SHORT COMMUNICATION

Qualitative and Quantitative Characteristics of Selected Bacterial Groups in Children with Inflammatory Bowel Diseases

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A b s t r a c t

The aim of the study was evaluation of qualitative and quantitative changes in bacterial ecosystem in 109 children with inflammatory bowel diseases. Stools obtained from patients were analysed for selected bacteria and concentration of faecal inflammatory markers (calprotectin, lactoferrin, M2-PK). The number of selected microorganisms depends on the level of clinical activity of disease and is correlated with faecal concentration of inflammatory markers. Differences in microflora disturbance, observed in patients with Crohn's disease and ulcerative colitis, may suggest different causes of development of both pathologies.

K e y w o r d s: Crohn's disease, faecal bacteria, IBD, inflammatory markers, M2-PK

Crohn's disease (CD) and ulcerative colitis (Colitis ulcerosa, CU), referred to as inflammatory bowel diseases (IBD) are conditions of which the aetiology has not been yet fully understood. Approximately 10–15% of patients with IBD do not meet criteria of CU or CD. Those cases are qualified as inflammatory bowel disease unclassified (IBDU) (Austin *et al.*, 2007).

The role of microbiological factors in the pathogenesis of IBD is advocated by numerous evidence. A renowned analysis by Sellon *et al.* (1998) indicated that introduction of physiological, non-pathogenic microflora to the environment of germ-free mice caused the development of colitis. In another experiment, development of inflammatory condition occurred following transfer of ileostomy content into the healthy part of the intestine (D'Haens *et al.*, 1998). Also, efficacy of antibiotics in therapy of IBD, and changes in composition of bacterial ecosystem in IBD patients and animal models may constitute evidence.

A search for a single pathogen leads to contradictory conclusions. Many microorganisms are being analysed, *e.g. Escherichia coli*, *Yersinia*, *Listeria*, but none of them have been isolated from all patients with IBD (Lakatos *et al.*, 2006; Wank *et al.*, 2014; Maukonen *et al.*, 2015). Some researchers associate causes of the disease with *Bacteroides fragilis*. Fragilisin – an endotoxin of high proteolytic activity produced by the strain – participat-

ing in the destruction of tight junctions, and in consequence, to loss of selectivity of the intestinal barrier and destruction of intestinal membrane. Kamińska *et al.* (2004) demonstrated that in children with moderate to acute IBD the discussed group of bacteria constituted a significant majority, and in the study by Andoh and Fujiyama (2006) those bacteria were even the only microorganism detected in all examined patients with CD. Also a role of *Clostridium difficile* and *Candida* spp. was widely discussed in relation to IBD (Gerard *et al.*, 2015; Monaghan *et al.*, 2015). Zwolińska-Wcisło *et al.* (2004) demonstrated the much higher level of *Candida albicans* occurrence in children with CU (78%) compared to healthy children (7%). The introduction of anti-mycotic agents caused a significant improvement in a patient's clinical condition. Noteworthy are data indicating the lower number of *Faecalibacterium prausnitzii* in patients with IBD (Gałęcka *et al.*, 2013). *F. prausnitzii* is extremely important for colonic homeostasis. Intestinal system of IBD patients is strongly quantitatively and qualitatively altered compared to healthy individuals, therefore maybe causes of IBD should be sought not in excessive proliferation of potentially pathogenic bacteria, but in reduction of count of beneficial microorganisms. It is commonly known that intestinal microbiota is a key factor in human health. The aim of our study was to determine if changes in the profile of microorganisms

Table I
Number of patients at individual levels of clinical activity
of disease

Diag-nosis	The level of clinical activity of disease				Total
	No activity	mild	moderate	severe	
UC	0	19	9	8	37
CD	18	9	12	8	47
IBDU	–	–	–	–	25
Total	18	28	21	16	109

UC – ulcerative colitis
CD – Crohn's disease
IBDU – inflammatory bowel disease unclassified

could be an useful marker preceding progression of disease, and correlate with inflammatory markers.

Hundred and nine children (64 boys and 45 girls) with IBD, hospitalised between 2009–2011, at the Department of Paediatric Gastroenterology and Metabolic Diseases of Poznan University of Medical Sciences participated in the study. All patients were between 3–16 years of age. Diagnosis was made on medical history, physical examination, laboratory tests, endoscopic and radiographic examinations as well as histopathological examinations. Activity of the disease was evaluated by the Paediatric Activity Index (PCDAI) for patients with CD and by the Truelove-Witts index (Ryzko and Woynarowski, 1995) for children with CU. Table I contains characteristic of each group. Patients in all study groups did not differ significantly in antibiotics and probiotics administration. Stool samples for microbiological culture and evaluation of inflammatory markers were collected from all children. Faeces were inoculated on a set of selective-differentiating and proliferative media. Count of the following bacteria was analysed: *E. coli* (including mycotic and lactose-negative strains), *Proteus* spp., *Pseudomonas* spp., other proteolytic bacteria (*Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp.), *Enterococcus* spp., *Bifidobacterium* spp., *Bacteroides* spp., *Lactobacillus* spp. (including hydrogen peroxide strains), *Clostridium* spp. and total bacterial count. Stools collected from patients were also analysed for yeasts and moulds. Concentration of analysed markers was evaluated using immuno-enzymatic tests (ELISA): M2-PK (ScheBo), calprotectin (Immundiagnostik) and lactoferrin (Techlab).

Stool samples were collected from each patient following obtaining an informed consent on participation in the analysis (patient or patient's guardian in case of children under the age of 16 years). The study obtained approval from the Ethical Committee at the Poznan University of Medical Sciences. All determinations were performed at the Institute of Microecology in Poznan.

Normality of distribution was verified using the Shapiro-Wilk test. Determination of the significance of differences between particular groups of patients was performed using a non-parametrical Kruskal-Wallis test. Spearman R correlation test was used for analysis of correlation between the level of clinical activity of the disease and microbial count. The same test was also used for analysis of correlation between bacterial count and various variables, including: patient's age, time elapsed since diagnosis and levels of inflammatory condition markers.

No statistically significant correlations were found between age of participating children and the count of analysed microorganisms. Similarly, time elapsed since diagnosis seem to have no effect on count of analysed microbiota.

A count of selected bacteria compared to established physiological norm was estimated to prepare characteristics of intestinal microflora of IBD patients. A median count of individual microorganisms in the test group of children was used. The percentage results of selected bacteria in particular diseases are presented in Table II.

Protective microflora. *Bacteroides* spp. (normal range: $\geq 10^9$ CFU/g of faeces). In all analysed pathologies count of those bacteria fell within the lower part of the normal range (median values: CU – 1×10^9, CD – 2×10^9, IBDU – 3×10^9); *Bifidobacterium* spp. (normal range: $\geq 10^9$ CFU/g of faeces). Only in children with IBDU the count of those bacteria fell within the normal range; and the value in patients with CU and CD was significantly reduced (median values: CU – 7×10^8, CD – 8×10^8, IBDU – 1×10^9); *Lactobacillus* spp. (normal range: $\geq 10^5$ CFU/g of faeces). – In all analysed pathologies count of discussed bacteria fell within the lower part of the normal range (median values: CU – 2×10^6, CD – 4×10^6, IBDU – 9×10^5). In all analysed pathologies count of those bacteria fell within the normal range; H_2O_2 – *Lactobacillus* (normal range: $\geq 10^5$ CFU/g of faeces). In all analysed pathologies the counts of the discussed bacteria fell within the lower part of the normal range (median values: CU – 8×10^5, CD – 6×10^5, IBDU – 1×10^5).

Immunostimulatory microflora. *E. coli* (normal range: $\geq 10^6$ CFU/g of faeces) – count of *E. coli* bacteria did not exceed the upper limit of normal range in any analysed pathologies (median values: CU – 4×10^7, CD – 7×10^7, IBDU – 2×10^7); *Enterococcus* spp. (normal range: $\geq 10^6$ CFU/g of faeces). In all analysed CD – 7×10^7, CI – 2×10^6).

Other proteolytic bacteria. *Clostridium* spp. (normal range: $\leq 10^5$ CFU/g of faeces). Count of *Clostridium* spp. fits within the normal range in all subgroups of included children (median values: CU – 0, CD – 2×10^4, IBDU – 0); Mycotic, lactose-negative bacteria of *E. coli* genus (normal range: $< 10^4$ CFU/g of faeces) – the bacterial count fell within the normal range in all pathologies (median values: CU, CD, IBDU – 0); *Proteus* spp.

Table II
Percentage distribution of the number of selected microorganisms in different diseases

Cell count	Percentage			
	Total	Diagnosis		
		CU	CD	IBDU
Bacteroides spp.: $< 10^9$	33.0	37.1	37.2	18.2
Bacteroides spp.: $10^9 - 10^{11}$	67.0	62.9	62.8	81.8
Bacteroides spp.: Total	100.0	35.0	43.0	22.0
Bifidobacterium sp.: $< 10^9$	64.0	65.7	67.4	54.5
Bifidobacterium sp.: $10^9 - 10^{11}$	36.0	34.3	32.6	45.5
Bifidobacterium sp.: Total	100.0	35.0	43.0	22.0
Lactobacillus spp.: $< 10^5$	15.0	11.4	9.3	31.8
Lactobacillus spp.: $\geq 10^5$	85.0	88.6	90.7	68.2
Lactobacillus spp.: Total	100.0	35.0	43.0	22.0
H2O2-*Lactobacillus*: $< 10^5$	38.0	31.4	39.5	45.5
H2O2-*Lactobacillus*: $\geq 10^5$	62.0	68.6	60.5	54.5
H2O2-*Lactobacillus*: Total	100.0	35.0	43.0	22.0
E. coli: $< 10^6$	23.0	20.0	27.9	18.2
E. coli: $\geq 10^6$	77.0	80.0	72.1	81.8
E. coli: Total	100.0	35.0	43.0	22.0
Enterococcus spp.: $< 10^6$	26.0	22.9	23.3	36.4
Enterococcus spp.: $\geq 10^6$	74.0	77.1	76.7	63.6
Enterococcus spp.: Total	100.0	35.0	43.0	22.0
Clostridium spp.: $\leq 10^4$	54.0	57.1	48.8	59.1
Clostridium spp.: $> 10^4$	46.0	42.9	51.2	40.9
Clostridium spp.: Total	100.0	35.0	43.0	22.0
E. coli lactose neg.: $< 10^4$	73.0	71.4	74.4	72.7
E. coli lactose neg.: $\geq 10^4$	27.0	28.6	25.6	27.3
E. coli lactose neg.: Total	100.0	35.0	43.0	22.0
Proteus spp.: $< 10^4$	88.0	80.0	88.4	100.0
Proteus spp.: $\geq 10^4$	12.0	20.0	11.6	0
Proteus spp.: Total	100.0	35.0	43.0	22.0
Pseudomonas spp.: $< 10^4$	100.0	100.0	100.0	100.0
Pseudomonas spp.: $\geq 10^4$	0	0	0	0
Pseudomonas spp.: Total	100.0	35.0	43.0	22.0
Other proteolytic bacteria: $< 10^4$	59.0	62.9	53.5	63.6
Other proteolytic bacteria: $\geq 10^4$	41.0	37.1	46.5	36.4
Other proteolytic bacteria: Total	100.0	35.0	43.0	22.0
Total bacterial count: $< 10^{11}$	89.0	94.3	88.4	81.8
Total bacterial count: $\geq 10^{11}$	11.0	5.7	11.6	18.2
Total bacterial count: Total	100.0	35.0	43.0	22.0

CU – ulcerative colitis
CD – Crohn's disease
IBDU – inflammatory bowel disease unclassified

(normal range: $< 2 \times 10^4$ CFU/g of faeces) – bacterial count fell within the normal range for all pathologies (median values: CU, CD, IBDU – 0); *Pseudomonas* spp. (normal range: $< 10^4$ CFU/g of faeces) – in all analysed pathologies counts of those bacteria fell within the physiological normal range (median values: CU, CD, IBDU – 0); Other, proteolytic bacteria – *Klebsiella, Citrobacter, Enterobacter, Serratia* (normal range: $< 10^4$ CFU/g of faeces). In all analysed patients counts of proteolytic bacteria fell within the normal range (median values: CU, CD, IBDU – 0); Total bacterial count (normal range: $10^{11}–10^{12}$ CFU/g of faeces) was reduced

in all discussed groups of patients (CU – 4×10^{10}, CD – 3×10^{10}, IBDU – 5×10^{10}).

Fungi. *Candida* spp. (normal range: $< 10^3$ CFU/g of faeces) – total count of yeasts was increased in all analysed subgroups of patients. The increase was most prominent in children with CD (median values: CU – 7×10^3, CD – 1×10^4, IBDU – 1×10^3). Physiological range of yeasts count was observed in only 32% of children with CU, 21% of children with CD and 50% of children with IBDU.

Another stage of the study was estimation if there is a correlation between count of analysed microorganisms and the level of clinical activity of the disease. Because of the insufficient data relating to the use of nonsteroidal anti-inflammatory drugs in the analyzed group of patients we did not asses their use at levels of calprotectin in the stools which is the limit of presented analysis. A negative correlation was demonstrated between the level of clinical activity of the disease and count of *Bifidobacterium* (p = 0.01; R = –0.28), *Bacteroides* (p = 0.006; R = –0.31) and total bacterial count (p = 0.002; R = –0.35). We have observed a higher number of mentioned bacteria in the lighter stage of the disease. The level of clinical activity of the disease was positively correlated with count of yeasts (p = 0.01; R = 0.28). Correlations between individual bacteria in particular types of IBD were estimated. In patients with CU no correlation between the clinical activity of the disease and count of analysed microorganisms was found. On the other hand, in children with CD at various stages of activity of the disease there were statistically significant differences regarding count of *Clostridium* spp. (p = 0.01), total bacterial count (p = 0.04) and count of yeasts (p = 0.02).

The analysed inflammatory markers were found to correlate with the level of activity of disease. Obtained results were compared to count of analysed faecal bacteria. A statistically significant, negative correlation was found between the number of *Bifidobacterium* and M2-PK concentration (p = 0.05), so we have observed higher number of *Bifidobacterium* and lower M2-PK concentration. Negative correlation was also found between the number of *Bacteroides* and concentration of all analysed markers (M2-PK p = 0.02; calprotectin p = 0.03; lactoferrin p = 0.01). A negative correlation was also observed between the total bacterial count and concentration of M2-PK and lactoferrin (M2-PK p = 0.02; lactoferrin p = 0.007), so we have observed higher number of total number of bacteria and lower concentration of M2-PK and lactoferrin. A positive correlation was observed between the number of *Candida* and concentration of M2-PK and lactoferrin (M2-PK p = 0.001; lactoferrin p = 0.003).

Considering that the role of microbiological factor in IBD aetiology seems almost certain (among other factors, such as: genetic predisposition and immunological condition) authors decided to evaluate presence and count of selected bacteria in a group of paediatric patients. Increasing incidence rate of the disease in that group of patients, diagnostic and therapeutic difficulties constitute one of the most important challenges of contemporary medicine. Early diagnosis of the disease and determination of its actual aetiology are necessary for introduction of efficient therapy and ensuring optimal conditions of growth and development for affected child. Existence of correlation between bacterial factor and analysed faecal inflammatory markers was also studied. This analysis involved count of individual microorganisms in patients with particular type of IBD and stage of clinical activity of the disease. That approach was caused by postulated differences in causing factors of CD and CU.

Contrary to speculations, the composition of microflora in the group of analysed paediatric patients with IBD was not significantly different from accepted quantitative normal range (Gibson and Roberfroid, 1995). The authors demonstrated a significant increase in the number of yeasts in the analysed group. Similar observations were made by Zwolińska-Wcisło (2004) studying a group of children with CU. In the cited study, it was shown that the incidence rate of significant mycotic colonisation was significantly higher in the study group compared to the control (78% *vs.* 7%). That observation is very important, because the authors evaluated that a real probability of occurrence of mycotic infections was growing proportionally to time of the disease. Therefore, systematic monitoring of yeasts count is necessary, as well as rational use of antibiotics in that group of patients (Rosińska *et al.*, 2007). The authors demonstrated a decrease in the count of *Bifidobacterium* in the analysed patients. An appropriate gut concentration of *Bifidobacterium* is obligatory for maintenance of homeostasis of the organism. Reduction in count of *Bifidobacterium* may cause an inflammatory condition. As stated in the literature, a reduction in count of *Bifidobacterium* is accompanied by excessive proliferation of anaerobic *Clostridium*. Those observations seem to be confirmed in this analysis. *Clostridium* count in children with CD was increased compared to other patients. Despite the fact that the count did not exceed the admissible physiological normal range in none of analysed pathologies, in children with CD the count was close to the upper limit of the normal range. Significantly lower counts of *Clostridium* were observed in children with CU and IBDU. Despite the lack of statistical significance, the observed differences in count of discussed microorganisms suggest the participation of separate microorganisms in the aetiology of both diseases. Another abnormality demonstrated in the group of participating children was the reduction

of total bacterial count. A conclusion on disorders of intestinal ecosystem may be drawn on that basis. As the strongest reduction was observed in relation to *Bifidobacterium*, it seems that reduction of total count may be attributed mostly to the protective group of bacteria. Maybe, elimination of observed dysbiosis and restoration of desirable bacterial conditions in the intestine is a prerequisite of successful therapy of IBD.

Another stage of the study was an evaluation of correlation between count of individual microorganisms and the level of clinical activity of the disease. No correlations of that kind were found in children with CU. However, in children with CD a significant correlation was detected between activity of the disease and total bacterial count, count of yeasts and *Clostridium* genus. This is another argument for the stronger influence of microbiota on development and/or maintenance of CD, compared to CU. Maybe, complexity of bacterial interactions, affecting development and maintenance of inflammatory condition, is a cause of lack of success of probiotic therapy in that pathology. Observed proliferation of proteolytic bacteria of *Clostridium* genus and of yeasts is an unfavourable condition, facilitating further development of pathogenic intestinal flora. Excessive development of proteolytic bacteria causes damage to intestinal epithelium (production of toxic metabolites) and increases pH value of intestinal environment, intensifying the disease. On the other hand, excessive count of yeasts, being usually a result of dysbacteriosis, leads to increased pool of secreted mycotoxins. Further analyses are necessary to evaluate a real correlation of microorganisms discussed here with exacerbation of patient's condition. Elimination of dysbiosis may contribute to improved health condition of a patient.

Analysis of correlation between bacterial count and the level of inflammatory markers demonstrated existence of numerous associations. Increased level of all inflammatory markers was accompanied by reduced count of *Bacteroides*, constituting the most numerous part of intestinal microflora. M2-PK and lactoferrin concentration was reversely proportional to count of protective bacteria, as well as to count of *Bifidobacterium* – the bacterial count decreased with increasing activity of the disease. Another word – the more intensified inflammatory process, the less protective flora is present, or otherwise: the lower the count of lactobacilli, the more intensified inflammatory process is. Count of yeasts and of proteolytic bacteria increased with increasing marker concentration. It remains to be established if increased quantity of produced proteolytic enzymes (participating in destruction of tissues) and of mycotoxins may be a direct cause of inflammation. A correlation between clinical activity of the disease and changes in intestinal ecosystem should be studied intensively in the analysed group of patients.

A correlation between activity of the disease, inflammatory marker levels, and intestinal bacterial count may contribute to the development of a new, effective scheme of diagnostics and evaluation of a patient's condition.

The final aetiological factor of IBD remains unknown. It is worth to emphasize that the cause of IBD can be either a pathogenic infection, or a reduction of tolerance of bacterial symbiotic antigens to initiate the pathogenic sequence. However, in analysed patients, both significant qualitative and quantitative changes of intestinal microflora were observed, being a valuable diagnostic and therapeutic indication. Observed microbiological changes may be associated both with a type of IBD and with its clinical activity. Basically, a trend for reduction of beneficial bacteria count is observed with simultaneous increase in count of potentially pathogenic microorganisms. Currently, determination if observed abnormalities are a cause or a result of the disease is a crucial problem. Evaluation of type of changes of intestinal ecosystem is important for development of effective therapy in IBD. An appropriate modification of microflora could play a role in therapy of exacerbation periods of the disease and maintenance of remission.

Conflict of interest
All of the authors have no conflict of interest to declare.

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