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).

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
Schaeffer 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 Merkoquant peroxide test strips (Merck) were
used to measure H2O2 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 H2O2 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
\[ 3 \times 10^6 \text{ CFU/ml} \] but after 24 h this density increased to \[ 1 \times 10^7 \text{ CFU/ml} \]. Uninoculated TSB broth was used as a negative
control. The amounts of H2O2 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:

\[
\sum \left( \frac{m}{p} \right) \times \frac{1}{1 \times 10^7}
\]

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

Statistical methods

Due to extremely skewed and far from normal/Gaussian
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
Both in adolescents with UC and CD, numbers of faecal samples of control adolescents, statistically higher stool samples obtained from patients with CD in comparison to were significantly higher than in controls. Furthermore in the examination done on the stool samples collected from patients significantly higher than in control group. Microbiological total numbers of bacteria of the higher counts of Gram-negative rod populations, especially of with UC in comparison to control group have shown statistically significantly higher than in control group.

**Enterobacteriaceae** 4.92×10^5 7.35×10^5* 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 1.36×10^7 7.68×10^6 7.29×10^7 1.15×10^7
**Streptococcus** 6.83×10^6 5.40×10^7 1.29×10^8** 2.14×10^8* 5.40×10^8 9.46×10^7
**Lactobacillus** 9.28×10^6* 1.10×10^7 3.37×10^7 6.35×10^7 1.02×10^8 2.14×10^8
**Bifidobacterium** 5.56×10^6* 1.47×10^7 8.62×10^6* 6.35×10^7 1.40×10^8 1.18×10^8
**Bacteroides** 2.00×10^6 1.00×10^7 1.24×10^7 1.44×10^7 8.04×10^7 1.41×10^8

* = 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).

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 infected 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
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 in comparison to the amounts produced by the same bacteria present in biopsies from adolescents of the control group.

**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.

<table>
<thead>
<tr>
<th>Species of tested bacteria</th>
<th>Mean amount of H$_2$O$_2$ produced by 10 strains of the same species in 4 hours (in mM)</th>
<th>Mean amount of H$_2$O$_2$ produced by 10 strains of the same species in 24 hours (in mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. avi</em></td>
<td>0.3±0.047</td>
<td>0.6±0.1414</td>
</tr>
<tr>
<td><em>E. durras</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>E. gallinarum</em></td>
<td>0.3±0.047</td>
<td>0.31±0.057</td>
</tr>
<tr>
<td><em>S. bovis</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. salivar</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. mitis</em></td>
<td>0.6±0.095</td>
<td>1.8±0.566</td>
</tr>
<tr>
<td><em>S. oralis</em></td>
<td>0.3±0.032</td>
<td>0.6±0.141</td>
</tr>
<tr>
<td><em>S. sanguis</em></td>
<td>0.3±0.032</td>
<td>0.9±0</td>
</tr>
<tr>
<td><em>L. rhamnosus</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>L. fermentum</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>L. delbrueckii</em></td>
<td>0.9±0</td>
<td>1.8±0</td>
</tr>
<tr>
<td><em>L. acidophillus</em></td>
<td>0.3±0.043</td>
<td>0.6±0.141</td>
</tr>
<tr>
<td><em>L. brevis</em></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**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.
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 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 commensal 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 antioxidative 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).

Acknowledgements: This study was partially supported by a grant no. 2PO5A 094 29 from the Polish Ministry of Research and Higher Education.

Conflict of interests: None declared.

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