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Association between fecal levels of Short-Chain Fatty Acids and serum Pro- and Anti-Inflammatory Cytokines in patients with Inflammatory Bowel Disease

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Abstract: Introduction: Inflammatory bowel disease (IBD) represents a group of chronic inflammatory disorders characterized by dysbiosis and altered short-chain fatty acid (SCFA) level. The association between individual SCFA levels and cytokine levels is unknown.
Objectives: We aimed to determine the fecal SCFA levels in patients with IBD in relation to disease severity and the serum levels of pro- and anti-inflammatory cytokines.
Patients and Methods: The study included 61 patients with IBD (inactive, 22; active, 39) and 16 controls. Fecal levels of organic acids (acetic, lactic, propionic, butyric, isovaleric, isobutyric, and valeric), serum levels of tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10), IL-17, and IL-22, complete blood count and C-reactive protein (CRP) were measured.
Results: Patients with active IBD had reduced butyric, acetic, valeric, and isovaleric acid levels and elevated lactic acid levels in stool. Hemoglobin levels were positively correlated with the levels of acetic and butyric acids (R = 0.266 and R = 0.346, respectively; P <0.05). In addition, CRP levels were inversely correlated with butyric acid levels (R = –0.573; P <0.05). Higher serum TNF-α levels were observed in patients with active IBD compared with controls (6.64 pg/ml vs 2.05 pg/ml, P <0.05). No relationship was noted between the SCFA profile and cytokine levels.

C o n c l u s i o n s: The study showed that determination of SCFA levels can be used to evaluate the activity of IBD. The relationship between individual SCFA and cytokine levels seems to be complex and requires further studies.

Keywords: butyric acid, C-reactive protein, tumor necrosis factor-alpha, short chain fatty acids, intestinal inflammation.

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Introduction

The gut microbiota, which consists of various microorganisms including bacteria, fungi, archaea, eukaryotic microbes, and viruses, plays an important role in human health. The microbiota is responsible for various processes in the body. Microbial diversity ensures optimal immune function and energy metabolism as well as maintains the integrity of the intestinal barrier [1]. Moreover, the gut microbiota supports digestion, facilitates the assimilation of individual compounds from food, and regulates the appetite [2]. These broad beneficial effects are possible thanks to bacterial metabolites, in particular short-chain fatty acids (SCFAs), which are formed in the intestine with the participation of dietary fiber [3].

SCFAs are organic acids with fewer than 6 carbons, including acetic, butyric, and propionic acids, which reach the highest concentration in the large intestine [3]. The most widely studied SCFA is butyric acid. It is the primary fuel for colonocytes and is known for its anti-inflammatory and anticancer properties [4]. Among SCFAs there are also valeric, isovaleric, and isobutyric acids, but they occur at lower concentrations and their role has been less well elucidated.

SCFAs act as a histone deacetylase inhibitor and by binding to G protein–coupled receptors that are expressed in various cells, such as colon mucosa, immune cells, and adipose tissue [5]. Therefore, SCFA affects the immune response and consequently alter the release of cytokines.

Inflammatory bowel disease (IBD) is a term for diseases that are characterized by chronic inflammation of the gastrointestinal tract and unfavorable alteration of the gut microbiota. The 2 main clinical types of IBD are ulcerative colitis (UC) and Crohn's disease (CD) [6]. The pathogenesis of IBD is complex and involves an interaction between genetic, microbial, immunological and environmental factors [7]. The complex network of cytokine levels plays an important role in pathogenesis and course of intestinal inflammation [8]. The main cytokines players in IBD are inter alia tumor necrosis factor α (TNFα), interleukin-10 (IL-10), IL-17 and IL-22 [7]. Pro-inflammatory cytokines, such as TNFα and IL-17, play a critical role in the intestinal homeostasis [8]. On the other hand, IL-10 is a typical anti-inflammatory cytokine with protective effect on intestinal barrier [9]. Despite the proven effects of SCFAs on cells

of the immune system, the relationship between level of SCFA and particular pro- and anti-inflammatory cytokines remains unknown.

The aim of this study was to determine the fecal SCFA profile in patients with IBD depending on disease activity (inactive vs active disease). Moreover, we investigated the relationship between SCFA profile, serum cytokine levels (TNFα, IL-10, IL-17, IL-22), and inflammatory markers such as red blood cell count, white blood cell count, hemoglobin and C-reactive protein (CRP) levels.

Patients and Methods

IBD was diagnosed using a combination of clinical, biochemical, endoscopic, and histological investigations according to the European Crohn's and Colitis Organisation guidelines [10]. Patients were classified into active and inactive IBD using the total Mayo Score for UC patients and the Crohn's Disease Activity Index (CDAI), colonoscopy and/or imaging studies for CD patients. Patients with UC were eligible if they had at least left-sided colitis, and in the CD group, if the disease involved the large intestine.

All patients underwent a clinical and dietary interview by a physician and dietitian, respectively. During the dietary interview, patients were asked about the diet in the previous 3 months. The control group included volunteers without a history of organic gastrointestinal disease or any alarming symptoms and who did not fulfill Rome IV criteria for irritable bowel syndrome. The exclusion criteria were as follows: any other organic intestinal disease, acute gastrointestinal infections, lactation, pregnancy, malignancy, severe chronic diseases, malabsorption syndromes, current TNF-α inhibitors therapy, intake of prebiotics, probiotics, or dietary supplements containing sodium butyrate, as well as total or partial parenteral nutrition.

Ethical approval

All participants gave written informed consent to participate in the study. The protocol of study was approved by the Bioethics Committee at Jagiellonian University in Kraków, Poland (no. 1072.6120.18.2018; as of February 23, 2018), and was run in accordance with the Declaration of Helsinki.

Biochemical analysis

We determined the serum levels of complete blood count and CRP. Biochemical tests were performed in the Laboratory of the University Hospital in Cracow (Poland) in accordance with the manufacturer's instructions. Blood samples were centrifuged at 1000 *g* for 12 minutes at a temperature of 4°C and then collected and stored at a temperature of −80°C until further assay.

Measurement of serum cytokine levels

Human cytokines (IL-10, IL-17, IL-22 and TNF-α) were measured using commercially available ELISA kits according to the manufacturer's protocol. The detection kits for IL-10, IL-17, and IL-22 were purchased from Shanghai Sunred Biological Technology Co. (Shanghai, China), while TNF-α was purchased from LDN Labor Diagnostika Nord GmbH & Co.KG (Am Eichenhain, Germany). We used: Human IL-10 catalog number 201-12-0103D, Human IL-17 catalog number 201-12-0143, Human IL-22 catalog number 201-12-0039, and TNF-α ELISA catalog number IL E-3100. An ELx 808 spectrophotometric microplate reader (BioTek, Winooski, Vermont, USA) was used to determine the optical density at 450 nm.

Measurement of fecal short-chain fatty acid levels

Fresh fecal samples were collected from all participants and stored at a temperature of −80°C until further assay. The preparation and subsequent extraction of fecal samples were performed in the Department of Food Chemistry and Nutrition, Faculty of Pharmacy, Jagiellonian University Medical College. The preparation of each stool sample comprises the following steps: 1) evaporation drying; 2) grinding; and 3) extraction process. Owing to the disease symptoms, the feces collected for analysis were often in a liquid or semi-fluid voluminous form and were reduced by evaporation. The samples were then lyophilized to minimize the loss of volatile analytes. The extraction of analytes from stool samples was performed in 2 successive stages: 1) ultrasound-assisted extraction of analytes and 2) shaking the sample with the extractant. Then, all obtained extracts were centrifuged and stored at a temperature of −20°C until further analysis.

Organic acids in stool samples were determined by means of capillary electrophoresis, using PA 800 Plus Pharmaceutical Analysis System (Beckman Coulter, Inc., Brea, California, United States), furnished with an ultraviolet spectrophotometric detector, at the Laboratory for Forensic Chemistry, Department of Analytical Chemistry, Faculty of Chemistry, Jagiellonian University. The analyzed compounds were separated in a fused silica capillary (I.D. 75 μm; total length, 60 cm/50 cm to the detector) at 25°C using a high voltage of −30 kV. Samples were injected hydrodynamically (3.45 kPa for 8 s). An indirect spectrophotometric detection was performed at 230 nm. The separation was performed using a background electrolyte consisting of methyl-β-cyclodextrin in a commercially available buffer (Anion Kit 5; Analis, Namur, Belgium). Median acid levels were expressed as microgram per gram dry weight of feces.

Statistical analysis

Descriptive statistics (medians, lower and upper quartiles) were calculated for all organic acids and cytokines in the study groups. The differences between groups were assessed using the Kruskal–Wallis test with the post hoc Dunn test, because the parameters had non-Gaussian distribution, as demonstrated by the Kolmogorov–Smirnov test. The Spearman correlation coefficients were calculated for the pairs of parameters. A probability level of less than 0.05 was considered significant. Statistical analyses were carried out using Graph Pad Prism v.3.02 (GraphPad Software, San Diego, California, United States). The STATISTICA v. 13.3. package (TIBCO Software Inc., Palo Alto, California, United States) was used for the graphic representation of data.

Results

Study population

The study included 77 participants: 22 patients with nonactive IBD (mean [SD] age, 36.8 [13.71] years), 39 patients with active IBD (mean [SD] age, 32.5 [10.9] years), and 16 controls (mean [SD] age, 31.7 [16.7] years). The clinical and demographic characteristics of the study groups are summarized in Table 1. The IBD group included 18 newly diagnosed patients.

Table 1. Clinical and demographic characteristics of patients with inflammatory bowel disease (IBD) and controls.

Parameter	**Patients with IBD** (n = 61)	**Controls** (n = 16)
Sex (male/female), n (%)	39/22 (64/36)	3/13 (19/81)
Age, median (min-max), y	32 (18–71)	23.5 (19–64)
Disease activity (inactive/active) n (%)	22/39 (36/64)	—
Body mass index, median (SD)	21.43 (4.17)	21.65 (4.07)

Fecal short-chain fatty acid concentration in patients with inactive and active inflammatory bowel disease as well as controls

The following organic acids were identified in stool samples: acetic, lactic, propionic, butyric, isovaleric, isobutyric, and valeric. Organic acid levels in patients with IBD classified according to disease activity are shown in Table 2 and Fig. 1. In patients with

Table 2. Concentrations of organic acids in patients with inactive inflammatory bowel disease (IBD), with active IBD, and controls.

Organic acid	Inactive IBD (n = 22)	Active IBD (n = 39)	Controls (n = 16)
Acetic, µg/g	1248.7 (755.8; 1862.1)	**906.3*** (596.5; 1454.4)	1827.5 (931.7; 2484.9)
Lactic, µg/g	505.6 (183.3; 1560.4)	**1593.3*** (584.2; 2796.9)	242 (29.6; 1241.6)
Propionic, µg/g	417 (214.4; 920.5)	299.2 (179.4; 556.9)	522.5 (317; 861.8)
Butyric, µg/g	334 (67.2; 500.9)	**54.7***/*** (24.3; 351.3)	473 (172.5; 767)
Isobutyric, µg/g	54.1 (38.6; 103.2)	29.3 (5.5; 82.4)	45.7 (33.3; 86.6)
Valeric, µg/g	5.5 (5.5; 38)	**5.5*** (5.5; 23.2)	30.2 (17; 58)
Isovaleric, µg/g	76.3 (5.5; 139.2)	**16.3****/*** (5.5; 53.4)	61.1 (37; 116.5)

Data are expressed as medians (lower and upper quartiles). * P <0.01 versus controls; ** P <0.05 versus controls; *** P <0.05 versus inactive IBD.

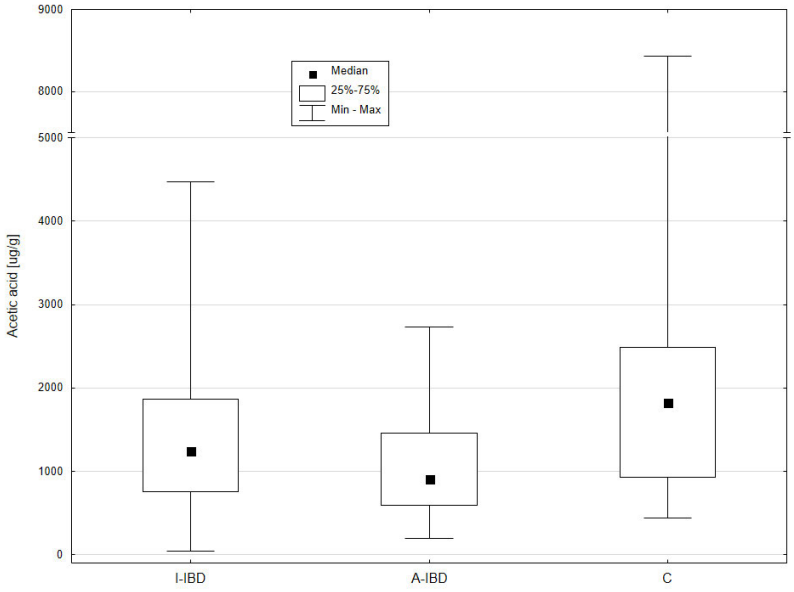

Fig. 1. Concentrations of fecal organic acids in patients with inactive inflammatory bowel disease (I-IBD), with active IBD (A-IBD), and controls.

IBD in remission, the highest median levels of organic acids were noted for acetic acid, while in patients with active IBD, for lactic acid. In the control group, the highest median concentrations were observed for acetic, propionic, and butyric acids.

Median acetic acid levels were lower in patients with active IBD compared with controls (906.3 μg/g vs 1827.5 μg/g; P <0.01). Valeric acid also showed lower levels in active IBD vs controls (5.5 μg/g vs 30.2 μg/g; P <0.01). In contrast, median lactic acid levels were higher in patients with active IBD than in controls (1593.3 μg/g vs 242.0 μg/g; P <0.01). Moreover, median butyric acid levels were lower in patients with active IBD than in those with inactive IBD (54.7 μg/g vs 334.0 μg/g; P <0.05) and controls (54.7 μg/g vs 473.0 μg/g; P <0.01). Additionally, median isovaleric acid levels were lower in patients with active IBD as compared with those with inactive IBD (16.3 μg/g vs 76.3 μg/g; P <0.05) and controls (16.3 μg/g vs 61.1 μg/g; P <0.05).

Correlation between fecal organic acid levels and biochemical parameters

Hemoglobin levels were positively correlated with the levels of acetic and butyric acids (R = 0.266 and R = 0.346, respectively; P <0.05). There was also a positively correlation between hematocrit levels and the levels of acetic and butyric acids (R = 0.281 and R = 0.373, respectively; P <0.05). Also, CRP concentrations were significantly positively correlated with lactic acid levels and inversely correlated with butyric acid levels (Table 3). There were no correlations between any of the organic acids and red blood cell or white blood cell count (data not shown).

Table 3. Spearman rank correlation coefficients (R) for correlations between organic acid and C-reactive protein levels (P <0.05).

Organic acid	**C-reactive protein**
	R
Acetic	–0.376
Lactic	0.534
Propionic	–0.323
Butyric	–0.573
Isobutyric	–0.320
Valeric	–0.427
Isovaleric	–0.395

Serum cytokine levels

Median TNF-α levels were higher in patients with IBD compared with controls (6.29 pg/ml vs 2.05 pg/ml, P <0.05). Moreover, median TNF-α levels were higher in patients with active IBD as compared with controls (6.64 pg/ml vs 2.05 pg/ml, P <0.05). No significant associations were found between any of the studied organic acids and serum cytokine levels.

Discussion

In this study, we assessed the fecal SCFA profile and serum cytokine levels in patients with IBD. Our results showed that individual SCFA levels are associated with the activity of IBD. Moreover, SCFA levels were correlated with biochemical parameters such as hematocrit, hemoglobin and CRP levels. We demonstrated that higher serum TNF-α levels are observed in patients with active IBD compared with controls. However, there were no correlations between any of the studied organic acids and serum cytokine levels.

This study showed that patients with active IBD have decreased fecal butyric acid levels. In addition, butyric acid levels were positively correlated with the levels of hemoglobin levels, and inversely correlated with CRP levels, which is the most widely used serum indicator of inflammation in IBD. Butyric acid is formed from carbohydrates by gram-positive anaerobic bacteria such us *F. prausnitzii* and *Roseburia spp* [11]. The acid acts as an important anti-inflammatory and immunomodulating agent [12]. Recent studies have shown that butyrate reduces the level of pro-inflammatory cytokines such as TNF-α, IL-6, IL-8 and increases the level of anti-inflammatory cytokines, including IL-10 [13]. The dynamics of butyrate production depends on the microbial ecology of the gastrointestinal tract. The large intestine is the main reservoir of microorganisms. It contains approximately 3.8×10^{13} bacteria and shapes the entire bacterial population [14]. Patients with colitis develop dysbiosis and show reduced butyric acid production. In addition, a correlation between a reduced amount of butyrate-producing bacteria and IBD activity was previously reported [13]. All this evidence is consistent with our findings. However, we did not observe any correlation between fecal butyric acid levels and serum cytokine levels.

Interestingly, we demonstrated that patients with active IBD have showed a reduced fecal valeric acid levels. Valerate-producing bacteria belong to the phylum Firmicutes, including members of *Clostridium* cluster I, the balance of which is disturbed in the course of IBD [15]. A recent study confirmed that the abundance of valerate-producing *Megasphaera* was positively associated with increased fecal valerate levels in IBD [16]. Of note, valeric acid is converted by the intestinal bacteria from other acids, including lactic and propionic acids, as well as from several amino acids [17, 18]. Hence, valeric

acid reduction in patients with active IBD may result both from reduced amounts of intestinal SCFA-producing bacteria and from the disruption of metabolic pathways. The role of valeric acid in IBD has been not fully elucidated, but it appears to have numerous beneficial effects on intestinal inflammation. Previous studies demonstrated that valeric acid modulates cytokine production by creating an anti-inflammatory environment (elevated IL-10 and reduced IL-17 levels), increases survival of regulatory B cells, and strengthens the intestinal barrier by regulating tight junction proteins [19]. However, we did not observe a relationship between valeric acid levels and the serum cytokine levels mentioned above. Unfortunately, in our study we did not determine the expression of cytokines in the colon mucosa, perhaps more significant correlations between inflammatory and anti-inflammatory factors and SCFA exist at the tissue, local and not systemic level. In addition, a recent study revealed an increase in fecal valerate levels following treatment of *Clostridioides difficile* infection [20]. Valerate acid was also shown to inhibit the growth of *C. difficile* [20]. All these findings suggest that reduced valerate acid levels may promote intestinal inflammation in patients with active IBD. We hypothesize that the normalization of valeric acid levels may lead to a reduction of inflammatory response and promote tissue repair in the course of IBD. However, the actual anti-inflammatory mechanism of valeric acid requires further studies.

Our study also revealed a reduction in acetic acid levels in patients with active IBD. Similar observations were reported in previous studies [13, 21]. In a healthy gut, acetic acid has the highest concentration of all SCFAs, and the ratio of acetate, propionate, and butyrate acid levels in the colon is about 3:1:1 [22]. This is in line with our observations in the SCFA profile of the control group. Interestingly, the corresponding ratio in our patients with IBD was about 6:2:1. In the analysis by disease activity, the ratio in patients with inactive IBD was similar to that in healthy controls, while in patients with active IBD, it reached about 18:6:1. This shows a significant advantage of acetate and propionate acids over butyrate acid in active disease. It was demonstrated that the gut microbiota is involved in the conversion between major SCFAs in the intestinal lumen. This metabolic cross-feeding appears to play an important role in forming the SCFA profile [23]. In previous research, all possible conversions between the main SCFAs were observed, but the conversion of acetate into butyrate was the most significant one [24]. Therefore, it is not only the depletion of butyrate-producing bacteria that may reduce the production of beneficial butyrate in patients with IBD. Despite previous research, the exact role of acetate in human gut health remains elusive. A recent study showed that increased acetate levels are required for the proper functioning of memory $CD8^+$ T cells [25]. Interestingly, the conversion of acetate to butyrate by the gut microbiota may play an important anti-inflammatory role [26]. Thus, if the balance of the gut microbiota is disturbed, the conversion may not be possible, leading to acetate accumulation and limiting its anti-inflammatory potential.

Another important issue is the phenomenon of nutrient cross-feeding. The SCFAs are metabolized both by the host organism and the intestinal microorganisms. Facchin *et al.* [27] demonstrated that sodium butyrate supplementation leads to an increase in the number of SCFA-producing bacteria in patients with IBD. Thus, the deficiency of SCFAs, particularly butyrate, may not only be a marker of inflammation but also a risk factor for the worsening of intestinal inflammation in these patients. Moreover, the disbalance between individual SCFAs may enhance dysbiosis, thus intensifying the inflammatory reaction.

TNF-α is a pleotropic cytokine involved in a wide range of pathological processes, including IBD. Accordingly, we observed higher serum TNF-α levels in patients with active IBD compared with controls. This finding may be of great interest for the use of the TNF-α assessment in disease monitoring. However, previous studies investigating the relationship between serum TNF-α levels and IBD activity have been inconclusive [28, 29]. It may be related to the complex dichotomous role of TNF-α in IBD. TNF-α can a variety of immunomodulatory effects on various immune effector cells, including neutrophils, macrophages, and T cells. It can have a number of anti-infective and metabolic effects [30]. Of note, we did not observe any relationship between individual fecal levels of SCFA s and serum cytokine levels. This may be due to the complex network between individual cytokines and their low concentration in serum. On the other hand, it could be related to the disadvantages of this study, such as a relatively small number of the participants studied and quite a large difference in sex between the study groups. Therefore, we are conducting further research in this area on a larger group of participants, including the determination of pro- and anti-inflammatory cytokines in colonic tissue.

In conclusion, our study showed that reduced SCFA levels may be a marker of active IBD. Moreover, the imbalance in the proportion of individual SCFAs may serve as an additional marker of intestinal inflammation and likely contributes to an exacerbation of inflammatory response. The relationship between cytokine levels and SCFA profile seems to be complex and requires further studies on a larger group of patients.

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Conflict of interest

None declared.

Contribution statement

O.K., A.D.-D., A.P.-G. and M.Z.-W. conceived the concept of the study. M.W., P.Z., and M.Z.-W. contributed to the design of the research. A.D.-D., O.K., A.P.-G., K.W., and M.Z.-W. were involved in data collection. A.D.-D., O.K., J.D.-I., P.P., A.W., A.T., A.P.B., M.W., K.W., P.Z., and M.Z.-W. performed the research. P.Z. analyzed the data. O.K., A.D.-D., A.P.-G., and M.Z.-W. wrote the paper. M.Z.-W. revised and edited the manuscript for final submission.

Abbreviations

CD — Crohn's disease
CRP — C-reactive protein
IBD — inflammatory bowel disease
IL — interleukin
SCFA — short-chain fatty acid
TNF-α — tumor necrosis factor-alpha
UC — ulcerative colitis

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