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M. STRUS[1], T. GOSIEWSKI[1], K. FYDEREK[2], A. WEDRYCHOWICZ[2], K. KOWALSKA-DUPLAGA[2], P. KOCHAN[1], P. ADAMSKI[3] P.B. HECZKO[1]

A ROLE OF HYDROGEN PEROXIDE PRODUCING COMMENSAL BACTERIA PRESENT IN COLON OF ADOLESCENTS WITH INFLAMMATORY BOWEL DISEASE IN PERPETUATION OF THE INFLAMMATORY PROCESS

[1]Chair of Microbiology, Jagiellonian University Medical College, Cracow, Poland; [2]Department of Pediatrics, Gastroenterology and Nutrition, Polish-American Children's Hospital, Jagiellonian University Medical College, Cracow, Poland; [3]Institute of Nature Conservation, Polish Academy of Science, Cracow, Poland

Bacteria in the gut play a central role in the initiation and progress of inflammatory bowel disease (IBD). This study was prepared to elucidate the role in the inflammatory process of the bacterial species which are able to produce hydrogen peroxide, present in samples taken from colon lesions in adolescents with inflammatory bowel disease. Fifty eight adolescents were enrolled into the study from January 2004 to October 2006 in Cracow, Poland. Biopsies and stool samples were collected. Bacteriological examinations and measurements of hydrogen peroxide production by enterococci, streptococci and lactobacilli were performed. For the first time it has been shown here that HP producing bacteria may contribute to increased amounts of hydrogen peroxide in the inflamed mucosa of Crohn's disease and ulcerative colitis patients. Moreover, we have been able to demonstrate an increase of total populations of aerobic bacteria but not anaerobes in the studied samples of mucosa of adolescents with inflammatory bowel disease which is an indirect evidence of higher oxygen tension present in inflamed tissues in IBD. We have also been able to demonstrate the direct relationship between presence of blood in stools of IBD adolescents and increased populations of *Enterobacteriaceae* but not streptococci in samples of colon mucosa. It is, therefore, possible that different products of *Enterobacteriaceae* and especially their lipopolysaccharides may also contribute to perpetuation of the chronic colon inflammation.

Key words: *hydrogen peroxide, inflammatory bowel disease, ulcerative colitis, Crohn's disease, aerobic bacteria, Lactobacillus*

INTRODUCTION

Bacteria inhabiting the human intestinal tract play a pivotal role in initiation and progress of inflammatory bowel disease (IBD). Some years ago, it was believed that these inflammatory processes are caused by a single etiological agent of either bacterial or even viral origin, but introduction of animal models to study pathomechanisms of IBD and the use of modern molecular methods led to a change of the basic idea on the involvement of bacteria in IBD. Now, it is commonly accepted that the complex commensal bacterial gut microflora is involved in the mechanisms of IBD together with genetic alterations and aberrated immune response of the host. Although gut commensal bacteria interact with the gut mucosa as a whole, there are quantitative changes in the composition of the gut microflora in IBD patients in comparison to healthy persons (1).

Bibiloni and colleagues (2) found significant differences in proportions between different bacterial species present in mucosal biopsies taken from patients with Crohn's disease (CD) and ulcerative colitis (UC). Also, the total numbers of bacteria isolated from the biopsies from UC patients were significantly higher than those from CD patients. Similar results stressing the increase of numbers of bacteria that adhered to colon mucosa in UC were also obtained by Kleessen *et al*. (3) and Swidsinski *et al*. (4, 5).

Bibiloni *et al*. (2) in their above mentioned study found for the first time that in biopsies from both UC and CD patients populations of lactobacilli were more numerous than in healthy controls. Both *Lactobacillus, Streptococcus* and *Enterococcus* genera are members of the gut commensal microflora possessing similar structures of the Gram-positive bacterial cell wall specifically recognized by the gut immune system. Unlike other inhabitants of the gut flora, some species of *Lactobacillus, Streptococcus* and *Enterococcus* genera are able to produce substantial amounts of hydrogen peroxide (HP), comparable to those liberated by stimulated phagocytic cells like macrophages and neutrophils (6, 7).

Both hydrogen peroxide and other reactive oxygen species (ROS) are dangerous to mucosal cells, easily penetrating through membranes and causing, depending on their concentration, their apoptosis and necrosis (8). The damaging effects of low concentrations of ROS on tissues are neutralized in physiological conditions by various anti-oxidant mechanisms, mainly by different enzymes: catalase, peroxidase, dismutase, *etc*. contained in tissues. On the other hand, in chronic inflammatory conditions like IBD, production of ROS by

numerous stimulated phagocytes is markedly increased and anti-oxidative mechanisms are impaired (6, 7). Up to now, it is commonly considered that increased amounts of ROS present in colon mucosa of IBD individuals originate from stimulated gut immune cells (9). This study was devoted to elucidate the role in the inflammatory process of these bacterial species present in samples taken from colon lesions in adolescents with IBD, which are able to produce hydrogen peroxide.

MATERIALS AND METHODS

Patients

Fifty eight adolescents (mean age 15 years; SD±4.13) were enrolled into the study from January 2004 to October 2006. There were 12 patients with UC (mean age 14.74 years; SD±2.9), 22 with CD (mean age 16.22 years; SD±3.8) in the study group and 24 control subjects (mean age 14.13 years; SD±4.4) who underwent colonoscopy because of chronic abdominal pain. All participants were hospitalized in the Department of Paediatrics, Gastroenterology and Nutrition of the Jagiellonian University Medical College, Cracow, Poland. The diagnosis of CD or UC was based on endoscopic, histopathological, immunological and radiological criteria. Histology was assessed blindly by an independent histopathologist. All patients with IBD were in the active phase of the disease. Presence of frank blood in stools was observed during sample collection and fecal occult blood in stools was diagnosed using commercial available quick immunoenzyme tests (Instalert, Innovacon, USA). The use of antibiotics 30 days prior to enrolment, infectious diarrhoea, malabsorption, immunodeficiencies and intestinal enteropathies were the exclusion criteria. The trial was approved by the Jagiellonian University Bioethical Committee (No. KBET/236/B/2002) and informed consent was obtained from all patients' legal guardians and/or patients over 16 years of age enrolled into the study.

Sampling of mucosa

Biopsy samples from IBD patients were obtained from the inflamed colonic mucosa. In the control group, the biopsy samples were taken from a normal sigmoid colon for the same assessments. The biopsy samples were transferred directly into Schaedler Anaerobic Broth (SAB) medium (Difco, BD, Franklin Lakes, USA) with 10% of glycerol. The samples were immediately snap frozen on dry ice and kept at –80°C until analysis. All procedures were performed as quickly as possible, using sterile instruments and ensuring the integrity of the intestinal tissue. The codes of the biopsy samples were blinded before performing microbiological analysis.

Stool sampling

Stool samples were collected during routine preparation of the colon for colonoscopy by using a phosphate laxative followed with saline enema. Stools were homogenized, pooled and kept frozen at –80°C in 10 ml volumes until tested.

Bacteriology

The frozen samples were thawed, weighed, homogenized in 1 ml of SAB and quantitatively analyzed for the main bacterial constituents by cultures on differential media in aerobic and anaerobic conditions. All these manipulations were done aseptically in the anaerobic chamber (MACS-MG 500 Work Station, DW Scientific, Shipley, UK) in $N(85\%)+H_2(10\%)+CO_2(5\%)$ atmosphere. Homogenized samples were serially diluted with SAB and 100 µl aliquots plated on the following media: McConkey Agar (Oxoid, Basingstoke, UK) for *Enterobacteriaceae,* Columbia Blood Agar (Difco) with 5% sheep blood for streptococci, Enterococcosel Agar (BBL, BD, Franklin Lakes, USA) for enterococci*,* MRS agar (Oxoid) for lactobacilli and other lactic acid bacteria (LAB), glucose-blood-liver (BL) agar for bifidobacteria and Wilkins-Chalgren Agar Base with supplements for *Bacteroides* (Difco). The dilutions were then spread over the plate surface by making use of glass rods and then the plates were incubated aerobically at 37°C for 24 hours, except for the cultures for anaerobic bacteria, which were kept in the anaerobic chamber for up to 4 days depending on type of the medium. The morphology of the grown colonies was analyzed under magnifying glass and several colony picks of each morphological type were subcultured on appropriate aerobic and anaerobic media and Gram-stained. After further incubation and culture purity checks, phenotypic identification was performed using commercial identification systems (API 20E, API20A, API50CHL, APIStaph, APIStrept: bioMerieux, Marcy l'Etoile, France; BBL Crystal ID System, BD, Franklin Lakes, USA). Identification of the isolates which were not successfully speciated by phenotypic methods was then confirmed by molecular identification based on PCR technique using primers listed in *Table 1*.

Measurement of hydrogen peroxide production by bacteria from Enterococcus, Streptococcus and Lactobacillus genera

Analytical Merckoquant peroxide test strips (Merck) were used to measure H_2O_2 production by randomly selected 10 strains of each species belonging to *Enterococcus*, *Streptococcus* and *Lactobacillus* genera on a detection scale between 0 and 100 mg/l. The tested bacteria were suspended in 2 ml of TSB broth (Difco) and cultured at 37°C in aerobic conditions. The measurements of H_2O_2 were done twice at 4 and 24 hours according to the procedure provided by the producer. The mean density of bacteria after 4 h was estimated as approximately 3×10^6 CFU/ml, but after 24 h this density increased to 1×10^7 CFU/ml. Uninoculated TSB broth was used as a negative control. The amounts of H_2O_2 were given in mM or mg/l.

These data were then used to calculate: (i) the total number of bacteria belonging to *Enterococcus*, *Streptococcus* and *Lactobacillus* genera (in CFU per gram of tissue) which produced hydrogen peroxide in samples of inflamed tissues of patients with UC or CD *vs*. control group and (ii) total amounts of HP produced by bacteria in samples in inflamed tissues of adolescents with CD in 24 hours in comparison to amounts produced in control patients. The following formula was used in this calculation:

$$\left|\sum \frac{(l_i m)/\, p_i}{1*10^7}\right|$$

m – sample mass (g); l – number of bacteria of *i* strain; *i* – strain isolated from sample; p – mean amount of hydrogen peroxide produced by 1×10^7 c.f.u./ml of *i* bacterial species during 24 hours.

Statistical methods

Due to extremely skewed and far from normal/Gausian distribution of the analyzed data, typical parametric statistical tests could not be used. For this reason comparisons between CD and control patients were conducted with Wilcoxon test. Moreover, small numbers of some samples made it impossible to use most

Table 1. Primers used for species identification of selected isolates from biopsy specimens.

Bacteria	Primer codes	Sequences	Source
Escherichia coli	BG1 BG2	CTTTGCCTGGTTTCCGUCACCAGAA AACCACCGCACGATAGAGATTCGGG	Kane *et al*. (25)
Bacteroides-Prevotella group	Bac32F Bac303R	AACGCTAGCTACAGGCTT CCAATGTGGGGGACCTTC	Bernhard *et al*. (26)
Lactobacillus acidophilus	Aci I Aci II	AGC TGA ACC AAC AGA TTC AC ACT ACC AGG GTA TCT AAT CC	Tilsala-Timisjarvi *et al*. (27) Walter *et al*. (28)
L. delbrueckii	Del I Del II	ACG GAT GGA TGG AGA GCA G GCA AGT TTG TTC TTT CGA ACT C	Tilsala-Timisjarvi *et al*. (27) Walter *et al*. (28)
L. fermentum	L fpr Ferm II	GCC GCC TAA GGT GGG ACA GAT CTG ATC GTA GAT CAG TCA AG	Tilsala-Timisjarvi *et al*. (27) Walter *et al*. (28)
L. plantarum	L fpr Plan II	GCC GCC TAA GGT GGG ACA GAT TTA CCT AAC GGT AAA TGC GA	Tilsala-Timisjarvi *et al*. (27) Walter *et al*. (28)
L. gasseri	GasI GasII	GAG TGC GAG AGC ACT AAA G CTA TTT CAA GTT GAG TTT CTC T	Tilsala-Timisjarvi *et al*. (27) Walter *et al*. (28)

Table 2. Numbers of bacteria (in CFU/g of tissue) cultured from biopsy and stool samples taken from adolescents with UC, CD and from the control group.

Groups (taxons) of bacteria	Adolescents with ulcerative colitis (n=12)		Adolescents with Crohn's disease (n=22)		Adolescents in the control group (n=24)	
	Tissue samples	Stool samples	Tissue samples	Stool samples	Tissue samples	Stool samples
Global numbers of the cultured bacteria	$5.83\times10^{8*}$	3.41×10^{8}	3.93×10^{8}	8.38×10^{8}	1.77×10^{8}	1.52×10^{9}
Enterobacteriacae	4.92×10^{5}	7.35×10^{7} *	2.17×10^{7}	2.70×10^{8} *	1.99×10^{7}	2.72×10^{7}
Enterococcus	1.80×10^{6}	4.55×10^{6}	1.36×10^{6}	7.68×10^{7}	7.29×10^{5}	1.15×10^{7}
Streptococcus	6.83×10^{5}	5.40×10^{6}	1.29×10^{8}**	2.14×10^{8} *	5.40×10^{6}	9.46×10^{7}
Lactobacillus	9.28×10^{7}*	1.10×10^{8}	3.37×10^{6}	6.35×10^{7}	1.02×10^{7}	2.14×10^{8}
Bifidobacterium	5.56×10^{6}*	1.47×10^{8}	8.62×10^{6}*	2.14×10^{8}	1.40×10^{8}	1.18×10^{9}
Bacteroides	2.00×10^{6}	1.00×10^{3}	1.24×10^{5}	1.44×10^{3}	8.04×10^{5}	1.41×10^{6}

* significant difference between UC and/or CD *vs*. control group (p=0.01 to 0.005)
** highly significant difference between UC and/or CD *vs*. control group (p=0.001 to 0.01).

popular non-parametric tests based on the ranges (Mann-Whitney, Wilcoxon, Kruscal-Wallis, Kolmogorov-Smirnoff). In such a case, average bacterial numbers in the groups had to be analyzed with less restrictive median test (10, 11).

RESULTS

Numbers of all cultivable bacteria present in homogenates of biopsies taken from mucosal sites with visible inflammation in adolescents with UC were significantly higher than those taken from control group (*Table 2*). Moreover in patients with UC the total numbers of bacteria of the *Lactobacillus* genus were significantly higher than in control group. Microbiological examination done on the stool samples collected from patients with UC in comparison to control group have shown statistically higher counts of Gram-negative rod populations, especially of *Escherichia coli*.

In the tissue samples collected from inflammatory lesions of adolescents suffering from CD, the numbers of streptococci were significantly higher than in controls. Furthermore in the stool samples obtained from patients with CD in comparison to faecal samples of control adolescents, statistically higher numbers of *Streptococcus* populations and *Escherichia coli* were observed. Both in adolescents with UC and CD, numbers of bacteria of *Bifidobacterium* genus appeared to be significantly lower in inflamed sites than in unchanged sites in controls.

Randomly selected 10 strains of each species belonging to *Enterococcus*, *Streptococcus* and *Lactobacillus* genera which were isolated from biopsies were tested for production of HP *in vitro*. It appeared that strains of *E. avium, E. gallinarum, S. mitis, S. oralis, S. sanguis, L. delbrueckii* and *L. acidophilus* produced measurable amounts of peroxide, which differed from 0.3 mM for most of the tested strains up to 1.8 mM liberated in 24 hours by *L. delbrueckii* and *S. mitis* (*Table 3*).

Numbers of all bacteria belonging to HP producing species of the *Lactobacillus*, *Streptococcus* and *Enterococcus* genera present in samples taken from inflamed sites of adolescents with UC and CD were summarized and compared with corresponding numbers of the same species in samples from control adolescents. As shown in *Fig. 1*, populations of HP producing bacteria were significantly ($p<0.01$) more numerous in inflamed tissues of adolescents with UC in comparison to control group. This phenomenon was not observed in adolescents with CD. To elucidate this discrepancy, an attempt was made to calculate the total amounts of HP produced by bacteria of the three genera in tested inflamed sites of all adolescents with CD in 24 hours in comparison to amounts produced by the control group using the formula presented in Materials and Methods section. As shown in *Fig. 2*, the total amounts of HP produced by all bacteria of the

Table 3. Mean amounts of hydrogen peroxide (in mM) produced *in vitro* in 4 and 24 hours by 10 randomly selected strains of species belonging to *Enterococcus*, *Streptococcus* and *Lactobacillus* genera isolated from colon mucosal biopsies and faeces obtained from adolescents with UC and CD.

Species of tested bacteria	Mean amount of H_2O_2 produced by 10 strains of the same species in 4 hours (in mM)	Mean amount of H_2O_2 produced by 10 strains of the same species in 24 hours (in mM)
E. faecalis	0	0
E. faecium	0	0
E. avium	0.3±0.047	0.6±0.1414
E. durans	0	0
E. gallinarum	0.3±0.047	0.31±0.057
S. bovis	0	0
S. salivarius	0	0
S. mitis	0.6±0.095	1.8±0.566
S. oralis	0.3±0.032	0.6±0.141
S. sanguis	0.3±0.032	0.9±0
L. rhamnosus	0	0
L. plantarum	0	0
L. fermentum	0	0
L. delbrueckii	0.9±0	1.8±0
L. acidophilus	0.3±0.043	0.6±0.141
L. brevis	0	0

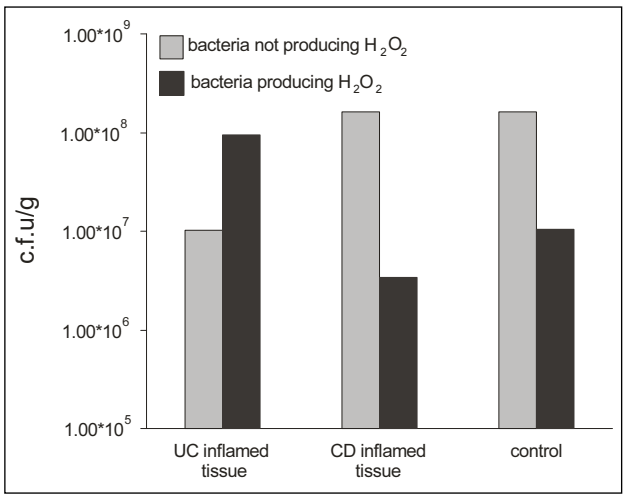

Fig. 1. Comparison of total numbers of hydrogen peroxide producing versus non-producing bacteria present in inflamed sites of colon mucosa in adolescents with UC and CD and those from the control group.

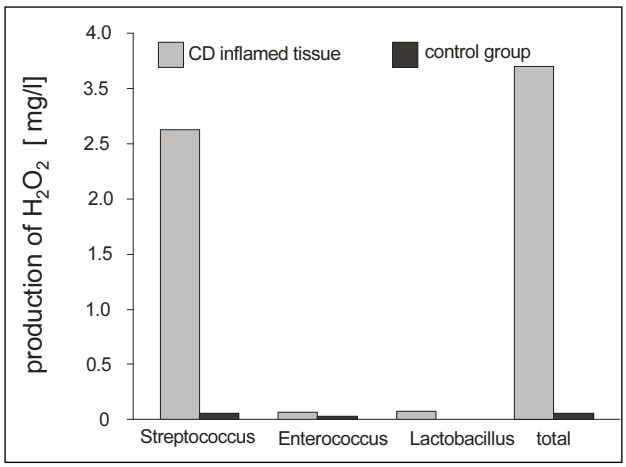

Fig. 2. Estimated total amounts of hydrogen peroxide produced in 24 hours by populations of bacteria belonging to *Streptococcus*, *Enterococcus* and *Lactobacillus* genera present in mucosal biopsies from adolescents with CD in comparison to the amounts produced by the same bacteria present in biopsies from adolescents of the control group.

Streptococcus genus in inflamed tissues were significantly higher ($p<0.048$) than those produced in non-inflamed mucosa of the control adolescents. This difference was not significant for bacteria of the remaining, previously listed genera.

Increased numbers of aerobic bacteria, mainly streptococci and lactobacilli present in inflamed mucosa of adolescents with UC and CD and decreased numbers of anaerobic bifidobacteria, as shown in *Table 2*, may indicate inverse proportions between aerobic and anaerobic bacteria present in inflamed mucosa in comparison to unchanged mucosa in controls. To prove this hypothesis, total numbers of cultivable bacteria belonging to anaerobic and aerobic taxons were compared in samples taken from adolescents with CD and UC and in biopsies from adolescents of the control group. As shown in *Fig. 3*, populations of aerobic bacteria significantly predominated those of anaerobic bacteria ($p<0.001$) in samples from inflamed sites of both UC and CD adolescents but not in samples from adolescents of the control group.

Since it was possible to demonstrate significantly increased numbers of aerobic Gram-negative rods of *Enterobacteriaceae* family in faecal samples of adolescents with CD and UC versus control groups (*Table 2*), a question was raised if these changes were related to some factors playing a role in pathology of IBD linked to inflamed mucosa, like blood extravasated from ulcerations. As shown in *Fig. 4*, presence of blood in stools of the studied adolescents was positively related to elevated numbers of the Gram-negative rods (especially of *Escherichia coli*) cultured from biopsies and to diminished numbers of

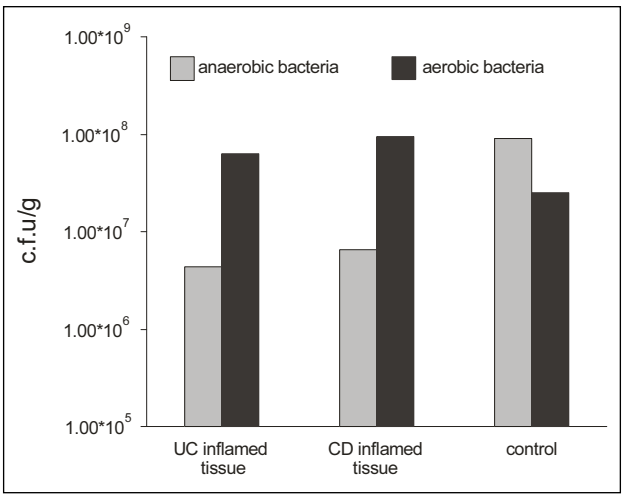

Fig. 3. Relationship between total numbers of anaerobic and aerobic bacteria present in inflamed mucosal biopsies from adolescents with CD and UC in comparison to appropriate numbers of bacteria present in biopsies from adolescents of the control group.

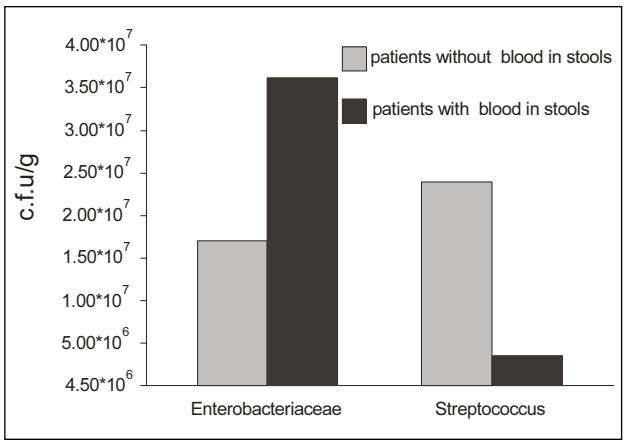

Fig. 4. Relationship between the presence of blood in stools and the total numbers of bacteria of *Enterobacteriaceae* family and *Streptococcus* genus in CD adolescents with (n=14) or without blood (n=8).

streptococci in biopsies taken from adolescents with frank and/or occult blood in stool samples ($p<0.0036$ and $p<0.0027$, respectively). This analysis was made only for CD patients since all stool samples of UC patients contained blood.

DISCUSSION

In this study, we have been able to show that bacterial content of the chronic inflammatory lesions in colon of adolescents show significant differences in total numbers and in composition of the adherent bacteria: *Lactobacillus* populations were elevated in adolescents with UC, while streptococci were more numerous in adolescents with CD in comparison to controls. As mentioned before, basically the same data on the increase of total bacteria and especially lactobacilli in biopsies from adult patients with both CD and UC were reported by Bibiloni *et al*. (2). However, in our studies, streptococci predominated over other bacterial groups in CD adolescents. Sartor *et al*. (12) was able to show an interesting mechanisms of evoking IBD in rats after submucosal injection of the *Streptococcus* peptidoglycan which resulted in pathological changes strongly resembling those in human CD.

Although it is generally accepted that CD and UC are two clinical forms of the IBD in humans, most probably pathomechanisms of these forms are different which influences the composition of the bacterial flora in inflammatory lesions, as described by Frank *et al.* (13). The review of Weersma *et al.* (14) on gene mutations leading to inflammation in CD and UC patients also suggests such a possibility.

It is of interest that both lactobacilli and streptococci, which are aerobic members of the commesal gut flora, show similar structure of the peptidoglycan in their cells walls but also share similar property: ability to produce hydrogen peroxide extracellularly. It has been shown here for the first time that HP producing bacteria may contribute to increased amounts of HP in the inflamed mucosa of CD and UC patients. It is known that increased concentrations of ROS in gut mucosa are harmful to the integrity of the epithelium (8). Most dangerous to the tissue is the chronically increased ROS flow originating from activated immune cells but also, as shown here, from HP producing bacteria adhering to IBD lesions.

Moreover, we have been able to demonstrate in this study an increase of total populations of aerobic bacteria but not anaerobes in the studied samples of mucosa of IBD adolescents which is an indirect evidence of higher oxygen tension present in inflamed tissues in IBD. This may be a reflection of the imbalanced anti-oxidative activity of the IBD mucosa (15).

There are many publications on IBD and UC, including somewhat related probiotic applications (16, 17, 18). Our studies done on mice models of IBD have indicated that the process is related to increased numbers of *Escherichia coli* in the colon (19). Similar increase was reported in IBD patients by two other groups (4, 20). Although we were unable to show more numerous populations of *E. coli* in tissue samples taken from adolescents with both UC and CD, we demonstrated higher populations of *Enterobacteriaceae* in faeces of IBD adolescents. It is generally known that IBD lesions cause extravasation of blood from ulcerated tissue and increased amounts of free heme particles present also in colon lumen and content. It is therefore possible that free iron ions may trigger gut populations of *E. coli* and other members of *Enterobacteriaceae* family to multiply more intensively. It is known that free iron ions are extensively trapped by *E. coli* siderophores in more aerobic conditions and cause increased multiplication of the bacterial populations (21-23). We have been able to demonstrate the direct relationship between presence of blood in stools of IBD adolescents and increased populations of *Enterobacteriaceae* but not streptococci in samples of colon mucosa. It is, therefore, possible that different products of elevated in such way populations of *Enterobacteriaceae* and especially their lipopolysaccharides may also contribute to perpetuation of the chronic colon inflammation (24).

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Author's address: Prof. Piotr B. Heczko, M.D., Ph.D., Head of the Chair of Microbiology, Jagiellonian University Medical College, 18 Czysta Street, 31-121 Cracow, Poland; Phone: +4812 633 25 67; Fax: +4812 423 39 24; E-mail: mbheczko@cyf-kr.edu.pl