Microbiological evaluation of jejunal aspirates and faecal samples after oral administration of bifidobacteria and lactic acid bacteria

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O.H. NIELSEN, S. JORGENSEN, K. PEDERSEN AND T. JUSTESEN. 1994. A double-blind placebo controlled investigation was carried out to study the effect of peroral colonization. Human volunteers were given mixtures of bifidobacteria and lactic acid bacteria. Measurements were made over a 1 week treatment period and for another week after the end of the treatment. Two different bacteriological preparations were used, one consisted of Enterococcus faecium and Bifidobacterium longum (a total of $6.4 \times 10^8$ cfu d⁻¹); the other consisted of Lactobacillus acidophilus, Bifidum, Lact. delbrueckii ssp. bulgaricus, and Streptococcus thermophilus (a total of $9 \times 10^9$ cfu d⁻¹). Together with a placebo preparation, they were given to 24 healthy controls (eight in each group). Microbiological examinations of jejunal aspirates showed that viable counts of most species were below the detection limit. However, the test preparation containing Ent. faecium and Bif. longum significantly reduced the anaerobe : aerobe ratio in faeces by a factor of three during treatment ($P = 0.03$), and increased it by a factor of 30 during the following week ($P < 0.02$). This study shows that peroral administration of certain bacterial cultures may affect the distal intestinal microflora.

INTRODUCTION

The normal intestinal flora is the largest bacterial reservoir in humans. The indigenous flora of the intestinal tract is composed of 400–500 different species of aerobic and anaerobic micro-organisms in a complex ecosystem. Faeces contain approximately $10^{10}$–$10^{11}$ cultivable bacteria per gram, with a normal anaerobe to aerobe ratio ranging between 1000 and 100 : 1 (Abrams and Bishop 1966; Savage 1977). Although the activities of the normal gut flora are not fully understood, there is evidence that alterations in its composition may result in lowered colonization resistance, and consequently in the promotion of pathogenic organisms (Fekety et al. 1980; Wiegertsma 1982). This may lead to serious secondary infections, especially in immunocompromised hosts. Furthermore, intestinal bacteria may play a role in the host’s acquisition of certain vitamins. Bleeding problems and symptoms of vitamin K deficiency associated with alterations of the gut flora have been reported by Finegold (1986).

The maintenance of a normal gut flora is therefore essential for the well-being of the host. Many factors may influence the gut ecosystem, including general state of health, diet, geographic location, pathologic conditions, surgery of the gastrointestinal tract and drug ingestion, especially during antimicrobial therapy.

Certain organic substances, such as acetic and lactic acids, are thought to inhibit proliferation of pathogenic micro-organisms (Rasic 1983). Both acids are produced by bifidobacteria and other members of the anaerobic flora, while lactic acid is a major product of lactic acid bacteria. Consequently, it has often been claimed that these organisms have a protective role in the gut (Rasic 1983). Accordingly, supplementation with microbial preparations containing lactic acid bacteria and bifidobacteria has often been used to stabilize the microflora of the gut, thereby treating or preventing various intestinal disorders (Gorbach et al. 1967; Colombel et al. 1987; Lidbeck et al. 1988; Sistonen et al. 1990; Black et al. 1991).

The double-blind placebo-controlled investigation reported was carried out to study the effect on the gut flora of feeding two different commercially-available preparations of lactic acid bacteria and bifidobacteria to normal
healthy volunteers, as judged from microbiological examination of jejunal aspirates and faecal samples.

**MATERIALS AND METHODS**

**Volunteers**

Twenty-four healthy volunteers (16 females, eight males), 19–59 years, were allocated to three study groups consisting of eight persons each, during a 2 month period (Table 1).

**Test cultures**

Two different commercially-available bacterial preparations were used: Group A, Idoform (Ferrosan A/S, Denmark) enteric-coated capsules containing lyophilized cultures of *Ent. faecium* and *Bif. longum*, contains ca 1·6 × 10⁸ colony forming units (cfu) per capsule (a total of 6·4 × 10⁸ d⁻¹; Group B, Trevis (Ferrosan A/S, Denmark) capsules containing lyophilized cultures of *Lact. acidophilus*, *Bif. bifidum*, *Lact. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, containing ca 3 × 10⁹ cfu per capsule (a total of 9 × 10⁹ d⁻¹).

**Experimental design**

Volunteers received 'double dummy capsules' from day 1 to day 7 in a dose of two capsules three times each day to mask the actual daily doses of Group A, two capsules twice each day, and in Group B, one capsule three times each day. Group C received placebo capsules containing glucose. After day 7 the treatment was discontinued.

None of the participating subjects received medication (except contraception), nor had they taken antibiotics for 3 months prior to the study. None had previously been subject to gastrointestinal surgery except appendectomy, and none had travelled outside the geographic area covering Europe within 6 months before study entrance. Furthermore, none of the volunteers received fermented milk products for 3 d before or during the investigation.

The volunteers were examined three times during the study on days 1 (before first dosage), 7 (within 3 h after administration of last dosage), and 15 at the out-patient clinic. Jejunal fluid aspiration was made by nasal intubation after 3 h fasting, as described by Rasmussen et al. (1983). The true position of the tube tip in relation to the intended position at the ligament of Treitz was verified by fluoroscopy. Faecal specimens (each of approximately 10 g) were collected and stored by the volunteers in sterile airtight containers at +4°C within 4 h before they were handed over to the out-patient clinic when they were immediately frozen to −80°C.

Jejunal aspirates were placed in sterile vials (2–3 ml), immediately flushed with N₂, hermetically sealed and subsequently stored together with the faecal samples at −80°C until further laboratory processing.

**Bacteriological examinations**

Bacteriological examinations were done by earlier published methods (Axelson and Justesen 1977; Pedersen and Tannock 1989; Pedersen and Jørgensen 1992). Faecal samples were transferred directly from −80°C to an anaerobic glove box with an atmosphere of 10% H₂, 10% CO₂ and 80% N₂ (Don Whitley Scientific Limited, Shipley, UK). Prereduced phosphate buffered peptone water (pH 7-2) in glass tubes containing 4·5 ml each were used to make serial 10-fold dilutions with 0·5 g of each sample. One set of prereduced anaerobic agar plates (chocolate agar supplemented with cysteine (0·05%) and vitamin K (0·0001%), Statens Serum Institute, Copenhagen) and one set of 5% blood agar plates were inoculated with 0·5 ml from each of the dilution tubes. The anaerobic agar plates were incubated in the glove box at 35°C under anaerobic conditions for 5 d. The blood agar plates were removed from the glove box and incubated aerobically at 35°C for 5 d.

Colonies were counted by choosing plates with 30–300 colonies, and expressing the results as cfu ml⁻¹. In all cases, the anaerobic counts exceeded the aerobic counts by at least one dilution step, allowing calculation of ratios between anaerobic and aerobic bacterial counts for each sample.

Jejunal aspirates were processed in the same way as faecal samples, except that colonies from the anaerobic plates were subsequently examined for their ability to grow aerobically.

In an additional experiment jejunal aspirates and faecal samples were suspended in peptone water as 10-fold dilutions (w/v) and further diluted in 10-fold steps before plating on agar media. Total intestinal lactobacilli were counted on Rogosa SL agar (Difco) incubated anaerobically (GasPak jars; BBL) at 37°C for 48 h. Lactobacilli were distinguished from bifidobacteria by colony and cell morphology. However, *Lactobacillus delbrueckii* ssp. *bulgaricus* does not grow on this Rogosa SL agar, but grows on Lee agar (Lee et al. 1974). This is a non-selective medium that

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**Table 1** The healthy volunteers in the three treatment groups (A, B, C)

<table>
<thead>
<tr>
<th>Group</th>
<th>Female/male</th>
<th>Age (medians and ranges)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>5/3</td>
<td>30 (23–59)</td>
</tr>
<tr>
<td>B</td>
<td>6/2</td>
<td>30 (19–43)</td>
</tr>
<tr>
<td>C</td>
<td>5/3</td>
<td>36 (23–41)</td>
</tr>
</tbody>
</table>
also allows the growth of several other bacteria. Lee agar plates were incubated anaerobically at 49°C for 48 h. These conditions prevented the growth of most other bacteria except certain enterococci, lactobacilli, *Bacillus* spp. and bifidobacteria. Total streptococci and enterococci were counted on *Mitis Salivarius* agar (Difco), containing nalidixic acid (15 μg ml⁻¹) instead of tellurite. Plates were incubated aerobically at 37°C for 48 h. Bifidobacteria were enumerated on a selective medium described by Beerens (1990). These plates were incubated anaerobically at 37°C for 72 h. Thus, *Lact. acidophilus* grew on Rogosa SL agar, *Bif. longum* and *Bif. bifidum* on Beerens’s medium, *Lact. delbrueckii* spp. bulgaricus, *Strep. thermophilus* and *Ent. faecium* on Lee agar, and *Ent. faecium* on *Mitis Salivarius* agar. Finally, *Escherichia coli* was counted on MacConkey agar plates (Difco) incubated aerobically at 37°C for 24 h.

**Ethics**

Informed consent after written and oral information was obtained in all cases, according to the second Helsinki Declaration. The investigation was approved by the Scientific Ethical Committee of Copenhagen County, in accordance with current legislation.

**Statistics**

Non-parametric statistics (medians, ranges, 25/75 percentiles, Wilcoxon’s tests for paired observations, and the Jonckheere-Terpstra test) were applied. A significance limit of 0·05 (2α) was used.

**RESULTS**

Analyses of jejunal aspirates showed that numbers of viable bacteria in most of the samples were below the detection limit of 10⁵ cfu ml⁻¹ in all three treatment groups (Fig. 1).

Faecal samples in all groups (A, B, C) at day 1 contained a median of 8 × 10⁹ cfu g⁻¹ (range 10⁸–7 × 10¹⁰) strictly anaerobic bacteria, and 6 × 10⁷ cfu g⁻¹ (range 10⁶–8 × 10⁹) aerobic species. No differences were observed between treatment groups in comparison with the total number of anaerobic or aerobic bacteria at day 1 (P > 0·05, Jonckheere-Terpstra test), making them eligible for further comparative analysis. Figure 2 shows the total number of anaerobic and aerobic cfu g⁻¹ of faeces at the start, after 1 week of treatment, and after another week without further treatment.

However, when the ratio between strict anaerobes and aerobic bacteria within each of the three treatment groups were compared on non-selective media, a statistically significant change was observed in treatment Group A, where the anaerobe : aerobe ratio diminished by a factor 3 (from a median ratio of 60 to 20) during the treatment period (day 1 to day 7) (P = 0·03, Wilcoxon’s test), whereas a 30-fold rise in this ratio (from a median ratio of 20 to 600) was observed from day 7 to day 15 (P < 0·02, Wilcoxon's test) (Table 2). No statistically significant differences in these ratios were observed from day 1 to day 15, or for treatment regimens B and C.

![Fig. 2 Number of cfu g⁻¹ of anaerobic and aerobic faecal bacteria in each of the three treatment groups (A, B, C) at start, and after 1 or 2 weeks. Median values and 25/75 percentiles are shown (n = 8 experiments in each group).](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>V₁ vs V₂</th>
<th>V₁ vs V₃</th>
<th>V₂ vs V₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0·03</td>
<td>0·45</td>
<td>0·02</td>
</tr>
<tr>
<td>B</td>
<td>0·25</td>
<td>0·38</td>
<td>0·89</td>
</tr>
<tr>
<td>C</td>
<td>0·11</td>
<td>0·21</td>
<td>0·17</td>
</tr>
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</table>

Visit 1 (start) (V₁), visit 2 (after 1 week of treatment) (V₂), and visit 3 (1 week after discontinuation of treatment) (V₃) (n = 8 experiments in each of the three treatment groups).
Faecal results from growth on five different media (MacConkey agar, mitis salivarius agar, Rogosa SL agar, bifido selective agar, and Lee agar) are shown in Fig. 3. A statistically significant increase was found in treatment group A for counts on mitis salivarius agar ($P < 0.03$, Wilcoxon's test) and Lee agar ($P < 0.03$, Wilcoxon's test) between commencing the experiment and up to one week of treatment, and a significant fall was seen in comparisons of counts at the start and day 15 on Lee agar ($P < 0.02$, Wilcoxon's test). No statistically significant differences were found in groups B or C.

**DISCUSSION**

Although lactic acid bacteria and bifidobacteria are considered to be important members of the indigenous flora of the large bowel, they are believed to exist as transients in the small bowel of humans (Gorbach *et al.* 1967).

As members of the indigenous gut flora, or when ingested as dietary adjuncts for therapeutic or prophylactic purposes, these bacteria have been ascribed various features beneficial to the host, and accordingly designated probiotics. Most reports have been anecdotal or derived from a small number of observations.

This paper describes the results of a double-blind study on two different preparations of lactic acid bacteria and bifidobacteria on the normal gut flora. Counts of bacteria in jejunal aspirates varied considerably between patients, and in most cases the numbers were below detection limits.

When growth was recorded from jejunal aspirates it was mixed, containing organisms belonging to various genera. It was therefore concluded that the ingested bacterial cultures were unable to colonize the jejunum, as a result of rapid removal from the upper small bowel. Previous experiments have shown that volunteers fed a commercial lactobacillus preparation had increased numbers of lactobacilli in jejunal aspirates within 3 h of ingestion (Robins-Browne and Levine 1981). These findings are consistent with the negative results in this study, where the patients for safety reasons (to avoid aspiration of jejunal contents) were fasted for 3 h before the intubation procedure. When the faecal flora was evaluated, a significant alteration was found in the treatment group with supplementation of *Ent. faecium* and *Bif. longum*, as judged from changes of the anaerobe : aerobe ratio, the decrease of which may be considered as a shift to a more abnormal bowel flora (Axelsson and Justesen 1977). In this group, a significantly increased number of enterococci was also detected on mitis salivarius agar after 1 week of treatment. This may be due to temporary colonization by the *Ent. faecium* strain. The ability of this strain to colonize the gut of mink and pigs and to change the enterococcal flora has previously been reported (Pedersen and Jørgensen 1992; Pedersen *et al.* 1992).

*Enterococcus faecium* is also able to grow on Lee agar, which may explain the simultaneous increase in the number of bacteria counted on this medium. No other significant results were obtained on any of the bacteriological media in any of the three groups, apart from an unexplained

![Fig. 3](image-url)
decrease in the counts on Lee agar at day 15 in group A. On both mitis salivarius and Lee agar, counts at day 7 were significantly higher than at day 1. At day 15, however, counts on both media were lower than on day 1. Whether this observation represents a rebound effect is not known.

In this study, faeces was stored in sterile airtight containers at −80°C until thawing inside the anaerobic glove box. According to Crowther (1971), this may cause a slight loss in viable counts. Without giving the exact number of faecal samples examined, Crowther (1971) found a decline in total counts from 100% to 99% after deep freezing with addition of a cryoprotectant, and to 94% without, which means a decline from e.g. 10^{10} to 9·4 × 10^9 cfu g⁻¹. In the present study all the faecal samples taken from the same patients at different times were handled in exactly the same way, so that a small loss in viability would be of the same magnitude and still make comparisons meaningful.

Lactobacillus supplementation to healthy volunteers was evaluated in a previous study with saliva (from the oropharynx) and faecal specimens. However, the daily dose was approximately 10 times higher than in our study, while the duration of treatment was identical (Lidbeck et al. 1987). These authors found no significant changes in the oropharynx flora, whereas in the faecal samples, significantly increased counts of lactobacilli on Rogosa agar plates were observed. No calculations of the total number of anaerobic and aerobic bacteria were performed. However, alterations of the faecal flora were observed until 9 d after the lactobacillus supplementation was stopped (Lidbeck et al. 1987). Thus, their study together with the present one indicates a temporary effect of peroral supplementation of bacteriological examination. 

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