THE FAECAL FLORA IN ULCERATIVE COLITIS

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In ulcerative colitis (Goligher et al., 1968; Fairburn, 1973) the processes in the bowel wall—hypersecretion, inflammation and haemorrhage—cause serious changes in the composition of the gut content, which may result in a disturbance of the bacterial flora. It is very improbable that bacteria are among the prime causes of the disease (Truelove, 1968; de Dombal, 1971), but their metabolic products, e.g., tyramine or lactic acid (Weijers and van de Kamer, 1965) might aggravate the symptoms and prevent recovery. Gorbach et al. (1968) concluded that the faecal flora of patients with ulcerative colitis was about normal, though a slight increase of coliforms was noted. Cooke (1967) and D. A. A. Mossel (personal communication) observed an increase in Streptococcus faecalis in the faecal flora of patients with ulcerative colitis. Incidental confirmation of this fact prompted the present study.

MATERIALS AND METHODS

Media. The transport medium and diluent was a solution containing yeast extract (Merck) 5 g, peptone (Oxoid) 1 g, cysteine hydrochloride 0.5 g, and NaCl 8.5 g per litre with a pH of 7.

For counting bifidobacteria and Gram-negative anaerobes, Reinforced Clostridial Agar (Oxoid) with 7.5% horse blood and 0.03% potassium ferriferrocyanide (China blue, Grüber & Co., Leipzig) was used (van der Wiel-Korstanje and Winkler, 1970). On this RCB medium bifidobacteria form raised opaque light to dark brown colonies; anaerobic Gram-negative rods form translucent light or dark blue colonies. Though several genera (Fusobacterium, Bacteroides, Spherophorus) can be differentiated, the whole group will be taken together in this paper and designated as Bacteroides, which in our material was always dominant.

Enterococci were counted on neutral red-kanamycin-milk agar (KMN), containing Tryptose (Difco) 15 g, meat extract (Oxoid) 3 g, NaCl 5 g, and agar (Davis) 18 g per litre; sterile skimmed milk 200 ml and aqueous solutions containing kanamycin 24 mg and neutral red 40 mg were added after sterilisation. This medium permits the separate counting of five varieties (or species, Jones, Sackin and Sneath, 1972) of enterococci, as confirmed by metabolic properties (table). All strains isolated on this medium reacted with anti-group D serum either after Lancefield extraction (90% of the strains) or after alcohol precipitation (Shattock, 1949).

Lactobacilli were counted on Rogosa agar, pH 5.4, and coliform bacteria on blood agar.

The production of hyaluronidase and chondroitinase was tested by the method of Smith and Willet (1968). Mucinase was detected on Brain Heart Infusion (Difco) agar with 0.28% hog gastric mucin (Koch-Light Lab. Ltd, Colnbrook, Bucks) added at 45°C before the

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plains were poured. After spot inoculation and incubation (48 h at 37°C) the plates were flooded with a solution of 1% calcium chloride, which precipitates the macromolecular mucin. Mucinase-positive strains produced clear zones around the inoculated spot.

The motility of Str. mobilis was tested on the medium of Ball and Sellers (1966). Samples of faeces were collected in quantities of about 1 g in weighed screw-capped bottles containing 19 ml of transport medium. Bifidobacterium and Bacteroides survived in this medium for at least 6 h. In practice, samples were analysed within 2 h. After homogenisation on a shaker and serial dilution in diluent, the relevant dilutions were inoculated according to Miles, Misra and Irwin (1938) on to RCB medium, Rogosa agar, KMN agar, and blood agar. The first two media were immediately incubated in anaerobic jars with 90% H2 + 10% CO2 and read after 72 h at 37°C; the other media were incubated aerobically and read after 24 and 48 h. Counts were expressed as log10 number of bacteria (colony-forming units) per g.

Production of lactic acid by streptococci from glucose, glucosamine, mucin, hyaluronic acid (a gift from Organon, Oss, the Netherlands), and N-acetyl neuramic acid (NANA) was studied in cell suspensions. The strains were grown in 100 ml of Todd-Hewitt broth with 0.1% of these substrates for 16 h at 37°C. Cells were sedimented for 30 min. at 16,000 g, washed twice with phosphate buffer (pH 6.8) and resuspended in 15 ml of the same buffer. The resulting cell suspensions contained 2-8 x 10^9 cells per ml; 4 ml of the suspension was incubated at 37°C with 0.4 ml of substrate (20 pmol glucose, glucosamine and NANA or 2 mg hyaluronic acid or mucin). After 30, 60 and 120 min. the cells were sedimented; if necessary mucin was precipitated with CaCl2.

Lactic acid was determined in the supernate with L-lactate:NAD oxidoreductase (EC. 1.1.1.27; from muscle; Boehringer, Mannheim, Germany) by measuring the reduction of NAD from the extinction at a wavelength of 340 nm in a spectrophotometer (Hohorst, 1965). Portions of 0-1 ml of supernate or blank were added to 2-2 ml of 0.5 M glycine buffer at pH 9 with 0.4 M hydrazine, 2.7 mM NAD, and 26 µg of enzyme. After 1 h at 25°C extinction was read. This method gave reliable and reproducible results. In the presence of mucin, however, the amount of lactic acid is underestimated by 20-30%, because part of the lactic acid is bound to the remaining mucin.

Antiserum against flagella of Str. mobilis. After growth in Todd-Hewitt broth for 24 h at 37°C, cells were centrifuged for 20 min. at 3000 g and resuspended in saline. The suspension was shaken on a Bühler shaker (E. Bühler, 7400 Tübingen-2, Western Germany) at 20,000 r.p.m. to separate the flagella and was centrifuged. The supernate was used for immunisation of rabbits. The resulting serum agglutinated the flagellar suspension as well as viable
Str. mobilis to a titre of 20,000. All strains of Str. mobilis but none of 30 other group-D streptococci were agglutinated by this serum.

**RESULTS**

*Normal flora.* Repeated counts of the faecal flora of healthy subjects produced results as in fig. 1. The flora was quite stable. The ratio of Bifidobacterium to Bacteroides was c. 1:1, and of anaerobes to aerobes <100:1. In 10 healthy subjects (45 samples) the variation from the mean (in logarithms) for the following groups of organisms was small: *Bacteroides* 9·71 ± 0·45, *Bifidobacterium* 9·52 ± 0·76, coliform bacteria 7·28 ± 0·80, enterococci 6·72 ± 0·80. The numbers of lactobacilli seemed fairly constant in each individual but varied between subjects (4–7·50) so that the calculation of a mean was unsatisfactory.

*Flora in ulcerative colitis.* A representative picture is given in fig. 2. The flora was unstable. The numbers of *Bacteroides* were about normal but bifidobacteria were reduced by a factor of 10–100. Enterococci were much increased. There generally was also an increase of coliforms. Lactobacilli were very variable. The increase of enterococci and coliforms and the decrease of bifidobacteria was a regular feature in patients with ulcerative colitis. As a measure of this feature a degree of deviation of flora (DDF) was calculated.

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**Fig. 1.—** Serial counts of faecal flora in a healthy subject: --- *Bacteroides*; ——— *Bifidobacterium*; —— *Lactobacillus*; ····· enterococci; —— x·x coliform bacteria.
Cn-10

Date and month (1970-1971)

For each sample the differences in the logarithms of the counts from the above (arbitrary) means were added, giving + signs to a decrease of anaerobes and to an increase of aerobes and — signs to inverse variations. In normal subjects the DDF varied around $0 \pm 1.26$. A DDF in the range of $-2.52$ to $+2.52$ (twice the standard deviation) may be considered eubiotic. In 10 patients with ulcerative colitis (67 samples) the DDF of single samples varied around a mean of $+4 \pm 1.64$ (range $0-9$). Though an occasional sample fell in the normal range (<2), in a series of samples from a patient with ulcerative colitis such samples were rare. The flora in ulcerative colitis can therefore be considered as disturbed (dysbiotic).

We observed quite different disturbances in a case of protein-losing enteropathy (DDF $-3.45$) and in three cases of blind-loop syndrome (overgrowth of Bac teroides and Escherichia coli, reduction of bifidobacteria and enterococci, DDF $+3.80$).

The increase of enterococci in the flora of patients with ulcerative colitis. The results of repeated counts of enterococci in 25 healthy subjects (85 samples) and 13 patients with ulcerative colitis (174 samples) are given in figs. 3a (distribution of samples) and 3b (distribution of subjects according to mean numbers). In normal subjects, enterococci were present in numbers from $10^5$ to $10^7$ organisms per g of faeces. In patients the numbers ranged from $10^7$ to $5 \times 10^9$ bacteria per g. In most patients ($84\%$) the number was in the range $10^8$ to $5 \times 10^9$ that is, a 100-fold increase.

The reliability of the differentiation of various group-D streptococci on KMN agar by colony size, colour and caseinolysis was confirmed by study of their metabolic properties (table). All strains reacted with anti-D serum. Some strains reacted with anti-D and anti-Q serum (Dr C. E. de Moor, National Institute of Public Health, Utrecht) and had the properties of Q strains (Nowlan and Deibel, 1967).

Forty strains were typed serologically by Dr J. M. N. Willers (see Willers and Michel, 1966). Only 15 were typable. Correlation between serotype and biochemical characters was not obvious. Eight of the nine typable strains from patients belonged to serotypes 1 and 4.
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Figs. 3a and 3b.—Frequency distribution according to numbers of enterococci of 85 faecal samples from 25 healthy subjects (white columns) and of 174 samples from 13 patients with ulcerative colitis (black columns): (a) distribution as percentage of samples; (b) distribution of mean numbers per subject as percentage of subjects.
All strains of *Str. mobilis* (Graudal, 1952; Lund, 1967; Slade and Slamp, 1972) were agglutinated by the antiflagellar serum.

The flora in patients with ulcerative colitis was characterised not only by a greater number of enterococci but also by a greater diversity of streptococcal types. This was already obvious from the variety of colonies on KMN plates, and was confirmed by biochemical identification of the strains isolated. In samples from healthy subjects one or two different types were generally found, whereas three or four different types occurred in most samples from patients. This was due not only to the simultaneous presence of more of the types also found in healthy subjects, but also to the appearance of *Str. mobilis* (fig. 4) which could be isolated only from patients and—by our methods—never from healthy subjects. In contrast, *Str. bovis* occurred regularly in healthy subjects but was rare in patients. When additional characters (hyaluronidase, mucinase) were taken into account, the variety of different types in patients was even greater.

*The breakdown of mucopolysaccharides by enterococci.* In healthy subjects most food is absorbed in the upper part of the digestive tract. The flora in the colon is living mainly from endogenous food sources (shed cells, mucus, etc.). The large increase of enterococci in ulcerative colitis suggested that they might be the cause of the increased amounts of lactic acid in the faeces (Weijers and van de Kamer, 1965). This lactic acid should then be produced from endogenous substrates.

![Figure 4](image-url)
The breakdown of hyaluronic acid, chondroitin sulphate and hog gastric mucin was studied in 46 independent isolates of enterococci from healthy subjects and 60 from patients with ulcerative colitis. The results are shown in fig. 5. Str. bovis did not hydrolyse any of these substrates. Mucin, which may be a natural substrate, was broken down by many strains from healthy subjects and patients. Still, it is remarkable that 85% of strains of Str. mobilis—occurring only in patients—disintegrated mucin. Chondroitin sulphate was dissimilated only by some strains of Str. faecalis. Hyaluronic acid was hydrolysed only by strains from patients.

![Graphs showing hyaluronidase, chondroitinase, and mucinase production](image.png)

**Fig. 5.**—Strains producing hyaluronidase, chondroitinase, and mucinase among 46 independent isolates of enterococci from healthy subjects (white columns) and among 60 strains from patients with ulcerative colitis (black columns) as percentage of the number of strains in each group.
Formation of lactic acid by enterococci. The plate method used to demonstrate mucinase shows only that the macromolecules are split to smaller molecules. To study whether enterococci can use the breakdown products as an energy source, the production of lactic acid from these substrates was estimated.

Fig. 6 shows the production of lactic acid by cell suspensions of two mucinase- and hyaluronidase-positive (muc+, hya+) and two muc+ hya− strains, grown with or without the substrate. All strains produced lactic acid from glucose, glucosamine, and mucin. The hyaluronidase-positive strains also produced lactic acid from hyaluronic acid. After adaptation, the rate of lactic-acid formation was much higher. Only one strain metabolised N-acetyl-neuramic acid.

DISCUSSION

The RCB medium is suitable for routine use because it permits a ready differentiation of several anaerobic organisms in the faecal flora. Some organisms are suppressed by China blue, however, and others may require still more exacting anaerobic techniques.

Even with this restriction, the difference between the normal flora and that in ulcerative colitis is quite clear (figs. 1 and 2). The decrease in bifidobacteria and the increase in group-D streptococci (fig. 3) are characteristic for ulcerative colitis. The diversity in the streptococcal flora is also a significant feature of a dysbiotic flora, as already recognised in the coliform flora of animals and patients with reduced colonisation resistance (van der Waaij, Berghuis-de Vries and Lekkerkerk-van der Wees, 1971). Further differentiation of anaerobes would probably show an analogous diversification in these groups. Furthermore, it is interesting that Str. bovis (mucinase−) was not found in the patients, whereas Str. mobilis (85% mucinase+), which was not isolated from healthy subjects by the methods used, came to the fore.

Hyaluronic acid is not normally present in the gut but this mesodermal polysaccharide is certainly open to attack in the ulcers in the gut wall. The breakdown of hyaluronic acid by many streptococci from patients and by none from healthy subjects (fig. 5) is probably due to a selection of strains that can adapt to hyaluronidase production.

The organisms hydrolysing these mucopolysaccharides can also use the breakdown products for lactic-acid production and thus presumably for growth (fig. 6).

It should be mentioned that nearly all bifidobacteria produce mucinase though the rate of mucin breakdown is much slower. In ulcerative colitis the increased slime production, the increased cell decay and the presence of unprotected mesodermal polysaccharide (hyaluronic acid) seem to cause a shift in the faecal flora, furthering growth and diversification of group-D streptococci. The high lactic-acid content in the faeces of these patients is almost certainly caused by the 100-fold increase of these streptococci.
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Fig. 6.—Production of lactic acid from glucose, glucosamine (N-ac-gluNH₂), mucin, hyaluronic acid, and acetylneuramic acid (NANA) by four mucinase + (muc +) strains including two hyaluronidase + (hya +) strains: (a) Str. faecalis, muc + hya +; (b) Str. mobilis, muc + hya +; (c) Str. mobilis, muc + hya -; (d) Str. faecalis, muc + hya -. ○---○ unadapted; o---o after adaptation. Only strain (d) metabolised NANA.
SUMMARY

Differential counts of the faecal flora of patients with ulcerative colitis showed a dysbiotic flora with a 100-fold increase of group-D streptococci and a reduction of bifidobacteria in comparison with the stable eubiotic flora of healthy subjects.

The increase in number in group-D streptococci was accompanied by an increase in variety. About four different varieties of enterococci were found in faeces from patients compared with one or two in samples from healthy subjects. The strains isolated from patients were more active in mucin breakdown, and only strains from patients were able to break down hyaluronic acid. Lactic acid could be formed from these substrates. The increased secretion of mucin in colitis and the presence of unprotected hyaluronic acid in ulcers seem to select these organisms which are probably the cause of the high lactic-acid content of the faeces in such patients.

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