The effects of probiotics on barrier function and mucosal pouch microbiota during maintenance treatment for severe pouchitis in patients with ulcerative colitis

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SUMMARY

Background
A total of 10–15% of patients with an ileoanal pouch develop severe pouchitis necessitating long-term use of antibiotics or pouch excision. Probiotics reduce the risk of recurrence of pouchitis, but mechanisms behind these effects are not fully understood.

Aim
To examine mucosal barrier function in pouchitis, before and after probiotic supplementation and to assess composition of mucosal pouch microbiota.

Methods
Sixteen patients with severe pouchitis underwent endoscopy with biopsies of the pouch on three occasions: during active pouchitis; clinical remission by 4 weeks of antibiotics; after 8 weeks of subsequent probiotic supplementation (Ecologic 825, Winclove, Amsterdam, the Netherlands). Thirteen individuals with a healthy ileoanal pouch were sampled once as controls. Ussing chambers were used to assess transmucosal passage of *Escherichia coli* K12, permeability to horseradish peroxidase (HRP) and 51Cr-EDTA. Composition and diversity of the microbiota was analysed using Human Intestinal Tract Chip.

Results
Pouchitis Disease Activity Index (PDAI) was significantly improved after antibiotic and probiotic supplementation. *Escherichia coli* K12 passage during active pouchitis [3.7 (3.4–8.5); median (IQR) was significantly higher than in controls [1.7 (1.0–2.4); P < 0.01], did not change after antibiotic treatment [5.0 (3.3–7.1); P = ns], but was significantly reduced after subsequent probiotic supplementation [2.2 (1.7–3.3); P < 0.05]. No significant effects of antibiotics or probiotics were observed on composition of mucosal pouch microbiota; however, *E. coli* passage correlated with bacterial diversity (r = −0.40; P = 0.018). Microbial groups belonging to Bacteroidetes and Clostridium clusters IX, XI and XIVa were associated with healthy pouches.

Conclusions
Probiotics restored the mucosal barrier to *E. coli* and HRP in patients with pouchitis, a feasible factor in prevention of recurrence during maintenance treatment. Restored barrier function did not translate into significant changes in mucosal microbiota composition, but bacterial diversity correlated with barrier function.

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INTRODUCTION

Ileal reservoirs [ileal pouch-anal anastomosis (IPAA)] have become the surgical treatment of choice for reconstruction of bowel continuity in refractory ulcerative colitis (UC) requiring proctocolectomy. Although IPAA improves quality of life for patients with UC, pouchitis, a nonspecific inflammation of the pouch, remains the most common post-operative long-term complication. As many as 46% of patients with UC have at least one episode of pouchitis within 5 years after surgery, and approximately 10–15% of the patients develop severe, chronic pouchitis that necessitates frequent use of antibiotics or even pouch excision.

Inflammation of the pouch is characterized by increased stool frequency, rectal bleeding, abdominal cramping, urgency and fever. The pathogenesis of pouchitis is still poorly understood and may result from several causes such as ischaemia, altered mucosal metabolism, bile acid cytotoxicity, recurrence of UC and genetic susceptibility. Allelic variations in genes encoding for IL-1 receptor antagonist (IL-1 RN), TNF and NOD2 have all been associated with an increased risk of developing pouchitis in UC patients. These factors are in various ways related to regulation of epithelial physiology and defence, thus suggesting a pathophysiological role for mucosal barrier function in pouchitis. In addition, there is evidence that implicates gut microbiota. First of all, antimicrobial therapy with antibiotics is an effective treatment. Secondly, several studies have reported a perturbation of the microbiota in pouchitis patients, using conventional culture-based methods, culture-independent approaches and high-throughput technologies.

Probiotics, defined as 'live microorganisms that when administered in adequate amounts exert a health benefit on the host', have been used extensively for prevention and treatment of various intestinal disorders, including maintenance of remission in UC. Furthermore, probiotics have been used successfully for the prophylaxis of pouchitis in newly constructed reservoirs for maintenance of remission in chronic pouchitis patients after induction treatment with antibiotics and in some studies for induction of remission.

The mechanisms by which probiotics exert their effect are highly complex and largely unknown, but several of these are directly or indirectly related to intestinal barrier function. In animal studies, the probiotic combinations VSL#3 and Ecologic 641 have been shown to prevent increased intestinal permeability to macromolecules in colitis and acute pancreatitis, respectively, by mechanisms involving stabilisation of the cytoskeleton and tight junction proteins. Moreover, probiotic supplementation decreased the permeability-related protein, zonulin, in faeces in healthy sportsmen.

We hypothesise that probiotic supplementation restores the mucosal barrier and thereby might reduce the risk of inflammation. Therefore, the aims of this study were to determine the effects of probiotic supplementation (Ecologic 825) on barrier function and mucosal pouch microbiota composition during maintenance treatment for severe pouchitis. Best evidence for positive effects of probiotics in pouchitis is in maintenance treatment following induction with antibiotics; we therefore chose to study mucosal barrier function before and after 4 weeks antibiotic + 8 weeks probiotic supplementation.

MATERIALS AND METHODS

Subjects and ethics

Sixteen UC patients (nine men and seven women, median age 48 years, range 32–71) with an IPAA with active pouchitis comprised the study group. Consecutive patients were recruited from the outpatient clinics of the Department of Surgery and the Department of Medical Gastroenterology at Linköping University Hospital. The study design was approved by the Regional Ethics Committee, Linköping, and informed consent was obtained from all patients included in the study.

Inclusion and exclusion criteria. Inclusion criteria: UC patients with an IPAA; clinically active pouchitis and a history of severe chronic pouchitis, defined as needing continuous antibiotic treatment or having had at least three relapses per year during the last 2 years.

Exclusion criteria: Crohn’s disease; use of steroids, azathioprine or other immunomodulatory or immunosuppressive drugs; allergy to antibiotics; coagulation disorders; previous surgery with implantation of foreign material.

Study group and control groups. Study group subjects underwent endoscopy with biopsies of the pouch on three occasions (see Figure 1): during active pouchitis, after 4 weeks of treatment with antibiotics, and after 8 weeks of oral supplementation of a multispecies probiotic product.
(Ecologic 825; Winclow Probiotics, Amsterdam, the Netherlands) as described below. Two of the patients in the study group were on continuous treatment of antibiotics and entered the study at the second endoscopic occasion.

Thirteen patients (12 men and 1 woman, median age 50 years, range 35–63), with UC who had had an IPAA for at least 2 years and with no clinical signs of pouchitis were assessed as a control for background reference. These patients underwent endoscopy with biopsies of the pouch on one occasion.

As a supplemental control group, three patients (two men, one woman, median age 47 years, range 37–53) with UC and active pouchitis at inclusion were kept on antibiotics for the whole 12-week study period and biopsies were taken as for the main study group.

Ecologic 825
The multispecies probiotics consisted of nine viable, freeze-dried probiotic strains: *Bifidobacterium bifidum* (W23), *B. lactis* (W51), *B. lactis* (W52), *Lactobacillus acidophilus* (W22), *L. casei* (W56), *L. paracasei* (W20), *L. plantarum* (W62), *L. salivarius* (W24) and *Lactococcus lactis* (W19). A single probiotic dose of 3 g [containing $2.5 \times 10^9$ colony-forming units (CFU) of bacteria per gram] was mixed in a glass of water and ingested twice daily.

Pouchitis Disease Activity Index
First presented by Sandborn *et al.*, Mayo Clinic, USA, the Pouchitis Disease Activity Index (PDAI) is an 18-point diagnostic instrument consisting of three principal component scores: symptom, endoscopy and histology. Patients with PDAI scores of 7 or more were diagnosed as having pouchitis.

Endoscopy
Bowel preparation was conducted the day before with 1 L of the orally given laxative Laxabon (BioPhausia AB, Stockholm, Sweden). After laxative treatment, only clear liquids were allowed until exam. Endoscopy of the pouch was conducted in an out-patient unit at the University Hospital, Linköping, Sweden. Eight to ten biopsies for research purposes were taken at the level of the pouch corpus. All biopsies were placed into 4 °C modified Krebs-Ringer bicarbonate buffer (KRB) and transported to the laboratory within 20 min. One of the tissue samples was sent for routine clinical histological assessment and later used for histological scoring according to PDAI.

Ussing chamber experiments
Pouch biopsies were mounted in modified Ussing chambers (exposed tissue area 1.76 mm$^2$; Harvard Apparatus, Holliston, MA, USA) as previously described and validated. Mucosal compartments were filled with 1.5 mL, 10 mM mannitol in KRB and the serosal compartment with 10 mM glucose in KRB. The KRB was pH adjusted to 7.4 at 37 °C, continuously oxygenated with O$_2$/CO$_2$ (95/5%) and stirred by gas flow in the chambers.

Experiments were performed in open-circuit conditions with assessment of potential difference (PD);
transmucosal electrical resistance (TER) and short-circuit current (Isc) were noted at the start of the experiment and at 2-min intervals thereafter, using a four-electrode system: Ag/Ag-electrodes (Ref 201; Radiometer, Copenhagen, Denmark) with 3 M NaCl/2% agar bridges for PD, and platinum electrodes for current as previously described.\(^4^2\) Samples of 0.3 mL from the serosal side were collected after 0, 10, 30, 60, 90 and 120 min. Previously, we reported that human intestinal biopsies have good viability in Ussing chambers, and are a validated technique to study transcellular uptake of protein antigens and paracellular permeability to marker molecules.\(^3^5\), \(^4^3\)

Transmucosal transport of macromolecules was assessed by measuring the transport of horseradish peroxidase as transcellular probe, and \(^{51}\)Cr-EDTA (3.25 μM; Perkin Elmer, Boston, MA, USA) as paracellular probe. The transcellular probe, Horseradish Peroxidase type VI (HRP mw 45 kDa, 10 μM, Sigma Chemical Co. St. Louis, MO, USA) was added on the mucosal side. Serosal samples were analysed using the QuantaBlu Fluorgenic Peroxidase Substrate Kit (Pierce, Rockford, IL, USA) as paracellular probe. The paracellular probe, \(^{51}\)Cr-EDTA, was added on the mucosal side and permeation measured by appearance of radioactivity in the 0.3 mL serosal samples [counted for 10 min in a 1282 Compugamma reader (LKB, Bromma Sweden)].

Bacterial passage was assessed by adding *Escherichia coli* K12, a chemically killed fluorescein-conjugated strain of *E. coli* (molecular Probes, Leiden, the Netherlands) to the mucosal compartments of the Ussing chamber (WinClove BV, Amsterdam, the Netherlands) at a final concentration of \(1 \times 10^8\) CFU/mL. At start, after 60 min and after 120 min, the entire volume of the serosal compartment was collected and analysed at 488 nm in a fluorimeter (Cary Eclipse; Varian, Victoria, Australia). One unit corresponds to \(3 \times 10^6\) CFU/mL.

**Microbiota analysis**

Pouch biopsies from eight patients within the study group (with a complete set of three samples per patient), and from 13 controls were selected for analysis of the microbiota composition using the Human Intestinal Tract Chip (HITChip) at Wageningen University in the Netherlands. The HITChip is a phylogenetic microarray containing over 4800 probes based on 16S rRNA gene sequences of over 1100 intestinal bacterial phylotypes, which are grouped into 131 genus-like taxa defined on the basis of 16S rRNA gene sequence similarity and representing all major intestinal phyla described for the human intestinal microbiota.\(^4^4\) Total DNA was extracted from pouch biopsies using the repeated bead beating method as previously described.\(^4^5\) Following, 16S rRNA genes were amplified, *in vitro* transcribed, labelled with Cy3 and Cy5 dyes and hybridised to the microarray. Hybridisations were performed in duplicate, and after washing and scanning of the microarray, data were extracted using the Agilent Feature Extraction Software version 10.7.3.1 (http://www.agilent.com). Analysis of the microarray was performed using a series of custom-made R scripts as previously described\(^4^4\) in combination with a custom-designed database, which runs under the MySQL database management system. Duplicate hybridisations with a Pearson correlation over 0.98 were selected for further analysis. Ward’s minimum variance method was used for the generation of hierarchical clustering of the total oligonucleotide hybridisation profiles by calculating a distance matrix between samples. To assess bacterial diversity, the Shannon diversity index was calculated based on the total oligonucleotide hybridisation profiles.

**Statistics**

Values are given as median (25–75th interquartile range) if not otherwise indicated. In Ussing chamber experiments, the \(n\) value represents the number of patients in study group and control group, with a mean value for each subject calculated from two to four biopsies for each treatment. Comparisons between groups were done using the Kruskal–Wallis one-way analysis of variance and Dunn’s multiple-comparison test. Differences were considered significant if \(P < 0.05\). Analysis was carried out using GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA).

To determine significant changes in microbiota composition between the study groups of individual genus-level groups, the Wilcoxon signed-rank test was used corrected for multiple comparisons with the Benjamini & Hochberg method. For comprehensive multivariate statistical analyses, Canoco software for Windows, version 5.00 was used.\(^4^6\) To assess correlation of specific microbial groups detected by HITChip with health status or treatment, redundancy analysis was used. The log-transformed hybridisation signals of the 131 genus-level groups were used as biological variables. As environmental variables health, inflammation, antibiotic treatment and probiotic supplementation were included. The Monte Carlo Permutation procedure, as implemented in the Canoco package, was used to assess statistical significance of the variation in data sets in relation to the environmental variables.
RESULTS

PDAI was improved after antibiotic treatment and probiotic supplementation

In total, 16 pouchitis patients were included in the treatment group and PDAI scores were collected at three time points: during active pouchitis, after antibiotic treatment and after probiotic supplementation (Figure 2). The PDAI scores of the pouchitis patients before treatment [0 (7.3–11.0)] were significantly reduced after antibiotic treatment [3.0 (2.0–5.5)] and there was no further change after probiotic supplementation [2.0 (1.0–5.0); PDAI score in the study group after antibiotic treatment compared to after probiotic supplementation (P = 0.40)]. The control group of 13 IPAA patients with no history of pouchitis had a PDAI of 1.0 (0.0–2.0), which was significantly lower than the study group after both treatments.

Active pouchitis was associated with an increased permeation of E. coli K12, which was normalised by probiotics

To assess the barrier function in the different study groups, mucosal permeability of the pouch was examined by mounting pouch biopsies in Ussing chambers. To explore the effects of mucosal inflammation on bacterial passage, permeation of E. coli K12 was quantified and presented in units (1 unit = 1.5 × 10^3 CFU/mL) (shown in Figure 3a). After 120 min, there was a significant difference in E. coli K12 passage of the inflamed pouches compared with healthy pouches [3.7 (3.4–8.5) vs. 1.7 (1.0–2.4), respectively, P < 0.01]. Antibiotic treatment did not result in restoration of the mucosal permeability, as there was no difference in E. coli K12 passage in the inflamed pouches compared with the pouches after antibiotic treatment [3.7 (3.4–8.5) vs. 5.0 (3.3–7.1) respectively]. Probiotic supplementation, however, was able to restore the mucosal permeability, as there was a significant decrease in bacterial passage after probiotic supplementation compared with the pouches after antibiotic treatment [2.2 (1.7–3.3) vs 5.0 (3.3–7.1), respectively, P < 0.05]. Probiotic supplementation was even able to normalise the mucosal permeability to the same level as the healthy pouches, as there was no difference in bacterial passage of the pouches after probiotic treatment compared with the pouches of healthy individuals [2.2 (1.8–3.3) vs 1.7 (1.0–2.4) respectively].

Escherichia coli passage in the three patients that took antibiotics for 12 weeks was 5.5 (3.8–10.5) U in active pouchitis, 2.9 (1.8–10.3) after 4 weeks antibiotic treatment and 3.3 (2.2–4.6) after 12 weeks of antibiotics.

The total PDAI score and the histological part of the PDAI score was compared with the values for E. coli passage in a linear regression analysis. We found no significant correlation in the different study groups (data not shown).

Increased flux of HRP in pouchitis was normalised by probiotics

In addition to bacterial passage, mucosal permeability was assessed by measuring the mucosal permeability to HRP during a 30–90 min period (Figure 3b). There was an increased permeation of HRP in the study group during active pouchitis [2.6 (1.6–5.1)] and after antibiotic treatment [2.3 (1.5–3.1)]. However, a significant normalisation of HRP flux was seen after the use of probiotics [0.6 (0.5–1.3)], which was to the same level as the healthy pouches, as there was no difference in HRP flux of the pouches after probiotic treatment compared with individuals with healthy pouches [0.6 (0.5–1.3) vs. 1.4 (1.2–1.9) respectively].

Pouchitis did not affect paracellular permeability

The paracellular probe ^51^CrEDTA was measured in the Ussing chamber experiment during 0- to 120-min time.
period \((\text{cm/s} \times 10^{-6})\). There were no significant differences between the study groups as shown in Table 1. Furthermore, there were no significant differences found in electrophysiology addressing the transepithelial resistance (TER) or short-circuit current (Isc) at start = 0 min.

**Probiotic supplementation did not influence mucosal pouch microbiota composition**

Pouch biopsies from eight individuals within the treatment group, from whom a complete set of three subsequent samples was available, and from the 13 individual in the healthy control group were selected for analysis of the microbiota composition using a comprehensive and highly reproducible phylogenetic array, the HITChip. HITChip analysis was done to determine possible differences in mucosal microbiota composition of inflamed pouches in comparison with healthy pouches. In addition, the effect of antibiotic treatment and probiotic supplementation on mucosal microbiota composition was assessed. For HITChip analyses, total DNA was extracted from all pouch biopsies and hybridised in duplicate onto HITChip arrays.

To address the differences between the different study groups, the bacterial diversity was determined per sample

**Table 1 | \(^{51}\text{CrEDTA} \) passage and electrophysiology**

<table>
<thead>
<tr>
<th>Study group</th>
<th>(^{51}\text{CrEDTA} ) ((\text{cm/s} \times 10^{-6}))</th>
<th>TER ((\Omega \times \text{cm}^2))</th>
<th>Isc ((\mu\text{A/cm}^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active pouchitis</td>
<td>2.9 (2.6–3.5)</td>
<td>35.0 (31.8–45.5)</td>
<td>23.3 (17.9–35.5)</td>
</tr>
<tr>
<td>After antibiotic treatment</td>
<td>3.2 (2.1–4.4)</td>
<td>31.8 (29.9–38.3)</td>
<td>23.2 (19.0–36.1)</td>
</tr>
<tr>
<td>After probiotic supplementation</td>
<td>2.8 (2.2–3.7)</td>
<td>35.9 (31.0–40.8)</td>
<td>30.8 (25.8–117.8)</td>
</tr>
<tr>
<td>Healthy pouch</td>
<td>3.1 (2.5–4.1)</td>
<td>34.4 (30.1–39.2)</td>
<td>28.1 (16.6–37.5)</td>
</tr>
</tbody>
</table>

No significant differences in permeation of \(^{51}\text{CrEDTA} \), transepithelial resistance (TER) or short-circuit current (Isc). Values are presented as median (25–75\text{th interquartile range}) and comparisons between groups were done using Kruskal–Wallis test.

**Figure 3 | Mucosal permeability in the different study groups.** Pouch biopsies were mounted in Ussing chambers and mucosal permeability was measured. (a) Permeation of chemically killed *Escherichia coli* K12 during 120 min after challenge (1 unit = \(1.5 \times 10^3\) CFU/mL). (b) HRP flux during 30- to 90-min time interval (pmol/cm\(^2\)/h). Values are presented as median (25–75\text{th interquartile range}). Inset: Individual, paired data points for (a) *E. coli* and (b) HRP flux in pouchitis patients with full technical success of experiments at all three time points. Act, active pouchitis; Ab, after antibiotics; Pro, after probiotics. Comparisons between groups were done using Kruskal–Wallis test and Dunn’s multiple-comparison test was used for calculation of significance: *P < 0.05 and **P < 0.01.
based on profiles of the intensity values for each HITChip probe (Figure 4). No significant differences were found in bacterial diversity of the mucosal pouch microbiota between the four different study groups.

To further study the potential influence of bacterial diversity on mucosal barrier function, bacterial diversity index was correlated with E. coli passage. There was a significant correlation between bacterial diversity and E. coli passage with \( r = -0.40; \ r^2 = -0.158; \ P = 0.018 \) (Figure 5).

The microbiota composition of the pouch biopsy samples was studied in more depth by averaging the final intensity values for each HITChip probe into 131 genus-level groups. However, for none of these genus-level groups, there was a significant difference in abundance between the inflamed and healthy. In addition, there was no significant effect of the antibiotic treatment or probiotic supplementation on the average abundance of each of these 131 genus-level groups. To look in even more detail at the differences in microbiota composition between the four study groups, a multivariate cluster analysis was done. In Figure 6, a redundancy analysis is depicted, which shows the pouch biopsy sample distribution according to microbiota composition and to the different environmental variables used. Even though it seems that the healthy pouch samples separate clearly from the other pouch samples in space, none of the variables used in the analysis (health, inflammation, antibiotic treatment or probiotic supplementation) revealed to be significant in sample separation. Nevertheless, genus-level groups belonging to Bacteroidetes and Clostridium clusters IX, XI and XIVa seem to be positively associated with healthy pouches. The shift in clustering of the samples after antibiotic treatment was mainly associated with changes in abundance of bacterial groups belonging to the Proteobacteria and Bacilli and negatively associated with members of Clostridium clusters XIVa. However, after probiotic treatment, the variation in microbiota composition of the previously inflamed pouches seemed to return to its original state before antibiotic treatment.

**DISCUSSION**

Multispecies probiotics have shown efficacy as maintenance treatment in pouchitis following IPAA surgery for UC. Therefore, it is now a recommended treatment for this condition in the guidelines from the European Crohn’s and Colitis Organization. The mechanisms of action are, however, to a large extent unknown. Although pouchitis may result from several causes, unveiling the mechanisms of how probiotics exert their effect may constitute the key of understanding not only the pathogenesis of pouchitis but also give us an insight in the mechanisms behind barrier dysfunction in intestinal inflammation.
Our study reveals increased permeation of *E. coli* in patients with active pouchitis, but unaltered permeability to 51Cr-EDTA. These results are well in line with earlier studies showing increased bacterial uptake despite normal paracellular permeability in IPAA. Treatment with antibiotics led to clinical remission and a normalised PDAI score, but did not affect the increased passage of *E. coli*. Our novel findings do, however, show that probiotics for 8 weeks normalised the bacterial passage of the mucosa in the pouchitis group to levels comparable to healthy pouches. On the other hand, our data, including the three patients on continued antibiotics for 12 weeks, do not rule out the possibility of a time aspect, with the improved bacterial translocation being part of the healing of mucosal inflammation rather than a direct consequence of the probiotic therapy. This makes further studies warranted, with larger patient groups randomised to probiotic supplementation or prolonged antibiotic therapy.

Addressing the question why some of the IPAA patients are more prone to develop pouchitis than others, one can argue that some of the IPAA patients and especially those with UC have a 'leaky gut' even before surgery. However, Keita *et al.* showed no increase in transcellular-, paracellular permeability or increased bacterial passage in human ileal samples from patients with active pouchitis. But untreated patients were included in that study, and we have confirmed increased luminal concentration of *E. coli* in active pouchitis, and increased bacterial passage in patients with UC. This was confirmed in a separate study at this institution.2, 48

UC compared with controls. This would support the notion that the increased permeability is acquired as a consequence of ileal pouch transformation. Although we have little knowledge about the mechanisms behind this colonic shift, terminal ileum, a high-flow organ is created into a neorectum and exposed to new conditions such as faecal stasis. It is fair to assume that this might influence changes in barrier function. In this study, we see a covariance between the increased bacterial passage and increased HRP flux again suggesting a transcellular mechanism of transport. This is further supported by our results showing that the inflammation of the pouch does not affect the permeability of the paracellular probe or the transepithelial resistance indicating an intact function of the tight junction complex.

Interestingly, previous studies have shown a 10-fold increased risk of developing pouchitis in patients with UC compared with FAP patients. The difference in the incidence of pouchitis might be found in the composition of the microbiota in the pouch in different individuals. In one study, Duffy et al. showed that 80% of pouch samples from patients with UC contained sulphate-reducing bacteria compared with none of the FAP samples. In a recent study, Zella et al. were able to demonstrate that the mucosal and luminal pouch microbiota of patients with UC-associated pouchitis is significantly different from that of healthy FAP or UC patients. They observed a decrease in the phyla Bacteroidetes and Proteobacteria in inflamed pouches, in addition to an increase in Firmicutes and Verrucomicrobia. Bacterial groups belonging to these phyla are generally thought to play a role in human health. In this study, however, close examination of mucosal pouch microbiota revealed that the microbial composition of healthy pouches was not significantly different from that of inflamed pouches. There was, however, a slightly, but nonsignificantly, higher bacterial diversity in the control group, and there was a significant correlation between degree of bacterial diversity and E. coli passage across the pouch mucosa. Moreover, based on multivariate analysis, variation in bacterial groups belonging to the Bacteroidetes and Clostridium clusters IX, XI and XIVa do appear to differentiate the healthy pouches from the inflamed pouches. Taken together, these findings suggest an important role of the microbiota in determining mucosal barrier function in ileal pouches.

Antibiotic treatment and probiotic supplementation did not have a profound impact on the microbiota composition. Antibiotic treatment had a small effect on the variation in the mucosal pouch microbiota composition between the groups, mainly correlated with changes in abundance of bacterial groups belonging to the Proteobacteria and Bacilli. However, after probiotic supplementation, the microbiota seems to have returned to its original state.

To supplement our results, future studies are needed, focusing on the ability of probiotic preparations as well as long-term antibiotic therapy to change luminal and mucosal bacterial microbiota over time and linked to the incidence of pouchitis. The variation in abundances of the bacterial groups within the four different groups was very high, which made it difficult to assess significant differences between groups with such a small sample sizes. Therefore, future studies should include larger study populations to overcome the inter-individual variation in microbiota composition. In addition, for future studies, it would be interesting to study the luminal pouch microbiota and the microbiota in the proximal portion of the small bowel as well, to assess the effects on probiotic intervention on pouch microbiota composition in more detail. Furthermore, it has been reported that a large proportion of the stool microbiota of UC patients with chronic or relapsing pouchitis is formed by bacterial groups that are normally not found in human faeces, such as members of the Caulobacteraceae, Sphingomonadaceae and Comamonadaceae. As the HITChip is specifically designed for bacterial groups found in the human intestinal tract, it might be possible that we have missed specific bacterial groups that are possibly linked to the pathogenesis of pouchitis.

Our study group consists of selected patients with a history of severe pouchitis. In our opinion, they reflect a common distribution in terms of age and gender of patients with pouchitis. Unfortunately not the same is seen in the group of healthy pouches where male gender is significantly dominant. However, there is nothing overt that would indicate a gender difference in barrier function in IPAA patients. A drawback of the study is the lack of an additional control group receiving prolonged antibiotic treatment for 12 weeks, instead of probiotic supplementation to assess the bacterial passage and microbiota composition further. As a supplement, we analysed biopsies from three patients with prolonged antibiotic treatment. They followed a similar pattern as the study group, although the bacterial passage was not fully normalised. Unfortunately, the subsequent recruitment of subjects to this group was severely hampered as almost every IPAA patient nowadays use some kind of probiotic supplementation regularly.

The use of the PDAI score proved to be strongly related to clinical activity of pouchitis. However, we
found no correlations among PDAI score, histological appearance and *E. coli* passage suggesting other mechanisms involved, for example, the diversity of the microbiota.

As described earlier, different probiotic strains have been shown to have several but different favourable effects on gut mucosa, mainly through enhancement of barrier function.\(^5\) Several studies imply further that combinations of different strains of probiotic bacteria are more effective than single-strain preparations.\(^52-54\) However, little is known about the optimal mixture in the treatment of different intestinal disorders.

Even though animal studies have shown promising results using probiotics in acute inflammatory disorders, the outcomes in human studies with similar settings have not been equally successful. The scientific support for the use of probiotics as prophylaxis and maintenance treatment in humans stands more solid. For instance, previous studies have associated probiotics with a reduction in infectious complications in patients undergoing elective abdominal surgery,\(^55, 56\) and maintenance treatment of UC.\(^26\) Further knowledge is needed to confirm in what manner probiotics should be used in the prevention and/or maintenance treatment of various abdominal conditions.

In conclusion, maintenance treatment with a probiotic mixture for 8 weeks after induction treatment with antibiotics restored the increased permeation to *E. coli* in patients with chronic pouchitis. This could be an important factor behind the prevention of recurrence during maintenance treatment with probiotics for this inflammatory condition.

**AUTHORSHIP**

Guarantor of the article: JD Söderholm.

**Author contributions:** MP contributed to research design, acquisition and interpretation of data in the work of Ussing chamber experiments, and drafted the manuscript. JG contributed to study design and acquisition and interpretation of data in the analysis of mucosal microbiota composition, and co-drafted the manuscript. CW contributed to study design and revised the manuscript. AC contributed to data acquisition and revised the manuscript. JG is currently an employee of Winclove Probiotics.

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