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SHORT COMMUNICATION

Faecalibacterium prausnitzii and Crohn's Disease – is There any Connection?

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Abstract

The aim of the study was evaluation of the correlation between the level of clinical activity of Crohn's disease (CD) and the number of *Faecalibacterium prausnitzii,* total number of bacteria and the concentration of selected SCFA in stool. 34 individuals diagnosed with Crohn's disease participated in this study in 2011. The disease activity was determined according to the CDAI. The number of *Faecalibacterium prausnitzii* and total number of bacteria were monitored by RT-PCR. The concentrations of SCFA were determined by gas chromatography. In CD patients, *Faecalibacterium prausnitzii* number and percentage of the total number of bacteria were greatly reduced. In patients with CD the percentage of acetate was elevated (70%), while the percentages of propionate and butyrate were significantly reduced (14.9% and 7.99%, respectively).

Key words: *Faecalibacterium prausnitzii,* Crohn's disease, SCFA

Inflammatory bowel diseases (IBD) are characterized by chronic inflammation of the gastrointestinal tract. The etiopathogenesis of the disease has not yet been fully elucidated. The disruption of the normal interaction between the microbiota and the intestinal immune system (GALT) appears to be one of the key causes of the disease (Danese and Fiocchi, 2006). The human intestinal ecosystem is rich in microorganisms. Due to the low oxygen content in the gut, it is composed mainly of anaerobic bacteria, and the average number of bacterial cells reaches 10^{11} per gram of feces (Flint *et al.*, 2007). Particularly beneficial to the proper functioning of the intestinal microbes are those that produce butyrate during fermentation of carbohydrates. Butyric acid and its derivatives – in the form of esters and salts – are essential for proper functioning of the intestine. These compounds are the most important energy sources for the colonocytes. As regulators of gene expression, butyric acid and its derivates affect cell proliferation, differentiation and apoptosis. Butyric acid has an anti-inflammatory activity and plays a role in maintaining the integrity of the intestinal barrier by strengthening cellular connections (tight-junctions). It improves the functioning of the protective barrier of the large intestine through the production of mucins and antibacterial peptides (Flint *et al.*, 2007; Van Immerseel *et al.*, 2010). The butyrate-producing bacteria that colonize the human gut form a functional rather than a homogeneous phylogenetic group. There are mostly Gram-positive *Firmicutes* which belong to clostridial clusters IV and XIVa. They are strict anaerobes and are generally regarded as difficult to grow in culture. For this reason, the detection of these bacteria is based mainly on genetic techniques analyzing their specific DNA sequences. The two numerous groups most related to the human butyrate producers are *Faecalibacterium prausnitzii* belonging to the *Clostridium leptum* (clostridial cluster IV) and *Eubacterium rectale/Roseburia* spp. which belong to the *Clostridium coccoides* (clostridial cluster XIVa). Research based on FISH and real-time PCR methods showed that each of these groups make up to 5–10% of the total number of bacteria detected in stool samples from healthy adult subjects (Louis and Flint, 2009).

Diet significantly affects the amount of butyrate produced. The administration of foods rich in starch and fiber increases both the concentration of acid produced and the number of *F. prausnitzii* and numbers of

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Table I
Number of patients according disease activity state and time of diagnosis

Number of patients N = 34	Disease activity state (according CDAI)			
	Remission	Mild	Moderate	Severe
	20	4	6	4
Time of diagnosis (years)	new diagnosis	≤ 2	2–15	
Number of patients N = 34	2	3	27	

clostridial cluster XIVa (Shen *et al.*, 2011). The diet also affects the pH in the proximal colon, which is also a key parameter in determining the production of butyrate. In addition, the stimulation of butyrate producers affects the metabolic cross feeding. It has been shown, that lactic acid produced *in vitro* by lactic acid bacteria is used by some species of the clostridial cluster XIVa to produce higher concentrations of butyric acid. This mechanism explains why sometimes the supply of lactic acid bacteria stimulates the production of butyric acid (Van Immerseel *et al.*, 2010; Louis and Flint, 2009).

Due to the reported anti-inflammatory effects, short chain fatty acids (SCFA), especially butyric acid, are seen as potential therapeutic agents in patients with IBD. An appropriate number of bacteria producing SCFA elicits a favorable effect on the state of the intestine. In an animal model, Sokol et al. reported a decreased number of these groups of bacteria in IBD (Sokol *et al.*, 2008).

The aim of the study was to evaluate the abundance of *F. prausnitizii* as a representative of the largest group of butyrate-producing bacteria and the number of individual SCFA in the feces of adult patients with IB

The study included 34 patients (19 females and 15 males) diagnosed with Crohn's disease, hospitalized in the Department of Gastroenterology, Nutrition and Internal Diseases, University of Medical Sciences in Poznan, in 2011. The patients were between 19 and 64 years of age (average age 34 years). Disease activity was determined according to the Crohn Disease Activity Index (CDAI). The patients were divided into the following groups: groups remission (CDAI < 150), a group of mild activity (CDAI 150–220), moderate activity (CDAI 220–450) and severe activity (CDAI > 450). In addition, all patients were divided based on the time of diagnosis: a newly made diagnosis, diagnosis made in the last 2 years, diagnosis made 2–15 years ago and diagnosis made more than 15 years ago. The numbers of patients, according the disease activity and time of diagnosis, are presented in Table I.

The material for the study was obtained from the feces of each patient. In order to determine the amount of *F. prausnitzii* and the total bacterial cell number in the feces, each sample was subjected to genetic analysis (Quantitative PCR-qPCR). The DNA was extracted using the Easy Mag DNA isolation system (BioMerieux). Primers were stringently selected using the Primer Q4 Designer program to avoid primer-dimer formation and yield 100- to 300-bp products. The primers are listed in Table II. Discriminating nucleotides were chosen to be at the 3' end of the primer. The standard line was based on actual counting of cultured bacteria and correlated directly to the Ct values of the qPCR. PCR amplification and detection was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems) in optical-grade 96-well plates sealed with optical sealing tape. Each reaction mixture (25 mL) comprised 12.5 mL of QuantiTect SYBR Green PCR Master Mix (Qiagen), 2 mL of primer mixes (10 pmol/mL each), 9 mL of sterile distilled water, and 1.5 mL of stool DNA (10 ng/mL). For the negative control, 2 mL of sterile distilled water instead of the template DNA solution was added to the reaction solution. A standard curve was produced using the appropriate reference organism to convert the qPCR values into numbers of bacteria per gram. The standard curves were prepared using the same PCR assay as used for the samples. The fluorescent products were detected in the final step of each cycle. A melting curve analysis was carried out after amplification to distinguish the targeted PCR products from the non-targeted PCR products. The melting curves were obtained by slow heating at temperatures of 55°C–95°C at a rate of 0.2°C/second, with continuous fluorescence collection. Real-time qPCR was performed in triplicate, and average values were used for quantification. PCR conditions were optimized based on those described in the literature (Matsuki *et al.*, 2002; Matsuki *et al.*, 2004; Franks *et al.*, 1998; Harmsen *et al.*, 2002). The amplification program used for all primers consisted of one cycle of 95°C for

Table II
16S rRNA gene-targeted group and species-specific primers used in this study

Target	Primer name	Primer sequence (5'–3')
Total bacteria	UniF340	ACTCCTACGGGAGGCAGCAGT
	UniR514	ATTACCGCGGCTGCTGGC
F. prausnitzii	PrausF480	CAGCAGCCGCGGTAAA
	PrausR631	CTACCTCTGCACTACTCAAGAAA

15 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds. In all assays, the amplification efficiency was > 90%, and the standard curve showed a linear range across at least 5 logs of DNA concentrations with a correlation coefficient > 0.9. The lowest detection limit of all assays was as low as 10–100 copies of specific bacterial 16S rDNA per reaction, corresponding to 104–105 copies per gram of wet-weight feces. All data was analyzed using ABI Prism software.

Stool samples to be analyzed for short chain fatty acids (SCFA): butyrate, acetate, propionate, were freeze-dried and subsequently analyzed using gas chromatography. The samples were weighed (~80 mg dry matter) and an extraction solution (1 mL) containing oxalic acid (0.1 mol/L), sodium azide (40 mmol/L), and an internal standard (caproic acid 0.1 mmol/L) was added. The solution was extracted for 60 min on a horizontal shaker and then centrifuged (10 min at 16,000 × g). Concentrations of the SCFA were determined in the supernatant using an Agilent 6890N gas chromatography with flame ionization detection equipped with a capillary column Innowax 30 m × 530 μm × 0.1 μm (Agilent). ChemStation software was used for data processing.

All statistical analyses were performed using Statistica 7.0. The normality of the data was checked using the nonparametric Kolmogorov-Smirnov test. Before analysis for treatment differences, data was subjected to the Levene test for homogeneity of variances. Depending on the normality of the underlying data, analysis of variance or the Mann-Whitney U test was used for statistical analyses. Spearman's rank correlation coefficient was calculated by analyzing the relationship between the number of microorganisms and the number of analyzed SCFA in the feces and the level of clinical activity of disease

The study was authorized by the Ethical Committee at the Karol Marcinkowski University of Medical Sciences in Poznan (the authorization number – 120/11).

There were no statistical significant correlations between: patient's sex, age, time of diagnosis and both the number of analyzed bacteria and SCFA amount. There were some significant differences in number of *F. prausnitzii* (but not in the total number of bacteria) and the amount of SCFA depending on the disease activity state. The numbers of analyzed microorganisms in various stages of the CD activity are shown in Table III.

In CD patients, the number of *F. prausnitzii* in the feces and its percentage of the total number of bacteria was greatly reduced. In healthy individuals, the number of reported bacteria is not less than 10^9 cells/g feces and they constitute more than 5% of the total number of bacteria (Schwiertz *et al.*, 2010). The authors demonstrated that even in patients in good health (remission) the number of microorganisms was only 10^8 cells/g of feces. The number of *F. prausnitzii* in feces differed significantly depending on the disease activity index ($p = 0.015$). The described correlation was negative: the higher the disease activity, the lower the number of bacteria.

The total number of bacteria in the feces in a properly functioning colon should be in the range of 10^{11}–10^{12} cfu/g of feces. In the examined group of patients, the number of microorganisms was significantly reduced (10^7 cells/g of feces). In all stages of the disease activity, the total number of bacteria in the feces did not reach the required physiological level.

The total amount of bacteria in the feces in each stage of the disease activity did not differ significantly, although the obtained probability was relatively high ($p = 0.09$). There were no significant differences in the number of microorganisms in the feces, depending on the time since diagnosis of CD.

The number of short-chain fatty acids in the feces of CD patients depending on the stage of disease activity is shown in Table IV.

Percentages of short-chain fatty acids of the total SCFA content in the feces should be relatively constant. The percentage of acetic acid should be 60%, propionic acid – 20–25%, butyric acid: 15–20% (Roy *et al.*, 2006; Rechkemmer *et al.*, 1988). Significant abnormalities were detected in the amount of individual SCFA in the feces of CD patients. It is believed that of all SCFA, the trophic effect of butyrate is the most essential. While the percentage of acetate was increased (70%), the percentage of propionate and butyrate in the feces of CD

Table III
Minimum, maximum and median concentrations of *F. prausnitzii* and total cell count in patients with Crohn disease depending on the state of disease activity.

Type of bacteria	The level of clinical activity of disease									p (≤ 0.05)
	Remission			Mild activity			Moderate activity			
	Min	Max	Median	Min	Max	Median	Min	Max	Median	
F. prausnitzii	0	9E + 08	2E + 07	0	1E + 06	0	0	2E + 08	0	p = 0.015
Total number of bacteria	1,2E + 08	6E + 10	3E + 09	1E + 08	5E + 08	3E + 08	1E + 08	4E + 09	1 + 09	p = 0.09

p – significant when ≤ 0,05

Table IV
Minimum, maximum and median concentrations [mM] of SCFA in patients with Crohn disease depending on the state of disease activity.

SCFA	The level of clinical activity of disease										
	Remission			Mild activity			Moderate activity			p (≤ 0.05)	Concentration in total SCFA %
	Min	Max	Median	Min	Max	Median	Min	Max	Median		
Butyrate	0.10	1.62	0.69	0.22	1.55	0.65	0.27	1.55	0.96	p = 0.57	7.99
Acetate	2.47	10.88	6.40	2.89	11.25	6.94	3.13	9.92	9.09	p = 0.57	70.34
Propionate	0.22	3.09	1.05	0.60	2.85	1.96	0.82	3.62	2.07	p = 0.05	14.98

SCFA – short chain fatty acids; p – significant when ≤0,05

patients was significantly reduced (14.9% and 7.99%, respectively). Moreover, the authors showed a positive correlation between the number *F. prausnitzii* on the one hand and the total number of bacteria and the amount of butyrate in the feces on the other hand (p = 0.001 and p = 0.003). In CD patients, both the number of the bacteria and butyrate were strongly reduced. A relationship between a decrease in the concentration of propionate and the deterioration of the patient condition (state of disease activity) was also demonstrated. The higher the degree of disease activity, the lower the concentration of propionate reported in the feces.

Bacterial dysbiosis in patients with inflammatory bowel disease (IBD) is considered one of the key mechanisms of the disease. Despite numerous studies of qualitative and quantitative changes in microbiota, the etiological agent of the disease could not be determined. For this reason, researchers became increasingly interested in *F. prausnitzii* and its possible involvement in the pathogenesis of IBD.

The aim of the presented analysis was to determine the role of *F. prausnitzii* and their metabolites in developing and maintaining inflammatory bowel disease.

For the first time the number of butyrate-producing bacteria, mainly *F. prausnitzii,* was confirmed by Sokol *et al.* (Sokol *et al.*, 2008) in an animal model of IBD. Schwiertz *et al.* showed that in children with CD the level of this microorganism is greatly reduced (Schwiertz *et al.*, 2010). The composition of microbiota in children differs significantly from the adult population. Therefore, this study assessed the number of selected groups of bacteria in the feces of adult patients diagnosed with CD. The number of butyrate-producing *F. prausnitzii* was significantly reduced in the study group, which is in agreement with the observations of other researchers (Sokol *et al.*, 2008; Schwiertz *et al.*, 2010). At the same time, a decrease in the level of butyrate in the total SCFA was observed. This confirms the observation that the microorganism under investigation is a major producer of butyric acid in the colon. Because butyrate is the main nutrient for colonocytes, a reduced level of butyrate leads to abnormal physiological properties of cells and their destruction. Consequently, it causes a loss of normal structure and selectivity of the intestinal barrier. These abnormalities are observed in IBD patients. It can be postulated that the reduction in the number of *F. prausnitzii* (and, consequently, the decline in the production of butyric acid) induces damage to the intestine. The reduced level of butyrate also has an adverse affect on the composition of human microbiota. Impaired homeostasis of colonocytes leads to the development of chronic inflammation. Numerous experiments performed on animals provide evidence that butyrate is beneficial to the gastrointestinal tract. Adding a butyrate supplement to the diet reduced mucosal ulceration and decreased the tendency to the formation of new lesions, increasing the thickness of the mucosa and increasing the number of cells lining the villi of the ileum. There was also an improvement in the number and height of the villi, increased number of cells in intestinal crypts, a greater increase in body mass and an improvement in physical condition (Kotunia *et al.*, 2004; Galfi and Bokori, 1990). Trophic effect of butyric acid on the digestive tract seems to be undisputed. Unfortunately, butyric acid in the form of food has a very unpleasant odor, which greatly limits the prospects for its use in nutritional therapy in IBD patients. Moreover, orally administered acid is immediately taken up by the epithelium of the upper gastrointestinal tract. It is therefore suggested that other methods of increasing the amount of butyrate in the gut should be tested. The interim solution is to supply the butyrate to the light of the colon. This results in a temporary regeneration of intestinal mucosa and improvement in the parameters of intestinal crypts. The long-term solution seems to be to supplement the microorganisms that produce butyric acid. Because *F. prausnitzii* is not considered a probiotic microorganism, it cannot be taken orally in the form of probiotics. This suggests that the administration of lactic acid bacteria can activate metabolic crossfeeding. This involves polysaccharide stimulation of the growth of butyrate-producing bacteria, resulting in an increased microbial activity. An increase in the number of lactic acid bacteria increases lactate production, and thus directly increases the amount of butyrate (through the conversion of lactate) (Van Immerseel *et al.*, 2010).

This occurs through the stimulation of microbial groups that are able to convert lactate to butyrate. Five different arrangements have been identified for the genes of the central pathway involved in butyrate synthesis. *F. prausnitzii*, having no butyrate kinase for butyrate production uses acetic acid pathway (butyryl – CoA: acetateCoA – transferase) (Louis and Flint, 2009). In this study, acetic acid was the only SCFA the level of which in the feces was significantly increased. Although this compound is a substrate for the production of butyrate, the increased amount does not result in an increase of the butyric acid level. Perhaps the number of *F. prausnitzii* was too low to ensure the proper level of the butyrate production, despite favorable conditions for its synthesis. On the other hand, high level of acetic acid can be regarded as an indicator to reduce the number of *F. prausnitzii*. With reduced butyrate-producing bacteria, acetic acid as a substrate is used, resulting in acid elevated level. The relationship between a reduced level of propionate in the feces, and an increase in disease activity also requires explanation. Perhaps the decrease in the amount of this acid also has a part in development of disease. In the present work, the reduction of the total number of bacteria also indicates a reduction in the number of other groups of bacteria which, in addition to *F. prausnitzii*, may be involved in the production of butyric acid. Conducting detailed studies of the participation of other butyrate producers in the development of IBD appears necessary. The reduction of *F. prausnitzii* and butyric acid demonstrated in this work indicates their important role in pathogenesis and/or maintaining the IBD. For this reason it is imperative to conduct further research for alternative methods of increasing the number of *Faecalibacterium* in the colon.

Literature

Danese S. and C. Fiocchi. 2006. Etiopathogenesis of inflammatory bowel diseases. *World J. Gastroenterol.* 12, 4807–4812

Flint H.J., S.H. Duncan, K.P. Scott and P. Louis. 2007. Interactions and competition within the microbial community of the human colon: links between diet and health. *Environ. Microbiol.* 9, 1101–1111

Franks A.H., H.J. Harmsen, G.C. Raangs, G.J. Jansen, F. Schut and G.W. Welling. 1998. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* 64, 3336–3345

Galfi P. and J. Bokori. 1990. Feeding trial in pigs with a diet containing sodium n-butyrate. *Acta Veterinaria Hungarica* 38, 3–17

Harmsen H.J., G.C. Raangs, T. He, J.E. Degener and G.W. Welling. 2002. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. *Appl. Environ. Microbiol.* 68, 2982–2990

Kotunia A., J. Woliński, D. Laubitz, M. Jurkowska, V. Romé, P. Guilloteau and R. Zabielski. 2004. Effect of sodium butyrate on the small intestine development in neonatal piglets feed by artificial sow. *J. Physiol. Pharmacol.* 55 (S2), 59–68

Louis P. and H.J. Flint. 2009. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *Microbiol. Lett.* 294, 1–8

Matsuki T., K. Watanabe, J. Fujimoto, Y. Miyamoto, T. Takada, K. Matsumoto, H. Oyaizu and R. Tanaka. 2002. Development of 16S rRNA gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl. Environ. Microbiol.* 68: 5445–5451

Matsuki T., K. Watanabe, J. Fujimoto, T. Takada and R. Tanaka. 2004. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl. Environ. Microbiol.* 70, 7220–7228

Rechkemmer G., K. Ronnau and W. von Englehardt. 1988. Fermentation of polysaccharides and absorption of short chain fatty acids in the mammalian hindgut. *Comp. Biochem. Physiol.* 90A, 563

Roy C.C., C.L. Kien, L. Bouthillier and E. Levy. 2006. Short-chain fatty acids: ready for prime time? *Nutr. Clin. Pract.* 21, 351–366

Schwiertz A., M. Jacobi, J.S. Frick, M. Richter, K. Rusch and H. Kohler. 2010. Microbiota in Pediatric Inflammatory Bowel Disease. *J. Pediatr.* 157, 240–244

Shen Q., L. Zhao and K.M. Tuohy. 2011. High-level dietary fibre up-regulates colonic fermentation and relative abundance of saccharolytic bacteria within the human faecal microbiota in vitro. *Eur. J. Nutr.* [Epub ahead of print]

Sokol H., B. Pigneur, L. Watterlot, O. Lakhdari, L.G. Bermúdez-Humarán, J.J. Gratadoux, S. Blugeon, C. Bridonneau, J.P. Furet, G. Corthier and others. 2008. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc. Natl. Acad. Sci.* 105, 16731–16736

Van Immerseel F., R. Ducatelle, M. De Vos, N. Boon, T. Van De Wiele, K. Verbeke, P. Rutgeerts, B. Sas, P. Louis and H.J. Flint. 2010. Butyric acid-producing anaerobic bacteria as a novel probiotic treatment approach for inflammatory bowel disease. *J. Med. Microbiol.* 59, 141–143