrate the principle of GC analysis (McNair & Miller, 1998)



Additional Figure 4. Schematic representation of gas chromatography separation (McNair & Miller, 1998)



Additional Figure 5. Schematic of gas chromatography

In gas chromatography, column property is the major factor affecting the effectivity of compound separation. Column stationary phase, length, inner diameter and film thickness affect column performance. From polar compounds to non-polar compounds, a variety of stationary phases can be used to cover the many compounds present in the sample. As mentioned above, the principle of GC separation is based on the affinity or how strong/weak

the interaction between the compound and column stationary phase. This is called selectivity (α). According to the law of "like to dissolve like", if a compound and a stationary phase have similar polarities, a strong interaction will occur between them. For example, a column with polyethylene glycol columns that have polar nature has high selectivity to polar compounds, such as alcohol. The length of the column determine the required time and the degree of peak separation. Longer columns provide better peak separation and can separate higher molecular weight compounds. Internal diameter determines the ability of column to withstand the sample concentration, and film thickness (thickness of stationary phase) also affects capacity and time (Restek, 2018).

Flame ionization detector (FID) is one of the most commonly used detectors in GC, especially for organic compounds (compounds that contain carbons). It uses the flame (air-hydrogen) to decompose the separated compounds as soon as it is separated. When the compound decomposes, it will release ions and electrons that can carry current through the detector. This will later be identified by a picoammeter to measure the ion released from organic species. The limitation of FID is that it has low sensitivity to highly oxygenated molecules due to the presence of oxygen in the structure.Usually, these molecules will be identified by other detectors (Krugers, 1968).

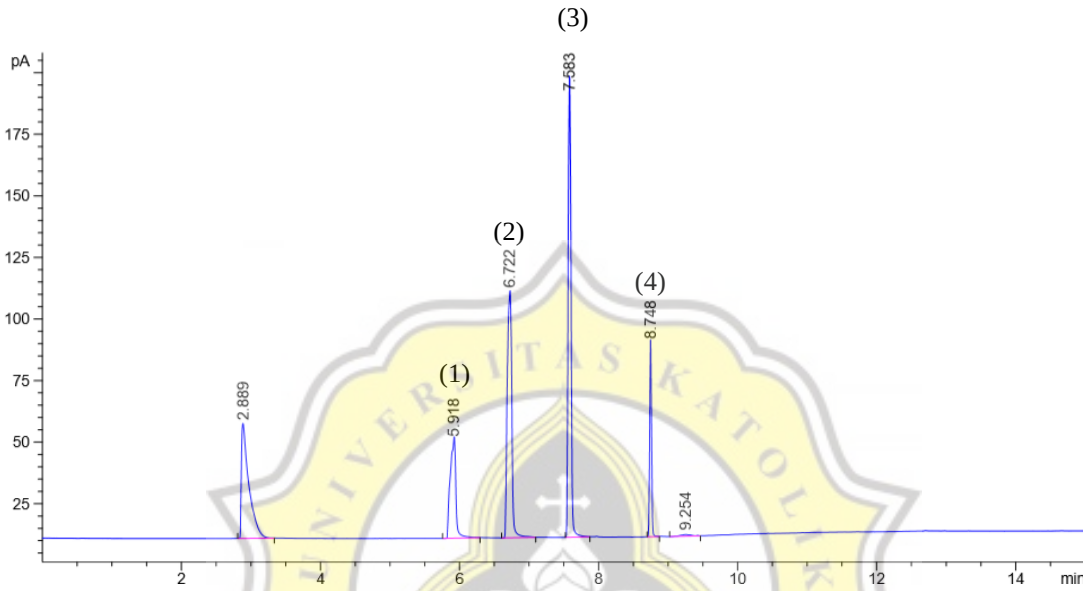
6.2.2. Sample preparation for measurement of short chain fatty acid

Both distilled water and saline water (0.9% NaCl; 0.02% NaN_3) are suitable solvents to extract SCFAs from fecal sample as SCFAs obtain high solubility in water (Lupton & Newmark, 1990).

After extracted with water or saline water, the sample is centrifuged at 3000 *x*g for 15 minutes at 4^{o}C and the brownish supernatant is taken and measured with GC. This method is known as direct injection as the sample was not treated or extracted extensively by organic solvent. The advantage of this is that the preparation is low cost and fast, therefore minimizing SCFA lost during preparation (Tangerman & Nagengast, 1996). In the preliminary test of this study, sulfuric acid and diethyl ether were used as extraction solvents to extract SCFAs from fecal sample according to Whitehead *et al*. (1976). However, no peak was observed in the GC, not even the internal standard, which indicated that SCFAs were degraded during extraction procedure. In addition, The yield of SCFA was too low to filter the sample with a 0.45 um filter membrane. As a result, the solution is insufficient and GC analysis cannot be performed.

6.2.3. Analysis of short chain fatty acid

The mixture of acetic acid, propionic acid, butyric acid and 2-ethylbutyric acid (as an internal standard) could be separated using the GC conditions in this study (Additional Figure 6). All SCFA standard solution had good linearity with R^2 value > 0.95. The R^2 values were 0.9964; 0.9979; 0.9786 for generated standard curves of acetic acid, propionic acid and butyric acid, respectively (Additional figure 1, 2, 3).



Note: (1) acetic acid; (2) propionic acid; (3) butyric acid; (4) 2-ethylbutyric acid. SCFA in the concentration of 60 µL/L and 2-ethylbutyric acid in 150 µL/L.

Additional Figure 6. Gas chromatography separation for short chain fatty acid standard solution

This GC conditions were set according to the method reported by Han *et al*. (2018), and modified the oven temperature. The oven was initially set to 90^{o}C, then gradually increased to 150^{o}C for the separation and finally increased to 220^{o}C. Such conditions could completely separate each SCFA, and each SCFA had a sharp peak, with the total running time of 15 minutes. However, the residue would cause column contamination after measuring the standard or sample, which indicated that using 220°C as the final temperature and holding it for 5 min was not enough to burn off the remaining sample. Raise the temperature to 240°C (the highest temperature the column can withstand), but there was still contamination.

Therefore, an additional washing stage was applied. After each sample / standard measurement, distilled water was injected at the initial oven temperature of 90°C, and then gradually increased the temperature to 150°C for 2 minutes. Each washing stage took 5 min. To ensure the elimination of residues, washing was repeated 5 times after each sample / standard measurement.

Report #10846442

INTRODUCTION BackgroundThe research of a chemopreventive agent for colorectal cancer had been widely conducted for decades, regarding its third position of the most diagnosed cancer in the world. Among cases, 70% was a sporadic case where the patient did not have any family history and genetic mutation on colorectal cancer, thereof it was proposed diet and lifestyle might be the factors that trigger cancer formation ADDIN (De Almeida et al., 2019). Diet, in specific, was the focus in most of the research as the major factor in triggering colorectal cancer ADDIN (Wong et al., 2019). This was related to the role of the colon as one part of the intestinal tract where all the fermentation occurs with the help of gut microbiota. Among dietary compounds, anthocyanin has been reported to depend on gut fermentation in order to be absorbed ADDIN (Ozdal et al., 2016). The breakdown of anthocyanin will result in the release of aglycone and sugar, from which sugar was an energy source for microbiota. Anthocyanin aglycone was also reported with its antibacterial activity toward pathogenic bacteria, thus the theory of anthocyanin consumption could affect gut microbiota composition was presented ADDIN (Zhang, et al., 2016). Other than that, this compound was known with high antioxidant activity and had been reported for other functional properties, such as inhibition to