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Microbiota-derived metabolites in colorectal cancer patients in preoperative period

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Abstract. – OBJECTIVE: Short-chain fatty acids (SCFAs) are microbial derived metabolites, which have multiple beneficial properties. The amount of SCFAs depends on several factors, such as age, diet (mainly intake of dietary fiber), and overall health condition. The normal proportion between SCFAs is 3:1:1 for acetate, proprionate and butyrate, respectively. In colorectal cancer (CRC) patients, microbiota alterations have been shown. Consequently, metabolome within the gut might change to a large extent. Therefore, the aim of this study was to analyse the content of SCFAs and the proportion between SCFAs in the stool obtained from CRC patients in preoperative period.

PATIENTS AND METHODS: This study included 15 patients with CRC in preoperative period. The stool samples were taken and stored at -80°C in the Fahrenheit Biobank BBMRI.pl, Medical University of Gdansk, Poland. The analysis of SCFAs from stool samples was conducted by means of gas chromatography.

RESULTS: This study included mainly males (66.67%, n=10). In all patients, there was abnormal proportion between SCFAs. The extremely higher concentration of butyrate was noted in 2 samples (13.33%) compared to the rest of patients. However, based on normal proportion between SCFAs, the results <1 for butyrate were noted in 93.33% of patients.

CONCLUSIONS: SCFAs pool is altered in CRC patients, among others characterized by low level of butyrate. It should be considered to administer butyrate supplementation to CRC patients especially prior to surgery to support an appropriate preparation to this treatment.

Key Words:
Short-chain fatty acids, Butyrate, Metabolome, Gut microbiota, Colorectal cancer.

Introduction

Currently, colorectal cancer (CRC) is still one of the most common diagnosed cancers worldwide. The link between CRC and gut microbiota-related aspects has been increasingly analysed during last years[1,2]. Gut microbes may promote or prevent carcinogenesis process[3] depending on their abundance, properties, and activity. Pathogens contribute to the development of CRC through several molecular mechanisms for instance *via* oxidative stress damaging DNA, activation of NF-κB signaling, promotion of inflammation, induction of E-cadherin lysis, genetic mutations in epithelial cells and many others[4,5]. *Fusobacterium nucleatum*, *Bacteroides fragilis*, *Enterococcus faecalis*, *Peptostreptococcus anaerobius*, *Porphyromonas gingivalis*, *Streptococcus bovis*, *Helicobacter pylori*, *Helicobacter hepaticus*, *Escherichia coli*, and *Streptococcus gallolyticus* are involved in colorectal carcinogenesis and they are known as colorectal cancer associated pathogens[1]. Notably, these microorganisms may affect carcinogenesis by different mechanisms. Gut-immune axis seems to be important in this context[6]. Interestingly, some of bacteria may reside various part of human body and due to virulence factors they may be involved in development of multiple conditions/diseases. For instance, *F. nucleatum* can reside in oral cavity and its abundance was detected in periodontal diseases; however, it is also associated with CRC occurrence[7].

Accumulating evidence suggests that microbiota-derived metabolites have also great impact on carcinogenesis process. Short-chain fatty acids

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(SCFAs), polyamines, and N-nitroso compound are key microbial metabolites[8]. Notably, SCFAs seem to be one of the most important parts of gut metabolome. SCFAs pool is made up of acetate (C2), propionate (C3), and butyrate (C4) predominantly[9,10,11]. They are produced by different gut microbes from fermentable non-digestible carbohydrates[12]. For instance, Bacteroidetes phylum and *Akkermansia mucuniphila* produce propionate[9]. Butyrate is produced by Fimicutes phylum[13] and the main butyrate-producer is *Faecalibacterium prausnitzii*, which similarly as *A. muciniphila*, is known as next-generation probiotic bacteria[14,15]. *Clostridium butyricum* is also butyrate producer and it strengthens gut barrier by increasing expression of tight junction proteins[16]. Acetate is synthesized by most of the anaerobic bacteria and serves as substrate to produce other SCFAs.

SCFAs provide many beneficial effects (Figure 1). For instance, SCFAs make up a source of energy for colonocytes and they promote the growth of colonic epithelium[17]. Butyrate maintains the intestinal barrier integrity through increasing the expression of claudin-1 and Zonula Occludens-1 (ZO-1) being a significant component of the tight junctions[9,18,19]. Butyrate affects immune system through for instance regulating the differentiation of colonic regulatory T cells[20-22] and it provides anti-inflammatory effects[19].

The concentration and the proportions between SCFAs may be altered by several factors, such as age, lifestyle (i.e., diet factors – mainly the content of fiber, the level of physical activity), diseases/conditions, and many others. The alterations in the concentration of SCFAs, which are measured from stool samples, can be linked to CRC. Most of the papers described the role of gut microbiome in the context of CRC development/treatment whereas the studies regarding the activity of gut microbiota are deeply undiscovered. Thus, the studies which assess the level of SCFAs and the proportion between SCFAs in case of CRC patients in preoperative period are also still strongly limited. Therefore, the aim of this study was to analyse the content of SCFAs and the proportion between SCFAs (normal is 3:1:1 for C2:C3:C4, respectively) in the stool obtained from CRC patients in preoperative period.

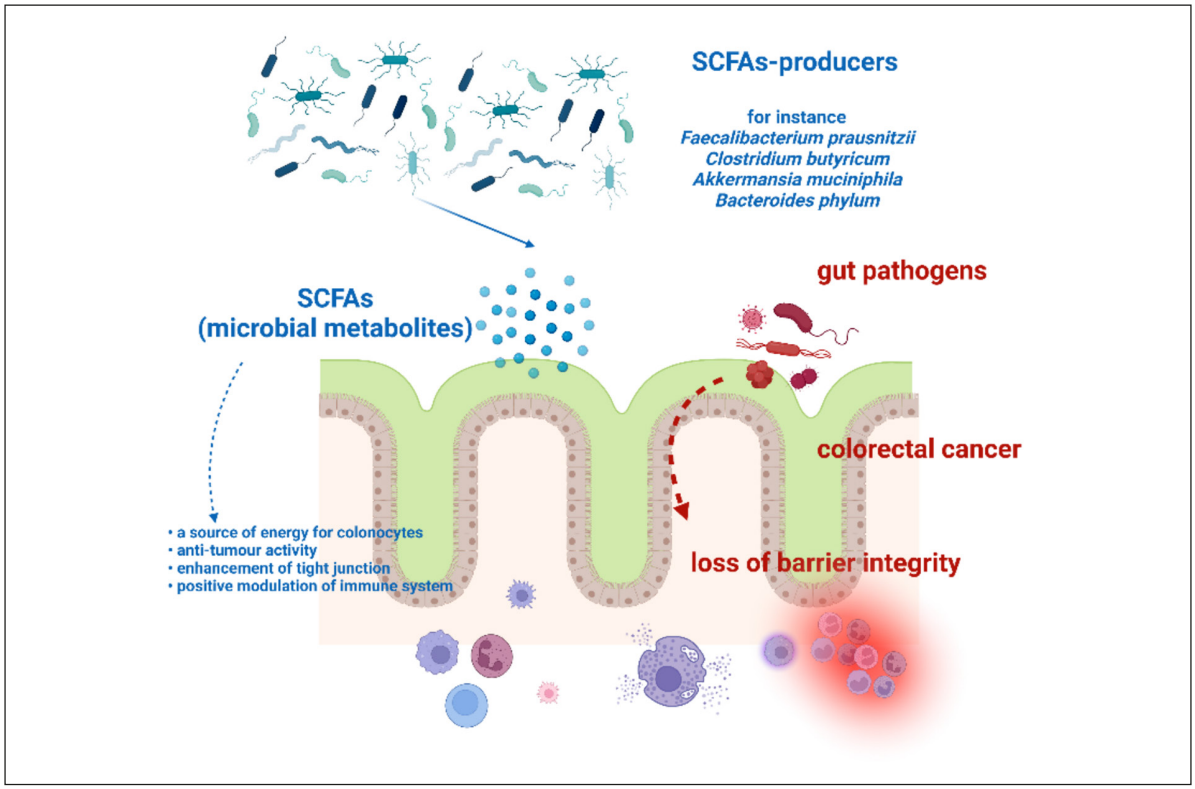

Figure 1. The beneficial effects of SCFAs. Own elaboration based on literature[14,17-19]. This figure was created using Biorender.com.

Patients and Methods

Participants (n=15) were recruited in the Department of Surgical Oncology (Outpatients care), Medical University of Gdansk, Poland. Inclusion criteria were age 18-80 year, the presence of CRC, preoperative period, and written consent to take the stool samples. Exclusion criteria regard: age <18-year, other type of cancer, the presence of any of inflammatory bowel diseases, pregnancy/postpartum period. This study has been approved by Independent Bioethics Committee for Scientific Research at Medical University of Gdansk, Poland NKBBN/129/2021, NKBBN/129-647, 703/2021, NKBBN/129-281/2022).

All of participants who met the inclusion criteria have received sterile tube for taking stool samples. They were instructed how to take them at home. Therefore, the stool samples were taken by patients at home and then stored in tubes in refrigerator (temperature -80°C) in the Fahrenheit Biobank BBMRI.pl, Medical University of Gdansk (Gdansk, Poland). After that, they were transported in dry ice to the Department of Biochemical Science, Pomeranian Medical University in Szczecin, Poland.

The assessment of SCFAs from stool samples was done by means of gas chromatography Agilent Technologies (Agilent Technologies, Santa Clara, US) 7890 A GC system with a Flame Ionization Detector (FID). A silica capillary column with a free fatty acid phase (DB-FFAP, 30 m x 0.53 mm × 0.5 mm) was used. Hydrogen was supplied as a carrier gas at a flow rate of 14.4 ml/min. The initial temperature was 100°C. It was held for 0.5 minutes, then raised to 180°C at a rate of 80°C/min and held for 1 minute. The temperature was then increased to 200°C (20°C/min) and finally held at 200°C for 5 minutes. The injection volume was 1 µl and the duration of each analysis was approximately 17.5 minutes. SCFAs were identified qualitatively by comparing the retention times to a standard, namely 2-ethyl butanoic acid. For quantitative analysis ChemStation Software (Agilent Technologies, Santa Clara, US) is used. The concentrations of individual acids were converted according to the internal standard.

Statistical Analysis

The statistical analysis was carried out using Microsoft Excel 2019 PL (Poland) and STATISTICA version 13.0. The basic parameters, such as average and standard deviation were calculated using Microsoft Excel 2019 PL.

Results

A total of 15 participants were recruited to this study predominantly males (n=10; 66.67%). The mean age of patients was 61.73±10.53 years (female 58.4±7.92 years, male 63.4±11.64 years). The results of the analysis of SCFAs content in stool samples are presented in Table I.

SCFAs such as acetic acid C2, proprionic acid C3, butyric acid C4, and valeric acid C5 were assessed in this study. The amount of 2 forms of butyric and valeric acid, such as branched (isobutyric C4i and isovaleric acid C5i) as well as linear (butyric C4n and valeric acid C5n) were analysed. The content of: acetate range from 41.53278 to 84.245 mmol/%, proprionate 5.648883 - 39.93942 mmol/%, isobutyric acid 0 - 5.673482 mmol/%, butyrate 0.945418 - 33.77541 mmol/%, isovaleric acid 0 - 10.4197 mmol/%, valeric acid 0 - 5.301064 mmol/%. Among all participants, the highest concentration (among all SCFAs) was noted in case of acetate (i.e., 84.245 mmol/%). The smallest amount, i.e., 0 mmol/% was observed in case of isobutyrate acid and both valeric as well as isovaleric acid.

The extremely higher concentration of C4n was observed in two cases – 13.33% (i.e., patients' number 1: 33.77541 mmol/% and number 8: 29.20398 mmol/%) compared to the rest of samples. Despite this fact, based on normal proportion between SCFAs, the results <1 for butyrate were noted in 93.33% of patients. In all patients there are incorrect proportion between SCFAs (based on that the normal proportion is 3:1:1 for C2:C3:C4, respectively) (Table II).

Discussion

Diet plays a significant role in the etiology of CRC[23,24]. Low intake of fiber, increased consumption of red meat and high-fat diet (especially with high content of saturated fatty acids) significantly contribute to the both development and progression of CRC[1,23,25,26]. An appropriate diet provides dietary fiber as well as omega-3 fatty acids, which increase the production of SCFAs and reduce the amount of secondary bile acids[27]. As a consequence, this type of diet promotes mucosal anti-inflammatory effects[27]. Dietary fiber is essential for the production of SCFAs in the gut. Pectin and starch increase the level of SCFAs in the gut whereas high-fat diet decrease their pro-

Table I. The analysis of SCFAs content in stool samples.

	[mmol/%]					
Patient number	**C2:0**	**C3:0**	**C4i (branched)**	**C4n (linear)**	**C5i (branched)**	**C5n (linear)**
1	41.53278	5.648883	5.673482	**33.77541**	10.4197	2.949741
2	67.47574	22.29351	0.913424	8.585407	0.731923	0
3	64.90102	16.54026	2.815648	10.24275	5.052697	0.447624
4	55.42902	21.77055	3.347534	12.42571	4.521115	2.506072
5	84.245	14.80958	0	0.945418	0	0
6	59.21988	19.83683	2.398944	12.38934	4.740803	1.414203
7	52.34433	29.54263	4.907851	4.432585	7.817878	0.954725
8	57.57091	13.2251	0	**29.20398**	0	0
9	51.26523	23.98846	2.466038	12.79901	4.180202	5.301064
10	72.69468	22.75378	1.689838	1.134855	1.726843	0
11	49.08491	39.93942	0	10.97567	0	0
12	69.37615	24.4978	1.528126	3.766191	0.831735	0
13	62.58446	25.60296	0.693974	10.95375	0.164851	0
14	72.64789	11.92372	1.571389	12.28855	1.568461	0
15	58.99588	12.3465	4.276769	15.60849	6.319227	2.453136
Average	61.29119	20.31467	2.152201	11.96847	3.205029	1.068438

Table II. The proportion between SCFAs.

[mmol/%]				Proportion (C2/C3/C4)		
Patient number	**C2:0**	**C3:0**	**C4**	**3**	**1**	**1**
1	41.53278	5.648883	19.72445	3.10381	0.42215	1.47404
2	67.47574	22.29351	4.749415	3.56944	1.17932	0.25124
3	64.90102	16.54026	6.529198	3.688795	0.940105	0.3711
4	55.42902	21.77055	7.886623	3.25723	1.279325	0.46345
5	84.245	14.80958	0.472709	4.232255	0.743995	0.02375
6	59.21988	19.83683	7.394143	3.42506	1.14729	0.42765
7	52.34433	29.54263	4.670218	3.023685	1.70654	0.269775
8	57.57091	13.2251	14.60199	3.37074	0.77432	0.85494
9	51.26523	23.98846	7.632522	3.092505	1.44707	0.46042
10	72.69468	22.75378	1.412347	3.752535	1.17456	0.072905
11	49.08491	39.93942	5.487834	2.59675	2.112925	0.290325
12	69.37615	24.4978	2.647158	3.593835	1.26904	0.13713
13	62.58446	25.60296	5.823864	3.32856	1.361695	0.309745
14	72.64789	11.92372	6.929967	3.96976	0.65156	0.37868
15	58.99588	12.3465	9.942627	3.62895	0.75946	0.61159

duction[20]. It is estimated that acetate, proprionate, and butyrate represent around ≥95% of SCFAs pool[28].

The amount of SCFAs varies from cecum to colon with the higher concentration in the proximal colon (70-140 mM) and lower in the distal colon (20-70 mM)[10]. An appropriate proportion between SCFAs is 3:1:1 for acetate (60%), proprionate (25%), and butyrate (15%)[29]. In the current study, we observed that all CRC patients have an abnormal proportion between these SCFAs. Similarly, in Ohigashi et al[30] study it was shown that CRC patients had an alteration in gut microbiota and decreased level of SCFAs. Recently, in another study[31] it was also noted that CRC patients have a reduced concentration of acetate (8.55 μg/mL), proprionate (5.61 μg/mL), and butyrate (3.79 μg/mL). All stool samples were analyzed using gas chromatography[31]. In our study, we also investigated the amount of branched SCFAs (BSCFAs), such as isobutyric acid and isovaleric acid. The range for isobutyric acid was 0 -5.673482 mmol/% and for isovaleric acid was 0 - 10.4197 mmol/%. Notably, BSCFAs are generated during the fermentation of branched amino acids, such as valine, leucine, and isoleucine[28].

Based on normal proportion between SCFAs, the results <1 for butyrate were observed in 93.33% of patients. Notably, Wang et al[32] have reported that the amount of butyrate producing bacteria is reduced in CRC patients. This result can be related to changes of the composition of gut microbiota. In these patients the dysbiotic alterations of gut microbiota may be linked to the tumour occurrence, side effects of anti-cancer treatment as well as poor diet, because often in case of cancer there is loss of appetite and disease-related malnutrition.

Butyrate, which is known as the most significant microbial metabolite among SCFAs, is characterized by wide range of beneficial properties. It may influence the cancer development and progression on many levels: preventing from carcinogenesis initiation, inhibiting cancer development as well as modulating and increasing the efficiency of treatment[20]. Firstly, it is the main source of energy for normal colonocytes. Although it induces the differentiation, apoptosis and inhibits cell proliferation in cancer cells, butyrate affects normal colonocytes in completely opposite way[33,34]. Butyrate preserves the intestinal barrier function, increases its integrity, decreases inflammation, and inhibits the bacterial translocation[20]. Due to these properties, it may improve the outcome of surgery and wound healing after resection of cancer[20]. In addition, improving the integrity of intestinal barrier may prevent from metastasis. However, it should not be ignored that recently published studies[35] indicate that bacteria (which are present in CRC patients) may promote tumorigenesis through butyrate secretion. Therefore, butyrate should be carefully administered as potential treatment to cancer patients[35]. It seems to be reasonable to analyse the content of butyrate in the stool prior to its supplementation.

Limitations

This study has some limitations. First of all, this study included 15 participants, thus it was conducted with small sample size. The stage of cancer was not collected, nevertheless all of patients were qualified to surgical treatment due to tumour occurrence. Another limitation is the fact that stool samples were taken at home by patients, thus there is a possible risk that they did not follow the instruction of taking stool samples precisely.

Conclusions

The normal proportion between SCFAs should be 3:1:1 for acetate, proprionate and butyrate, respectively. In the current study, we demonstrated that all CRC patients in preoperative period have incorrect proportion between above mentioned SCFAs. In case of 2 samples (13.33%), the extremely higher concentration of butyrate was noted compared to the rest of patients. Despite this fact the results <1 for butyrate was observed in 93.33% of participants. It can be linked to the dysbiotic alterations of the composition of gut microbiota regarding reduced level of butyrate producing bacteria in CRC patients.

The results of this study revealed the possible directions for the future which can be also introduced in clinical practice. The supplementation of butyrate to CRC patients especially prior to the surgery should be considered. It is important to support the preparation of patients to the surgical treatment. However, the future studies should focus on finding predictive biomarkers of butyrate efficiency, taking into consideration that in certain conditions it may also stimulate cancer progression. Therefore, it could be useful to analyse both, the composition of gut microbiota and at the same time, the metabolites of bacterial fermentation.

Conflict of Interest
The Authors declare that they have no conflict of interests.

Ethics Approval
This study has been approved by Independent Bioethics Committee for Scientific Research at Medical University of Gdansk, Poland (number 129/2021).

Informed Consent
All patients provided a written consent to take part in this study.

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