

PROBIOTICS

Prebiotic carbohydrates modify the mucosa associated microflora of the human large bowel

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Background and aims: The mucosa associated flora of the large intestine is important in determining mucosal function although what controls its composition is unknown. This study has determined the effect of the prebiotic carbohydrates oligofructose and inulin on the mucosal flora.

Methods: An in vitro chemostat model of both planktonic and surface associated bacteria was used followed by an intervention study in 29 subjects undergoing colonoscopy.

Subjects: Fourteen subjects, recruited from colonoscopy waiting lists, supplemented their diet for two weeks with a mix of 7.5 g of oligofructose and 7.5 g inulin. Fifteen subjects were recruited at the time of colonoscopy and given no supplement. Multiple endoscopic biopsies were taken from the caecum, transverse and descending colon, and rectum. The mucosal flora was characterised by culture and to species level by cellular fatty acid profiles. Cell proliferation was assessed by immunohistochemical staining for minichromosome maintenance protein 2, Ki67, and proliferating cell nuclear antigen.

Results: In vitro prebiotics increased surface counts of bifidobacteria from 6.6 to 7.3 log₁₀ colony forming units (CFU) per slide ($p < 0.0006$) with no significant changes in planktonic bacteria. In the feeding study, prebiotics increased mucosal bifidobacteria (log CFU/g mucosa (SEM)) in both the proximal (control 5.3 (0.4) v prebiotic 6.3 (0.3)) ($p = 0.059$) and distal (control 5.2 (0.3) v prebiotic 6.4 (0.3)) colon ($p = 0.01$). Lactobacilli were also increased (3.0 (0.1) v 3.7 (0.2)) ($p = 0.02$) in the proximal and 3.1 (0.1) v 3.6 (0.2) ($p = 0.04$) in the distal colon, respectively). There were significantly more eubacteria in fed subjects but no changes in total anaerobes clostridia, bacteroides, or coliforms, nor in proliferation indices.

Conclusion: Prebiotic carbohydrates can change the composition of the mucosa associated flora significantly.

Bacteria that inhabit the large bowel can be divided into planktonic, namely those that could be considered to be free living in the lumen of the bowel, and the mucosa associated flora (MAF). There is a large body of literature describing the planktonic flora of the colon and its functions but much less is known about mucosal populations. While the planktonic flora clearly play an important role in digestion through fermentation,^{1,2} vitamin synthesis,³ metabolism of xenobiotics,⁴ and provide a barrier to invading pathogens,⁵ the mucosal flora, because of their location, are increasingly thought to be critical in determining epithelial health through their ability to exclude pathogens, stimulate development of the immune system, and moderate inflammatory responses.^{6–9}

The growth and metabolism of established planktonic flora is controlled largely by dietary substrates that escape digestion in the small bowel, and probably also by endogenous secretions of the gut.^{10–11} What influences the MAF is largely unknown although there is clearly cross talk between epithelial cells, bacteria, and the gut immune system.^{12–15} The role of diet has not been studied, but carbohydrates such as oligofructose, inulin,^{16,17} and galacto-oligosaccharides¹⁸ are known to stimulate selectively the growth of bifidobacteria and lactobacilli in the planktonic flora of the colon (prebiotic effect) and thereby contribute to barrier function.^{6,19,20} If the composition of the MAF was also to be moderated by diet, then the potential exists for contributing to epithelial cell health.

In the present study, we have used an in vitro model of the large bowel that allows both planktonic and surface associated bacteria to be studied and have used it to show that the prebiotic carbohydrates oligofructose and inulin selectively stimulate the growth of bifidobacteria in the

surface associated flora. We went on to feed these carbohydrates to healthy patients scheduled to have a colonoscopy and examined their effect on the MAF in all regions of the large bowel.

PATIENTS AND METHODS

Fresh faecal specimens were obtained from six healthy volunteers (aged 24–55 years) who had no previous history of gastrointestinal disease and had not taken antibiotics for at least three months prior to the study. Faeces were collected separately from urine in a plastic bag suspended in a metal frame over a toilet and processed immediately.

Prebiotic feeding study

Twenty nine subjects were recruited from the colonoscopy waiting list at Addenbrookes Hospital. Exclusion criteria included: known or suspected inflammatory bowel disease, diarrhoea, colorectal carcinoma, and exposure to antibiotics in the preceding three months. Written consent was obtained from each patient, and the study approved by both the Medical Research Council Dunn Nutrition Ethics Committee and the Local Research Ethics Committee.

Prebiotics

Carbohydrates used were inulin (Raftiline HP) and oligofructose (Raftilose P95). Both were supplied by Orafiti (Tienen, Belgium). Raftiline HP contains 99.5% inulin extracted from chicory roots. It comprises molecules of glucose-fructose (GF)_N type, with N ranging from 5 to 60,

Abbreviations: MAF, mucosa associated flora; G, glucose; F, fructose; Mcm-2, minichromosome maintenance protein 2; PCNA, proliferating cell nuclear antigen; LI, labelling index

and an average degree of polymerisation of 10. Raftilose P95 contains oligofructose 93.2% (GF_N and F_N, β(2, 1) in a ratio of 2:1) and glucose + fructose + sucrose 6.8%.

All chemicals, unless otherwise specified, were supplied by Sigma Ltd (Poole, Dorset, UK).

Batch culture experiments

Faecal slurries were prepared by homogenising fresh faeces in anaerobic culture media and sieving using a 250 µm aperture. The culture media contained (g/l): soluble starch 5.0; pectin 1.0; xylan 1.0; guar gum 1.0; arabinogalactan 1.0; casein 5.0; peptone 2.5 (Oxoid, Basingstoke, UK); tryptone 2.5 (Oxoid); yeast extract 4.5 (Oxoid); porcine gastric mucin type II 2.5; NaCl 4.5; K₂HPO₄ 0.4; MgCl₂ 0.15; NH₄Cl 0.4; KCl 0.25; cysteine 0.8; vitamin B₁₂ 0.005; haemin 0.01; and Tween 80 1.0. A vitamin solution (1 ml/l) was added which consisted of (mg/l): menadione 1.0; biotin 2.0; pantothenate 10.0; nicotinamide 5.0; *p*-aminobenzoic acid 5.0; and thiamine 4.0.

Duplicate batch culture fermenters (Soham Scientific, Ely, UK) were fitted with a mucin bait (that is, a series of glass slides suspended in a glass rack within the culture). These slides were precoated with an agar/mucin mix (1% agar (Oxoid), 3% porcine gastric mucin type I). The fermenters (working volume 300 ml) were inoculated with 10% (w/v) faecal slurry and 7.5 g/l oligofructose, and 7.5 g/l inulin was added to one vessel. The vessels were continually stirred, sparged with oxygen free nitrogen, and maintained at a pH of 5.8 and temperature of 37°C. Samples of liquid culture (1 ml) and surface associated cultures (one coated glass slide) were removed from each fermenter after 0, 3, 6, 12, and 24 hours and serially diluted in an anaerobic cabinet (10:10:80; H₂:CO₂:N₂ atmosphere) with pre-reduced half strength peptone water. This model system for studying biofilm populations is described in detail elsewhere.²¹ Triplicate plates were then inoculated with 0.1 ml samples and incubated aerobically or anaerobically, as appropriate, at 37°C. Bacteria were counted on Wilkins-Chalgren agar (Oxoid; total anaerobes), nutrient agar (Oxoid; total aerobes), Wilkins-Chalgren agar plus gram negative supplements (Oxoid; bacteroides), Beerens agar (bifidobacteria), Rogosa agar (Oxoid; lactobacilli), and perfringens agar plus supplements (Oxoid; clostridia). Subsequently, bacteria were characterised to the genus level on the basis of morphology, Gram reaction, spore production, and metabolism using API 20A (Bio-Merieux, Lyon, France).²²

Feeding study

Twenty nine volunteers were recruited. Fourteen subjects (eight men and six women; mean age 59 years (range 35–72)) supplemented their usual diet with a mixture of 2.5 g inulin and 2.5 g oligofructose three times a day for two weeks prior to their booked colonoscopy appointment. All kept a daily diary of supplements taken and recorded any side effects. Indications for colonoscopy were: previous colorectal

polyps, a family history of colorectal carcinoma, iron deficiency anaemia, and rectal bleeding. Fifteen control subjects were recruited at the time of their colonoscopy appointment (six men and nine women; mean age 59 years (range 31–81)). Indications for colonoscopy were previous colorectal polyps, family history of colorectal carcinoma, iron deficiency anaemia, and rectal bleeding. All 29 subjects followed a low fibre diet the day prior to the procedure and took Picolax (sodium picosulphate and magnesium citrate; Ferring Pharmaceuticals, Feltham, Middlesex, UK), one sachet 24 hours and one 16 hours before the colonoscopy, which was performed under midazolam (Roche Products Ltd, Welwyn Garden City, UK) and pethidine sedation. Endoscopic biopsy samples were taken from macroscopically clean mucosa (no visible faecal material) using sterilised forceps from the caecum, transverse colon, descending colon, and rectum.

Bacteriological analysis of biopsies

Biopsy samples from the caecum and descending colon were transferred immediately into sterile pre-weighed bijoux bottles containing half strength peptone water. These were weighed and immediately removed to an anaerobic cabinet where samples were homogenised by hand and serially diluted and plated out as described above with the addition of MacConkey agar No 2 (Oxoid).

Bacterial isolates were characterised to species level on the basis of their cellular fatty acid profiles. Cell pellets from 30 ml of overnight culture in peptone yeast extract broth were saponified in 3.75 M NaOH in aqueous methanol (50% vol/vol). Fatty acids were then methylated by addition of two volumes of 3.25 N HCl in aqueous methanol prior to extraction. The Microbial Identification System (MIDI Inc. Newark, Delaware, USA) described previously²³ was used to generate a cellular fatty acid profile for each isolate and identification obtained by comparison with the Moore Standard Library for anaerobic bacteria (<http://www.midi-inc.com/pages/databases.html>).

Short chain fatty acids were measured as previously described.¹⁶

Proliferation markers

Other biopsies from the caecum, transverse colon, descending colon, and rectum were fixed in formalin and processed to paraffin. Proliferation in colorectal epithelial cells was assessed by immunohistochemical staining for the cell cycle markers minichromosome maintenance protein 2 (Mcm-2), Ki67, and proliferating cell nuclear antigen (PCNA), as described previously.^{24, 25} Stained sections were examined by a single observer (SJL) using a Nikon Eclipse E600 microscope. For every sample, the frequency of expression of each marker was determined in 2–4 well orientated complete crypts by calculating a labelling index (LI), representing the percentage of epithelial cell nuclei that stained positively in 2

Table 1 Mean viable bacterial counts (mean (SEM)) from six batch cultures at 12 hours incubation with and without 1.5% w/v added prebiotic

Bacteria	Planktonic counts (log ₁₀ CFU/ml culture)		Surface counts (log ₁₀ CFU/slide)	
	Control	+Prebiotic	Control	+Prebiotic
Total aerobes	6.9 (0.3)	6.8 (0.2)	4.9 (0.5)	5.0 (0.4)
Total anaerobes	9.7 (0.2)	9.6 (0.3)	7.8 (0.3)	7.6 (0.3)
Bacteroides	9.5 (0.2)	9.4 (0.3)	7.5 (0.3)	7.2 (0.2)
Bifidobacteria	8.4 (0.1)	8.8 (0.2)	6.6 (0.2)	7.3 (0.2)*
Lactobacilli	3.5 (0.6)	4.1 (0.3)	2.2 (0.3)	2.5 (0.4)
Clostridia	6.5 (0.3)	6.2 (0.4)	4.8 (0.6)	4.4 (0.5)

Significant difference: control versus prebiotic, **p*<0.006

Table 2 Short chain fatty acids, lactate, and succinate concentrations (mean (SEM) mM) in batch culture media with and without 1.5% w/v added prebiotic over 24 hours

Time (h)	Acetate		Propionate		Butyrate		Valerate		Caproate		Lactate		Succinate	
	Con	Pre	Con	Pre	Con	Pre	Con	Pre	Con	Pre	Con	Pre	Con	Pre
0	13 (1.2)	13 (1.2)	5 (0.7)	5 (0.7)	3 (0.7)	3 (0.7)	0.7 (0.2)	0.7 (0.2)	0.7 (0.2)	0.7 (0.2)	1.7 (0.4)	1.7 (0.4)	1.2 (0.5)	1.2 (0.5)
3	43 (4.1)	76 (13.5)	17 (3.3)	22 (4.9)	14 (2.9)	17 (3.3)	2.3 (0.7)	2.2 (0.6)	1.3 (0.5)	1.4 (0.4)	4.8 (1.9)	12 (3.0)	3.3 (2.4)	5.3 (2.7)
6	69 (4.9)	160 (20.4)	28 (3.3)	48 (16.7)	29 (4.5)	43 (8.2)	7 (2.4)	4.2 (1.4)	3.6 (1.8)	2.1 (1.1)	3.1 (1.9)	14 (8.0)*	3.0 (2.4)	5.4 (3.4)
12	90 (4.1)	200 (13.9)*	40 (2.4)	65 (18.4)	42 (2.0)	89 (5.3)*	18 (4.9)	20 (4.9)	7.4 (2.3)	11 (6.5)	3.5 (2.9)	26 (9.4)*	4.0 (3.4)	7.4 (6.8)
24	99 (5.3)	208 (13.5)*	45 (3.7)	57 (8.9)	54 (4.1)	96 (2.4)*	23 (5.3)	17 (4.5)	10 (2.9)	23 (10.2)				

Con, control; Pre, prebiotic.

Significantly different from control, * $p < 0.001$.

to "n" well oriented complete crypts. (LI values were compared using the Wilcoxon signed rank test for paired data.)

Data and statistical analysis

For each experiment, data were analysed using the Excel statistical package. For the bacteriological and cell proliferation analysis, estimation of probability of difference between groups was undertaken by the Student *t* test.

RESULTS

Batch cultures

Surface associated populations were established on mucin coated slides after three hours of incubation. However, the results shown in table 1 are at 12 hours when the bacterial growth rate in the system was high and, as can be seen from the short chain fatty acid concentrations (table 2), a stable system existed. Total anaerobe to aerobe ratio was similar in both surface and planktonic populations at 100:1. The effect of the added prebiotic mix was to increase numbers of bifidobacteria. This prebiotic action was significant only for surface counts which increased by fivefold in the supplemented vessel, and by 2.5-fold in the planktonic phase (NS). Surface lactobacilli increased twofold and clostridia decreased twofold but these changes were not statistically significant.

Short chain fatty acid concentrations increased progressively over the first 12 hours of the batch culture and then showed very similar levels at 24 hours. Concentrations of acetate and butyrate were significantly higher with prebiotic at 12 hours and 24 hours, as might be expected with the extra substrate provided. The molar ratio of acetate:propionate:butyrate was 52:23:24 at 12 hours in control vessels and 56:18:25 with prebiotic, which were not significantly different. Lactate concentrations also increased with prebiotic, as did caproate, but no such changes were seen with valerate or succinate.

FEEDING STUDY

Compliance and tolerability

The prebiotic supplement was well tolerated by subjects although all reported an increase in flatulence (mild-moderate in 10, severe in four). Other side effects observed were bloating, (mild-moderate in seven, severe in two) and a laxative effect (mild-moderate in 10, severe in one), as noted in previous studies.¹⁸⁻²⁶ Scores were similar for both weeks of the supplementation period. Compliance with the supplement was excellent, with subjects taking on average 97% of the supplements (range 81-100%).

Effect on mucosal flora

Overall, total anaerobe counts were unaffected by the addition of the prebiotic to the diet (table 3). A prebiotic effect was seen in both the right and left colon. Mucosa associated bifidobacteria were detected in 12 of 14 of the supplemented group compared with seven of 15 of the control group. The effect of the prebiotic was to give a difference in mean counts of 1 order of magnitude ($1 \log_{10}$) greater. Analysis of bacterial populations at the species level revealed *Bifidobacterium angulatum* as the dominant bifidobacteria in both groups, comprising 71% of the isolates from the supplemented and 50% of those from the control group. Mucosal lactobacilli were detected in eight of 14 of the fed subjects compared with only one subject in the control group. Overall numbers were 0.5-0.7 \log_{10} greater with the prebiotic. Eubacteria, the second most numerous faecal isolates,²⁷ were detected in 10 of 14 supplemented subjects and five of 15 control subjects. Eubacteria showed large differences between the groups with 1.5-1.6 \log_{10} greater numbers with the prebiotic. All of these changes were statistically significant. In contrast, numbers of mucosal coliforms and thus total aerobes were lower by 0.6 and 0.5 logs with the prebiotic although the range of counts was very wide (4.2-8.8 in the control group, 3-8.2 in the supplemented group) and the result was not statistically significant. Prebiotic feeding did

Table 3 Effect of supplementing diet with 15 g/day prebiotic mix on mucosa associated flora of the right and left colon

Bacteria	Proximal colon		Distal colon	
	Control	+Prebiotic	Control	+Prebiotic
Total aerobes	6.4 (0.4)	5.9 (0.4)	6.4 (0.3)	5.9 (0.4)
Coliforms	6.2 (0.4)	5.6 (0.4)	6.2 (0.3)	5.7 (0.4)
Total anaerobes	8.5 (0.2)	8.6 (0.2)	8.7 (0.1)	8.6 (0.1)
Bacteroides	8.1 (0.3)	8.3 (0.2)	8.3 (0.2)	8.5 (0.2)
Bifidobacteria	5.3 (0.4)	6.3 (0.3)*	5.2 (0.3)	6.4 (0.3)†
Eubacteria	4.5 (0.3)	6.0 (0.4)‡	4.6 (0.3)	6.1 (0.3)§
Lactobacilli	3.0 (0.1)	3.7 (0.2)*	3.1 (0.1)	3.6 (0.2)**
Clostridia	5.1 (0.3)	4.9 (0.3)	5.0 (0.3)	4.9 (0.3)

Values are expressed as mean (SEM) in log CFU/g mucosa.

Significance of difference, control versus prebiotic: * $p = 0.059$, † $p = 0.01$, ‡ $p = 0.003$, § $p = 0.002$,* $p = 0.017$, ** $p = 0.04$.

not change total bacteroides numbers and bacteroides species distribution. Overall, numbers of clostridia were unaffected by the prebiotic supplementation although marked species differences were noted, with *Clostridium innocuum* dominating the supplemented group (66% of all clostridia identified) but representing only 10% of control clostridia, 50% of which were identified as *Clostridium coeleatum*, a species not detected in the supplemented group. Total anaerobe numbers remained steady and the anaerobe:aerobe ratio was approximately 100:1 in both groups.

Proliferation indices

The mucosa was macroscopically normal in all subjects and this appearance did not change with the prebiotic supplement. For all biopsies, more cells were stained with the antibody to MCM-2 protein than with those to PCNA and Ki67. As shown in figs 1-3, the prebiotic supplementation had no effect on labelling indices for any of the markers of mucosal proliferation at the four sites of the colon studied.

DISCUSSION

These studies have shown clearly, both by the use of an in vitro model and in vivo, that the composition of surface associated flora can be influenced by substrate and hence by diet. Moreover, the dietary changes required are very small; only a few grams of sugar-like carbohydrates were added to subjects' food each day. Equally important is the nature of the change in the flora. An increase in bifidobacteria and lactobacilli in the gut, either by the use of pro or prebiotics, is currently considered a healthy objective. There are no previously reported studies in humans of any dietary factor that can be shown to alter the composition of the MAF. In a recent paper, Kleessen and colleagues²⁸ showed in human flora associated rats that feeding an oligofructose/inulin mixture increased counts of mucosa associated bifidobacteria in the colon and improved mucosal architecture and mucus secretion.

In the in vitro model, the overall composition of the flora was the same whether in the planktonic or surface associated phase (table 1), although bacterial densities were lower at 12 hours on the agar-mucin surface. A clear bifidogenic effect was seen on the mucus baits with no notable changes in other genera or in the planktonic bacteria. This absence of a selective effect on the planktonic bifidobacteria may reflect the fact that truly free living (that is, not surface associated) bacteria do not exist in the luminal phase of the large bowel (see below). Alternatively, the use of multiple carbohydrate substrates in the batch culture medium, in addition to oligofructose and inulin, may have reduced the importance of the prebiotics in determining the pattern of overall growth in the mixed culture.

Short chain fatty acid concentrations increased substantially with the additional prebiotic substrate but no change in molar ratios was seen and thus these carbohydrates cannot

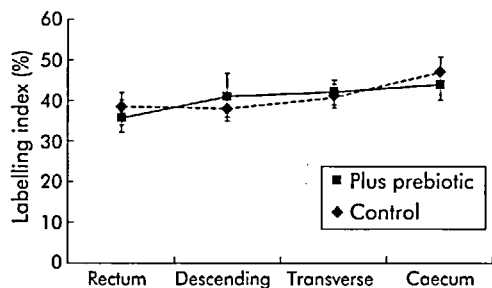


Figure 1 K167 labelling index from four sites of the colon in control subjects and those fed prebiotic. Values are mean (SEM).

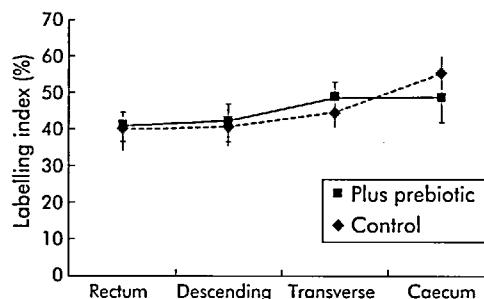


Figure 2 Proliferating cell nuclear antigen labelling index from four sites of the colon in control subjects and those fed prebiotic. Values are mean (SEM).

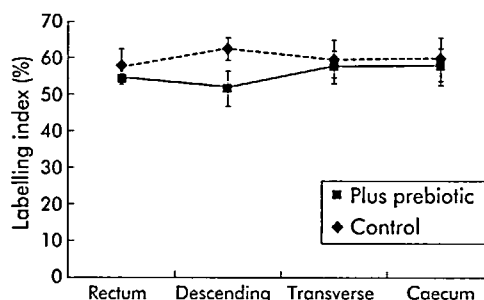


Figure 3 Minichromosome maintenance protein 2 labelling index from four sites of the colon in control subjects and those fed prebiotic. Values are mean (SEM).

be seen as providing selective increases in butyrate over other short chain fatty acids, although overall they are a good source of butyrate. These findings are similar to those observed in human studies of prebiotic feeding and faecal composition.¹⁶ The increase in lactate with added prebiotic is probably a consequence of the presence of rapidly metabolisable substrate in excess.

In the in vivo study, MAF reflected very much the bacterial composition that has been reported for planktonic and faecal populations. This is perhaps not surprising because the notion that a true planktonic flora exists in the large intestine is probably incorrect. Most, if not all, of the flora in the hind gut that are not mucosa associated are attached to particulate matter.^{29,30} Macfarlane and colleagues (in 1995 and 1997) showed that the composition of the flora attached to particles in the large bowel is very similar to that which is "free living" but that metabolically they are different. Increased activities of polysaccharidases, glycosidases, proteases, and arylamidases, and mucus degrading enzymes are seen in the particle associated species, probably reflecting the nature of the underlying surface material which will largely comprise plant cell walls, entrapped in a viscoelastic gel of the many mucins that are secreted into the gut. End products of fermentation also differ, with surface associated communities producing more acetate than planktonic flora.³¹

Addition of only 15 g/day of inulin and oligofructose produced significant changes in the MAF in both the proximal and distal colon. Most importantly, counts of bifidobacteria increased by 1 log₁₀ or more, the principal species being *B angulatum*. The increase in this particular genus is the classic prebiotic effect demonstrated previously in vitro³²⁻³⁴ and in vivo in faecal flora.¹⁶⁻¹⁸ The essence of prebiotic, however, is the selectivity of the effect. In the present study, the absence of significant increases in bacteroides, clostridia, coliforms, and total aerobes and anaerobes is reassuring. Numbers of lactobacilli also

increased significantly, but this genus, like bifidobacteria, is thought to be part of the beneficial or protective flora of the gut. We also observed significant changes in eubacteria, an observation not previously noted in studies of prebiotics. It is not clear why this should occur in MAF and not planktonic or faecal flora. However, it is known that bacteria living in biofilms on surfaces show altered substrate use and efficiency of metabolism as well as resistance to host defence mechanisms and antibiotics.³⁵⁻³⁷ Metabolic consortia build up and substrate use becomes efficient. It is possible therefore that eubacteria benefited from cross feeding of carbohydrate residues from the initial actions of bifidobacteria and lactobacilli on prebiotics. Many eubacteria are saccharolytic.

Inulin and oligofructose are fructans that are not hydrolysed by pancreatic enzymes and escape digestion in the small bowel.³⁸⁻⁴⁰ Bifidobacteria have relatively high amounts of β -fructosidase that is selective for β 1-2 glycosidic bonds in fructans.⁴¹ Subsequent transport mechanisms and sites of hydrolysis may also be faster. After oligosaccharide hydrolysis, monomers then serve as an efficient growth substrate for the bifidus pathway of hexose fermentation.⁴² In addition, the inhibitory effects of bifidobacterial growth on other colonic organisms⁶ are likely to assist in the competitive influence that occurred with oligofructose and inulin. Lactobacilli can also ferment FOS,⁴³ and human feeding studies have shown that faecal populations of these bacteria can be increased in some subjects when fed prebiotics.^{18 44} Data presented in this study show that lactobacilli appear to compete equally effectively for prebiotic substrates at the mucosal surface.

In any study of the gut microflora, methodology is always an important issue. Culture based methods are known to introduce bias towards organisms that are easier to culture and recently, studies using molecular techniques have improved our knowledge of bacteria that are more difficult to culture and which form a significant portion of the microbiota. Culture based methodologies will never account for the entire microbial population, are slow, require immediate sample handling and identification by a variety of morphological, metabolic, and biochemical techniques. Phenotypic methods of identification are unlikely to match the discriminatory power of some genotypic tools. However, culture based methods are well established, cost effective, sensitive, and allow direct comparison with the many similar studies reported in the literature. They give valuable information of the likely metabolic potential of the overall population. Molecular analyses are important in contributing to the gaps in our knowledge but no method is infallible and other factors need to be considered with these newer methods. They are also subject to bias—for example, bacteria that are more susceptible to lysis, probe permeability, and amplification will be more readily detected. There are still a very limited number of probes available at species level. FISH methods are almost impossible to quantify and molecular techniques in general have lower sensitivities. Techniques such as DGGE (molecular fingerprinting) are not quantitative, and the metabolic contribution of "molecular species" cannot be determined. Both methodologies continue to contribute to our understanding of the large bowel ecosystem but it is important to consider the limitations of each technique, particularly when comparing populations of absolute cell numbers to those given as a proportion of the total bacteria present. Similar population sizes have been found by both *in situ* hybridisations and bacterial cell culture for organisms such as bifidobacteria and eubacteria, but the size of the total microflora is underestimated by the latter method, which would alter the proportional value.^{45 46} However, it currently remains prudent to consider evidence

using both methodologies and this study clearly shows a significant change in the culturable portion of the MAF.

Widely differing results for the composition of the MAF have been reported. This is likely to be partly because of the different, and changing, methods referred to above, now used in bacteriology. Swidsinski and colleagues⁹ found low total counts, by conventional culture techniques, of MAF in 40 control subjects although these bacteria were similar in composition to faecal (planktonic) flora. However, biopsy specimens were vigorously washed before culturing. Much higher MAF counts were found in patients with active ulcerative colitis in this study. Schultz and colleagues⁷ also observed very low counts of MAF in nine controls undergoing colonoscopy. Specimens were not washed but were fixed in formalin embedded in paraffin and bacteria detected by non-radioactive detection of rRNA by *in situ* hybridisation. Again, high counts were found in ulcerative colitis patients. Poor staining in the controls was ascribed to the MAF being inactive or dead, poor antibody penetration, and the barrier effect of mucus. Others have shown a numerous and diverse MAF in colonic or rectal mucosal from either sudden death victims⁴⁷ or from colonoscopic and rectal biopsies of healthy subjects.^{8 48-51} In these reports, however, standard culturing techniques were used and biopsies were either not washed or washed gently to remove visible adherent faecal material. Similarly, Zoetendal and colleagues⁵² took biopsies at colonoscopy from the colons of 10 individuals and used 16S RNA probes to analyse bacterial diversity on paraformaldehyde fixed samples. Bacterial counts up to 10^6 were found in seven of 10 subjects but showed fewer species diversity than samples of faecal flora. Whether these bacteria are largely in the mucus layer, rather than adherent to epithelial cells, is a likely possibility but their proximity to the mucosal and thus interaction with epithelial and immune cells is an important interface. In situations where mucus secretion is thought to be abnormal, epithelial adherent populations exist.^{7 9}

Alterations in the MAF had no effect on cell cycle entry by mucosal epithelial cells at four different sites in the large bowel. Our observation that Mcm-2 was present in significantly greater numbers of epithelial cells than the traditionally used proliferation markers Ki67 and PCNA is consistent with data from other studies.^{25 53} MCM proteins are the most sensitive markers available of cell cycle entry, being abundant in nuclei throughout the cell cycle and lost rapidly following differentiation, as occurs in large bowel crypts.²⁵ It should be noted that Mcm-23, Ki67, and PCNA all essentially provide information concerning the cell cycle state of the stained tissue and more subtle differences in rates of cycling would not be identified using our approach.

Does the composition of the MAF matter? Almost certainly yes. Gut flora clearly interact with the mucosal immune system¹¹⁻¹⁵ which is important in the process of developing tolerance to commensal flora. In mucosal disorders such as ulcerative colitis, this tolerance may have broken down.⁵⁴⁻⁵⁶ Changing the composition towards a flora with increased bifidobacteria and lactobacilli is thought to be beneficial to the host.⁵⁷ These genera contain few if any pathogenic species and are a major part of the large bowel defence against invading pathogenic organisms through their capacity to secrete peptides that inhibit pathogen growth.^{5 58 59} When given as probiotics, lactobacilli adhere to the colonic and rectal mucosa^{46 60} although probably do not permanently colonise the gut epithelium.⁶¹ Probiotic bacteria are clearly established as being a benefit in the prevention of antibiotic associated diarrhoea^{62 63} and reducing the duration of infectious diarrhoea in children.⁶⁴ Animal models indicate that they may have a protective effect against the development of colorectal cancer^{65 66} an effect that can be strengthened by combination with a prebiotic.⁶⁷

The health benefits of prebiotic carbohydrates have yet to be established. Apart from some benefits to calcium absorption, no clear value has been shown.⁶⁸ However, research in this area is relatively recent while the benefits of probiotics have been known for 100 years.

Moving the composition of the MAF towards one with increased numbers and more diverse species of bifidobacteria and lactobacilli should be beneficial to colonic health. If this can be achieved by a small change in the carbohydrate composition of the diet, then new possibilities emerge for public health and for preventive strategies by the gastroenterologist.

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EDITOR'S QUIZ: GI SNAPSHOT

Answer

From question on page 1582

The strong immunohistochemical staining with c-kit is in keeping with a malignant epithelioid gastrointestinal stromal tumour (GIST). The tumour was inoperable on computer tomography, demonstrating extensive local spread (including the left lateral segment of the liver). GISTs are rare but nevertheless the commonest mesenchymal tumours to arise in the gastrointestinal tract, with an annual incidence of 10 per million per annum. They are characterised by expression of the receptor tyrosine kinase KIT (Cd117), and this expression is often associated with gain of function mutations. Glivec (ST1571, imatinib mesylate; Novartis Pharmaceuticals, Basel, Switzerland) is a selective tyrosine kinase inhibitor that has proved to be effective in achieving tumour shrinkage in up to 60%, and symptomatic benefit in 80% of patients with metastatic disease. Predicting the clinical behaviour of GIST tumours remains imprecise and survival often depends on the ability to achieve complete resection.

Our patient with a gastric GIST with liver metastases had a complete histological resolution after six months of Glivec therapy. This was confirmed by immunohistological examination after the patient underwent total gastrectomy with D2 radical lymphadenectomy and metastectomy of segment VI of the liver. The previous metastatic liver deposits were mucoid and cystic in appearance with no viable tumour cells and the gastric primary was replaced by hyalinsed fibrous tissue and macrophages with some spindle cells indicating where the tumour had previously been. Immunohistochemistry was performed on all lesions and was completely negative for CD117 and CD34. The response to Glivec treatment is variable, and the location and nature of the KIT oncogenic mutations influence the likelihood of clinical response to various KIT inhibitor therapies.

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