Altered profile of gut microbiota and the level short chain fatty acids in colorectal cancer patients

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Abstract. The gut microbiota plays as a real organism and alterations in its composition have been found in patients with colorectal cancer (CRC). This study aims to investigate the relationship between the microbiota structure and the level of SCFA in CRC subjects compared to non-CRC subjects. This case-control study included fourteen subjects with CRC compared to fourteen non-CRC subjects. Their stool samples were analyzed for SCFA [acetate, propionate and butyrate acids] using gas chromatography and an exploration of the diversity of the bacteria using 16S rRNA gene denaturing gradient gel electrophoresis [DDGE]. PCR-DGGE analysis from CRC subjects indicated that there were decreasing numbers of Bifidobacterium found in the stool of CRC subjects compared to non-CRC subjects. The concentrations of acetate, propionate and butyrate acids had significantly lower with mean 8.55 µg/mL, propionate 5.61 µg/mL and butyrate acids 3.79 µg/mL respectively. Among these three SCFA, the level of propionate and butyrate acids were statistically significant [p <0.05]. In conclusion, dominant band from Bifidobacterium groups vanished in all subjects with colorectal cancer and we found different spectrums of Bifidobacterium in CRC subjects compared to non-CRC subjects. SCFA concentrations also appeared to be lower in CRC patients.

1. Introduction
Colorectal cancer is the fourth most frequent cancer worldwide [1]. The occurrence of colorectal cancer is 5-10 times higher in most developed countries [2]. In Indonesia, colorectal cancer disease causes the increasing evidences of cancer-related mortality in recent years. Anatomical Pathology Division at Faculty of Medicine of University of Indonesia reported that there were 36.75 % of colorectal cancer patients who are less than 40 years old based on their epidemiological data in 1996-1999 [3]. Colorectal cancer is a disease from an accumulation of genetic mutation, epigenetic, the dysregulation of their communication in signaling pathways, and gut microbial contributions. Microbial involvement in colorectal cancer (CRC) is now well established [4]. Understanding the role of the microbiota might be as important as understanding the microbiota itself, moreover it might support the diagnostic and mechanistic insight to learn more about the composition of microbiota.
Composition of the gut microbiome relies on many diverse factors including the age, diet, genetic composition of the host, gender, geographic location, and health status of an individual. These factors result in the development of the indigenous microbiota [8].

Short Chain Fatty Acids (SCFA) are the result of anaerobic microbial fermentation in the large intestine and affect the health of the colon. SCFA are mainly produced as microbial metabolites, acetate, propionate, and butyrate acids. The formation of butyrate and other SCFA presumably act as chemo preventive products of microbial fermentation in the intestine [8,9]. The relation between functional characteristics, such as SCFA and the incidence of CRC has not been extensively elaborated [10]. This study aims to analyses the relationship between the composition of gut microbiota and the level of SCFA in CRC patients compared to non-CRC patients.

2. Materials and Methods
2.1 Participants and sample collection
Samples were taken from fourteen CRC patients and fourteen non-CRC patients from Gastroenterology-Hepatology Department at Dr. Zainoel Abidin General Teaching Hospital Banda Aceh, Indonesia. The patients were included in the study based on the following criteria: 1] Patients is eighteen years old and older; 2] Indonesian citizens proven by their identity cards; 3] Patients with colorectal cancer diagnosed by pathological examination; 4] Other patients who are not diagnosed with colorectal cancer; and 5] Patients who are willing to cooperate in the study. The patients must not receive antibiotic treatment within a month prior to the colonoscopy and must not receive chemotherapy and/or radiotherapy in the last six months. The patients should not consume yoghurt and take laxative medication in the last five weeks.

Stool samples, collected from each participant, were labelled and stored at -20ºC freezer by the researchers. The microbiological analysis was performed by specialized Microbiological Service at Faculty of Veterinary Medicine, Syiah Kuala University

2.2 Gut microbiota analysis
Growth bacteria colonies were taken for DNA isolation with PowerSoil® kit, DNA isolation was conducted based on previous method with some modifications [11]. From the extracted DNA, the 16S rRNA will be amplified by Polymerase Chain Reaction (PCR) using universal bacterial primers. Forward or reverse primer from every primer sets was added with GC-clamp on 5’ side to increase detection from PCR products with DGGE. Polymerase chain reaction (PCR) was conducted using GoTaq green master mix (Promega). Programmed-PCR was done as follow: 940C for 3 minutes; 30 cycles of 940C for 30 second, 550C for 30 second and 720C for 1 minute; and final extension of 720C for 5 minutes. Product of PCR was analyzed using DGGE (Biorad). DGGE analysis was conducted based on manufacturer’s protocols in combination with several previous studies [12,13,14] with some modifications.

2.3 Gas chromatography analysis of fecal SCFA concentration
SCFA concentrations were assessed from the stool samples using gas chromatography as mentioned previously in the method section [15,16]. The levels of acetate, propionate and butyrate acids were reported in µg/ml and %.

2.4 Statistical analysis
The exact chi-square test and Student t-test were used to make group comparisons, and non-parametric statistics was used in variables without normal distribution. P-values < 0.05 were considered as statistically significant.

3. Result and Discussion
A total of 28 participants joined in the study. Table 1 demonstrated the characteristics of the participants. CRC groups consisted of 10 male subjects and 4 female subjects, while non-CRC group consisted of 9 male subjects and 5 female subjects. Their age means (±SD) was 53.8± 13.3 years old for CRC and 50.0 ± 17.6 years old for non-CRC. CRC group showed lower level of hemoglobin, BMI and albumin compared to non-CRC group. 79% of the colorectal cancer is located in the rectum, while the other 21% is located in the descending colon.
Table 1. Characteristics of participants in this study

| Variable                  | Colorectal cancer mean (SD) | Non – colorectal cancer mean (SD) |
|---------------------------|-----------------------------|----------------------------------|
| Sex                       |                             |                                  |
| Male                      | 10 (72%)                    | 9 (64%)                          |
| Female                    | 4 (28%)                     | 5 (36%)                          |
| Age (years, mean)         | 53.8 (13.3)                 | 50.0 (17.6)                      |
| BMI (kg/m², mean)         | 20.21 (± 2.65)              | 23.6 (± 1.91)                    |
| Hemoglobin (g/dl, mean)   | 10.6 (± 2.1)                | 12.3 (± 1.2)                     |
| Leukocytes (10³/mm³)      | 8.20 (± 2.11)               | 8.14 (± 2.75)                    |
| Platelet (10³/mm³)        | 318.69 (± 101.40)           | 255.83 (± 107.47)                |
| Hematocrite               | 34.47 (± 5.32)              | 36.19 (± 3.82)                   |
| Albumin (g/dl, mean)      | 3.24 (± 0.71)               | 3.91 (± 0.53)                    |

Colonoscopy
- Ca Rectum: 11 (79%)
- Ca Colon Descending: 3 (21%)
- Colitis Infection: 10 (72%)
- Colitis Infection + Hemorrhoid Interna: 4 (28%)

Table 2. Bivariate analysis of the relationship composition gut microbiota between CRC and non-CRC patients

| Group      | Enterobacterium (+/- 370 bp) | Bifidobacterium (+/- 500 bp) | Total | P* |
|------------|------------------------------|-----------------------------|-------|----|
|            | n   | %  | n  | %  | n  | %  |     |    |
| CRC        | 14  | 100| 0  | 0  | 14 | 100| <0.001 |
| Non CRC    | 0   | 0  | 14 | 100| 14 | 100| 1    |
| Total      | 14  | 50 | 14 | 50 | 28 | 100|      |

*Chi-square test

Analysis of PCR-DGGE from CRC patients and non-CRC patients showed a different appearance (Figure 1). The result of DGGE from PCR product using primer for universal bacteria (line 1), Enterobacterium (line 3) and Lachnospiraceae (line 4) groups resulted in similar band between colorectal cancer patient and non-CRC patient. The different appearance was found on PCR product using primer for Bifidobacterium groups (line 2). Patients with colorectal cancer did not show any band appearances. In contrast, non-CRC patients were showed one clear band with size + 500bp. Result of PCR-DGGE did not show any dominant band from Bifidobacterium groups in all patients with colorectal cancer. Vice versa, all patients that only evidence several symptoms related to colorectal cancer were shown one dominant band of Bifidobacterium groups. The summaries of dominant band appearance from all samples were shown in table 2.

The mean of fecal concentrations of acetate, propionate dan butyrate were significantly lower in patients with CRC compared to non-CRC. From the table 2, the results showed that the mean concentration of acetate is 8.55 µg/mL, propionate is 5.61 µg/mL and butyrate acids is 3.79 µg/mL respectively (all P < 0.05) (Figure 2).
Nowadays, microbiome has shown its important role for human health. SCFA are the main products of anaerobic microbial fermentation in the large intestine and could affect colonic health. In this study, dominant band from Bifidobacterium groups vanished in all subjects with colorectal cancer and we found different spectrums of Bifidobacterium in CRC subjects compared to non-CRC subjects. SCFA concentrations also appeared to be lower in CRC patients.

### Table 3. Between-group comparison of SCFA level in CRC and Non-CRC Patients

| Variable          | Colorectal cancer patients (N=14) | Non-colorectal cancer patients (N=14) | P-value |
|-------------------|-----------------------------------|--------------------------------------|---------|
|                   | Mean±SD                           | Median (min-max)                      |         |
| Acetate Acids     | 8.55 ± 3.06                       | 8.54 (3.44-12.50)                    | 0.038   |
| Propionate Acids  | 5.61 ± 1.95                       | 6.24 (1.82-7.82)                     | 0.008   |
| Butyrate Acids    | 3.79 ± 2.04                       | 4.24 (1.20-8.15)                     | 0.002   |

Colorectal cancer (CRC) is one of the most frequent cancer-related mortality worldwide and its incidence has increased rapidly in the past few years [17,18]. Recently, the 16S rRNA gene sequencing approach has been widely used as an effective tool to globally analyze the microbial community. Multiple studies have demonstrated that breakdown of the intestinal microbiota structure can promote carcinogenesis and development of CRC [19]. Flanagan et al demonstrated a significant association between Fusobacterium nucleatum level and patient outcome suggesting that F. nucleatum may have value as a prognostic indicator [20]. Boleij et al, 2015. found that the detection of Bacteroides fragilis toxin [BFT], which was produced by Enterotoxigenic Bacteroides fragilis [ETBF], increased in the mucosa of patients with late-staged CRC [21]. Another study suggests that the disappearance of Bifidobacterium can be a parameter to detect colorectal cancer [22].

Figure 1. The 16S-RNA profile identified by PCR-DGGE analysis. Different band appearance was obtained from the sample, especially from the PCR product that using primers to amplify bacteroides groups. M= marker; P=patient with positive colorectal cancer (CRC); C= patient with negative CRC; Line 1= Universal bacteria; Line 2= Bifidobacterium; Line 3= Enterobacterium; Line
4=Lachnospiraceae Band from Bifidobacterium groups (line 2) vanished in all subjects with colorectal cancer.

In our study, subjects with colorectal cancer showed the disappearance of dominant band of Bifidobacterium groups. There is a significant association between the levels of SCFA and the composition of the microbiota. The high luminal concentrations resulting from fermentation can lower the pH of the colon [5.5–6.5 in proximal colon where fermentation is the highest, compared to pH 6.5–7.0 in the distal colon] and inhibit the growth of Gram-negative Enterobacteriaceae including familiar pathogens Salmonella spp. and Escherichia coli. In particular, butyrate has been reported to have protective effect against development of colitis and colorectal cancer [23]. The level of SCFA in faecal samples have been shown to be associated with some diseases such as Inflammatory Bowel Disease (IBD), Irritable Bowel Syndrome (IBS), Cardiovascular Disease (CVD), diarrhea, and cancer [8]. Therefore, the increasing of SCFA production and potentially a greater distribution of SCFA, specifically butyrate, to the distal colon may result in a protective effect [24]. In our study, we found that the SCFA level is decreased significantly in CRC compared to non-CRC patients. Due to the relatively small number of participants, the result of this study is limited. Larger study populations would be more reliable and represent the population more accurately. Another limitation of this study is that we cannot rule out the environmental factors such as diet and everything related to microbiota.

![Figure 2](image-url)

Figure 2. The level of short chain fatty acids in the colorectal cancer group and non-colorectal cancer.

4. Conclusion
This study showed that the dominant band from Bifidobacterium groups vanished in all subjects with colorectal cancer and we found different spectrums of Bifidobacterium in CRC subjects compared to non-CRC subjects. In addition, SCFA concentrations also appeared to be lower in CRC patients.

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