Enteral feeding reduces metabolic activity of the intestinal microbiome in Crohn’s disease: Observational study

Christopher Walton¹, Maria Pilar Bilbao Montoya¹, Dawn P Fowler¹, Claire Turner², Wenjing Jia³, Rebecca N Whitehead³, Lesley Griffiths³, Rosemary H Waring³, David B Ramsden³, Jeffrey A Cole³, Michael Cauchi¹, Conrad Bessant¹, Sally J Naylor⁴, John O Hunter¹.⁴

¹– Cranfield University, 2– The Open University Milton Keynes, 3– University of Birmingham, 4– Addenbrooke’s Hospital Cambridge,

Correspondence to: Professor J O Hunter, Box 262, Addenbrooke’s Hospital, Hills Road, Cambridge CB2 0QQ

Key words: Crohn’s disease, Enteral Feeding, Nutrition, Colonic microflora, Metabolomics, breath analysis, faecal analysis, Enterometabolic disorder, E028 extra, Modulen IBD
Abstract

Background

Enteral feeding will induce remission in as many as 80-90% of compliant patients with active Crohn’s Disease (CD) but its method of action remains uncertain. This study was designed to examine its effects on the colonic microbiome.

Method

Healthy volunteers and patients with CD followed a regimen confined to enteral feeds alone for one or two weeks respectively. Chemicals excreted on breath or in faeces were characterised at the start and at the end of the feeding period by gas chromatography mass spectrometry (GC/MS).

Results

One week of feeding in healthy volunteers caused significant changes in stool colour and deterioration in breath odour, together with increased excretion of phenol and indoles on the breath. Feeding for two weeks in patients with CD produced significant improvements in symptoms and a decrease in the concentration of C-reactive protein. The faecal concentrations of microbial products including short chain fatty acids (SCFAs), and potentially toxic substances including 1-propanol, 1-butanol and the methyl and ethyl esters of SCFAs showed significant falls.

Conclusion

A significant change occurs in the production of microbial metabolites after enteral feeding in both healthy volunteers and patients with CD. Many of those detected in CD are toxic and may feasibly lead
to the immunological attack on the gut microbiota, which is characteristic of IBD. The reduction in the production of such metabolites after enteral feeding may be the reason for its effectiveness in CD.
Introduction

Despite the increasing frequency of Crohn’s disease, its treatment remains unsatisfactory. Many of the therapeutic agents used have unpleasant or even dangerous side effects and some are very expensive. The continuing perception of CD as a relapsing and remitting disorder emphasises the difficulty in maintaining long term control. A complete cure remains elusive.

Reports of a positive response to dietary manipulation in CD have emerged from several sources.\textsuperscript{1-8} \textsuperscript{2-4} weeks of total enteral feeding has been reported to reduce remission in 85-90\% of compliant patients suffering active CD. \textsuperscript{1-6}. Lack of understanding of the method of action of enteral feeds in CD has however, discouraged their use.\textsuperscript{2-8}

Enteral feeds are nutritionally complete liquid mixtures of pre-digested foods presenting nitrogen as amino acids, oligopeptides or a single protein, carbohydrates as simple sugars, typically malto-dextrins, and fat as a single oil, (eg. Rapeseed oil), together with minerals and vitamins. They are nutritionally complete.

Suggestions as to the method of action of enteral feeding are many, but it is now known that bowel rest \textsuperscript{7} and the reduction of potential food allergens \textsuperscript{2-4} are incorrect. Enteral feeding is unlikely to have therapeutic benefit by producing immunosuppression as it is ineffective in the treatment of ulcerative colitis. \textsuperscript{3} Reduction in inflammation can be detected before any improvement in nutritional state begins,\textsuperscript{8} and the suggestion that dietary particles might be important was not supported by a controlled trial.\textsuperscript{9}

The increasing evidence that inflammation in CD is provoked by an immune response targeted against the intestinal microbiome implies that manipulation of the metabolic activity of the microbiota might have a role in the treatment of this disease \textsuperscript{10,12} We and others have recently demonstrated an association between Crohn’s disease and the profile volatile organic compounds obtained from breath and faecal headspace.
samples. These measurements are a useful indication of changes in the gut microbiome, being simple, rapid and non-invasive. We have used this approach here in a study of the effects of enteral feeding. It has been suggested that food intolerance, as distinct from food allergy, might reflect an interaction between unabsorbed food residues and the intestinal microbiome. As the nutrients contained in enteral feeds are absorbed high in the small intestine, they supply little in the way of energy substrates to microorganisms in the lower bowel. This might lead to changes in microbial metabolism which in turn could lead to a reduction in inflammation. The present studies were designed to investigate this possibility.

Methods

Study 1 Healthy volunteers

Volunteers were recruited from students of either sex aged 18-65 years, at Cranfield University who were in good health and eating a normal diet. A total of 12 subjects was recruited aged 23-32, of which 8 were female. Subjects suffering conditions possibly requiring specific diets e.g. irritable bowel syndrome (IBS), or coeliac disease were excluded. Other exclusions were pregnancy or lactation, a course of antibiotics in the previous six weeks, bacterial products such as pro- or pre-biotics and any chronic medication other than oral contraceptives.

Subjects were randomly allocated to take either E028 extra (Nutricia UK Liverpool), or Modulen-IBD (Nestle Ltd, Croydon UK), for 7 days with all other foodstuffs excluded except water ad libitum. Nutritional requirements were calculated for each individual using Schofield’s equation. After 7 days subjects returned to normal diets for 21 days before commencing the alternative enteral feed for a further 7 days. The two feeds were administered 4 weeks apart in order that they were taken at the same stage of the menstrual cycles of female volunteers. During enteral feeding, subjects were asked to record how much feed they consumed and to complete symptom score sheets recording on a daily basis stool frequency, consistency and colour and any changes in breath odour. Weights were recorded and
breath samples taken before the study and after each week of enteral feeding. This trial was an open, randomised controlled study performed at Cranfield University and approved by the ethics committee of Cranfield University and the NHS Cambridge local research ethics committee.

Volunteers were provided with a sheet depicting a range of faecal colours ranging from dark brown to bright green (copies supplied to the editor) and asked to assess the stool colour, consistency and frequency. They were asked to record daily changes in breath odour which was assessed subjectively on a scale from 1 (odourless) to 4 (extremely unpleasant).

Bio-VOC samplers were used according to manufacturer’s recommendations to obtain a one-litre end-tidal breath sample after breakfast on the first day of each feeding period. Samples were injected onto Thermal desorption tubes containing 1:1 Tenax TA and Carbotrap adsorbents (Markes International, Llantrisant, UK).

**Study 2 Patients with Crohn’s disease**

Patients aged 18-65 years were recruited in the department of Gastroenterology, Addenbrooke’s Hospital, Cambridge. A total of 17 patients each provided a faecal sample before treatment with enteral feed E028extra and again when they went into remission. At recruitment, all had symptoms of active disease. The diagnosis of CD was made by standard diagnostic criteria and the severity of symptoms was assessed using the Harvey and Bradshaw Index. The concentration of C-reactive protein (CRP) in serum samples obtained at each visit was determined by the Biochemistry Department of Addenbrooke’s Hospital to provide an objective measure of disease activity.

Any patients who had received antibiotics in the previous 6 weeks were excluded. Some were taking medication including 5-aminosalicylic acid compounds and/or azathioprine which had been insufficient to control their symptoms, but none had received previous dietary treatment. They were asked to continue such medication during the period of feeding with elemental diet. Non-fasting morning samples of faeces were obtained before starting two weeks treatment with E028 extra (Nutricia Liverpool UK) with
amounts again being calculated by Schofield’s equation. A further faecal sample was obtained at the end of this period. Samples were delivered to the hospital on the same day as passed with a maximum delay before freezing of 4 hours. They were stored at -40°C until transferred to the laboratory for analysis.

Ethical permission for this study was granted by the Leeds West LREC (Ref: 07/Q1205/39).

**Laboratory analysis**

An internal standard solution comprising 50 ng deuterated (D8) toluene (Supelco Cat no 48,593) in methanol was added to each tube according to the manufacturer’s instructions (Markes International Ltd, Llantrisant, UK). Head space samples were analysed by automated thermal desorption gas chromatography/mass spectrometry. A Perkin Elmer system was used for analysis combining a TurboMass MS 4.1 Autosystem XL GC and Automatic Thermal Desorption system (ATD 400 PerkinElmer, Wellesley MA). The gas carrier was CP-grade helium (BOC gases Guildford UK) passed through a combined trap for removal of hydrocarbons, oxygen and water vapour. A wall-coated Zebron ZB624 chromatographic column was used with dimensions 60 x .04 x 0.25mm (internal diameter), the liquid phase comprising a 0.25 µm layer of 6% cyanopropylphenyl and 94% methylpolysiloxane.

Thermal desorption tubes were initially purged for 2 minutes to remove air and water vapour and then desorbed for 5 minutes at 300°C. The automatic thermal desorption valve temperature was set at 180°C and TD tubes were desorbed onto the secondary cold trap, which was initially maintained at 30°C. Once desorption was complete, the secondary trap was heated to 320°C using the fastest available heating rate and then maintained for 5 minutes. The effluent was transferred to the gas chromatograph through a transfer line heated to 210°C. The gas chromatograph oven was maintained at 50°C for 4 minutes after injection and then raised at a rate of 10°C/min to 220°C and then held for 9 minutes. Eluted products were transferred to the mass spectrometer via a line heated to 240°C. Electron ionisation (70eV) was used. Full scan mode was selected with mass-to-charge ratios from 33 to 350 m/z with a scan time of 0.3 second and 0.1 second interscan delay to produce a total ion count (TIC) chromatogram.

**Study 2**
Samples were transferred to the laboratory packed in dry ice inside insulated containers and on arrival were stored at -80°C until analysis.

Aliquots (5ml) of the defrosted samples were placed in gas sampling bags which were then sealed and filled with hydrocarbon-free air and incubated for 10 minutes at body temperature. A portable air pump was then used to draw 500ml of headspace through TD tubes packed with 50% Carbotrap and 50% Tenax. Full details have been published elsewhere.

Data and statistical analysis

Study 1

Compound identification was achieved using Automated Mass Spectral Deconvolution and Identification (AMDIS version 2.62) software and the National Institute of Standards and Technology mass spectral library. Quantification was achieved by comparing the area of each compound peak with the peak area associated with the known amount of d8 toluene.

Concentration data proved to be heavily right-skewed, therefore a non-parametric approach was adopted. A McNemar test was used to determine whether the probability of a compound to be present before or after the diet was significant. When present a Wilcoxon Rank Test was used to see if the compound was present in different quantities. Raw TIC data (i.e. a matrix of time vs. ion abundance) were also subjected to Principal Components Analysis (PCA) using Matlab (version 6/5 Mathworks Inc USA incorporating functions from the PLS Toolbox version 2.0 Eigenvector Research Inc USA).

Study 2

Compound identification and quantification were carried out as for study 1. In any given faecal headspace sample, automated mass spectral deconvolution and identification (AMDIS) would identify between 100-300 different compounds and it was therefore found necessary to select a subset of those we observed to render statistical analysis tractable. Three approaches were followed to provide a list of what we have termed ‘candidate compounds’. The list comprised first compounds that appeared to be most abundant
from inspection of the results obtained using AMDIS; second compounds that appeared to discriminate between patient groups by visual inspection of a subset of pre treatment sample chromatograms and third compound selected on the basis of a search of the relevant literature. An initial generic list was made including short-chain fatty acids (SCFAs) and their derivatives, phenolic compounds and indoles and sulfides. This list was then refined according to publications dealing more explicitly with VOC profiles in disease. A final list of compounds was obtained in this way.

Results

Study 1

Of the 12 volunteers recruited, two females withdrew before the feeding commenced. During the first feeding period 2 withdrew after 2 days feeding, one (female having E028) because of persistent hunger and the other (male having Modulen-IBD) because of insomnia attributed to an empty stomach. Eight subjects completed the first phase. A further subject (male Modulen-IBD) withdrew after 4 days in the second phase because of malaise and headaches.

Stool consistency and frequency showed no change. There was a consistent change in stool colour from browns towards green on E028 extra ($r=0.639$, $p<0.05$ Spearman test), and a similar but less marked effect was seen after Modulen-IBD ($r=0.598$, $p<0.05$). Faecal colour had returned to normal by the start of the second feeding period.

All subjects showed deterioration in odour on E028 extra and 5 out of 6 on Modulen-IBD. One volunteer did not record his breath changes on a daily basis. A Spearman test showed a significant difference between the odour of the breaths of the volunteers before they started and the last day of the diet (E028 extra $r=0.575$ $p<0.05$, Modulen-IBD $r=0.574$ $p<0.05$). Subjects breath odour had returned to normal at the
start of the second feeding period. Numerical results are presented as mean with upper and lower quartiles. The frequency distributions for all compounds were found to be highly skewed with a proportion of nondetects; therefore, a nonparametric statistical approach was adopted.

Over 140 compounds were seen in the breath analysis including aldehydes, ketones, saturated and non-saturated hydrocarbons, organic acids, alkenes, alcohols and furans. The compounds also varied between volunteers. As at least one third of compounds were known to be environmental contaminants, e.g. benzene, toluene, xylene, we concentrated on two marker compounds known to be bacterial metabolites, phenol and indole.

The mean alveolar gradient for indole on a normal diet was $0.034 \pm 0.029$. There is little change following Modulen-IBD $0.041 \pm 0.028$ (NS). After E028 it rose to $0.149 \pm 0.099$ (NS) The differences between the values after diet did not differ significantly from those before, but the aveolar gradient after E028 was significantly higher than that after Modulen-IBD ($P<0.03$).

The mean level of alveolar gradient for phenol on the breath on a normal diet was $0.024 \pm 0.017$. After Modulen-IBD it rose to $0.055 \pm 0.025$ (NS). After E028 the levels were $0.229 \pm 0.152$ (p<0.05). The increase after E028 was significantly greater to that after Modulen-IBD $P=0.035$ After 3 weeks of normal eating, breath chemicals had in every case returned to levels indistinguishable from those present at the start of the first period of enteral feeding.

Results

Study 2:

At the start of treatment all 17 patients had active disease as confirmed by a Harvey and Bradshaw index of $>6$ and raised concentration of C-reactive protein (CRP) in the blood. 9 patients were receiving no medication, 4 were taking 5ASA compounds, 2 were taking 5ASA with Azathioprine, 1 taking
Azathioprine alone and 1 taking Azathioprine and Prednisolone. Patients were asked to continue the same medication throughout the study and this was not changed in any way, remission being achieved in all cases by the addition of enteral feed. The mean Harvey & Bradshaw (H&B) before treatment was 6.88 ± SD 2.93 falling to 4 ± SD 5.50 after treatment, (p<0.05). The initial mean CRP was 36.0 ± SD 41.3mg/L falling to 8.11 ± SD 3.59 after treatment (p<0.05).

The results of GC/MS faecal analysis are summarised in Table 1. Many compounds of known bacterial origin were present in the initial sample. These included propanoic and butanoic acids, para-cresol, indole, dimethyl disulphide and phenol. The concentrations of the SCFAs fell dramatically after enteral feeding. No difference was discerned in the fall of concentrations of bacterial metabolites in those subjects receiving enteral feeds alone, and those who continued their previous medication. Thus the results of all the patients were analysed together.

There were also however, a number of potentially toxic compounds present. These included the alcohols, 1-propanol and 1-butanol as well as the methyl and ethyl esters of propanoic acid and butanoic acid. After treatment, the amounts of these compounds also fell significantly. The SCFA-esters disappeared virtually completely and there was a significant fall in the concentration of 1-propanol and 1-butanol. However, other chemicals including those derived by bacterial breakdown of amino acids, phenol and indole did not change significantly (table 1).

**Discussion**

The present study demonstrates changes in chemicals of microbial origin in both healthy controls and in patients with CD after administration of enteral feeds. Our first study confirms reports of stool colour change during treatment with the development of breath odour. It is probable that this was the result of the cessation of the normal microbial breakdown of biliverdin (green) to stercobilin (brown).
We also attempted to assess bacterial activity by determination on the breath of known bacterial metabolites that might be absorbed into the blood stream from the colon. Many chemicals are present in breath and urine and we detected 140. Their origins of many are poorly understood. We therefore concentrated on changes in the excretion of two chemicals whose synthesis by the microbiota is well understood, namely phenol and indole.\textsuperscript{14,15}

Phenol and indole are produced by the microbial conversion of tyrosine and tryptophan respectively. Much less is produced when carbohydrate fermentation is continuing in the colon. Conversely, when carbohydrate was withdrawn from the diet, phenol production from endogenous protein sources such as intestinal secretions and exfoliated cells was increased\textsuperscript{17,18}

In the present study, phenol and indole identified on the breath showed a significant increase in concentration after feeding with Modulen-IBD and an even greater increase after E028extra, which rapidly returned to base line on resumption of a normal diet. This is consistent with a switch in colonic fermentation to a protein-based pattern, as an effect of ingesting carbohydrate in the form of maltodextrins - simple sugars that are absorbed high in the small intestine - rather than complex carbohydrates that may pass down to be fermented by the colonic flora. Indole is malodorous and may contribute to the unpleasant breath odour reported by our volunteers.

The term ‘enterometabolic disease’ has been suggested for non-infective conditions arising from abnormal fermentation by the colonic microbiota\textsuperscript{13}. Patients with IBS have a similar abnormal gut flora to that seen in CD\textsuperscript{11,20,23} and have a markedly increased excretion of a bacterial product, hydrogen. This was dramatically reduced, with highly significant reduction in symptoms, when patients were switched from a standard diet to an exclusion diet, suggesting that the diet reduced microbial activity\textsuperscript{24}. Support for this concept was provided by the demonstration of reduced hydrogen excretion in patients with IBS.
again with significant improvement in symptoms, when microbial activity was reduced by administration of antibiotics or by enteral feeding.\textsuperscript{25}

Is seems possible that CD like IBS may be an ‘entero-metabolic disorder’,\textsuperscript{13} and that enteral feeding is effective because it reduces the metabolic activity of an abnormal colonic flora?

There is strong evidence that the host microflora provokes an immunological response in CD. Duchmann and his colleagues showed that monocytes from the peripheral blood and the lamina propria were activated when incubated with preparations of faecal bacteria from other subjects, but not by such preparations derived from the faeces of the host. Monocyte activation occurred only when host faeces was incubated with cells obtained from the lamina propria from sites of active CD. No activation was seen in monocytes obtained from areas where no active CD was present, suggesting that monocytes in areas of active CD were specifically targeted against the host microflora.\textsuperscript{10}

This finding has been supported by later studies that demonstrated that the great majority of microorganisms found in the faeces of patients with IBD were coated with immunoglobulin, including IgA, IgG and IgM, whereas in normal subjects or those with IBS, less than 20\% were so affected\textsuperscript{12}. Furthermore, a significant reduction in the number of microorganisms coated with immunoglobulin was seen after 2 weeks treatment with corticosteroids in UC, and a similar response occurred in CD after a two week course of elemental feeding. This suggested that the immune response to the flora had been significantly reduced, an interpretation supported by the finding that patients with CD and UC in long term remission had similar numbers of coated bacteria to those seen in healthy controls.\textsuperscript{12}

No specific pathogen has as yet been confirmed as being the cause of CD, but it has been demonstrated that the faecal flora is abnormal with an overgrowth of facultative anaerobes and reduction in the numbers of important beneficial species such as \textit{Lactobacilli} and \textit{Bifidobacter}.\textsuperscript{11,20} Although previous studies of the effects of enteral feeding on the composition of the bacterial flora in CD, had been inconclusive,\textsuperscript{21,22}
a recent study of the entire gut mucosal microbiome in a child with CD before and after nutritional therapy showed that the flora, initially markedly abnormal, returned after therapy, to a pattern very similar to that found in a healthy control. Likewise, it has also been shown that enteral nutrition in CD may reduce the levels of certain bacteria within the *Firmicutes*. These bacteria are important producers of SCFAs and this report is in keeping with our discovery of reduced SCFA production.

Unfortunately, it was not possible in the present study to perform complex studies of changes in the gut microbiome, but changes in bacterial metabolites serve as valuable markers of its metabolic activity. SCFAs have an important function in the colon especially butanoic acid which is a major source of nutrition for colonocytes. They are produced by the microbial fermentation of undigested complex carbohydrates entering the caecum and the fall in faecal SCFA concentration found after enteral feeding in our patients with CD was consistent with reduction in colonic fermentation.

Such a reduction in fermentation might be beneficial if it resulted in less production of toxic metabolites. There were highly significant falls in the concentrations of number of chemicals including 1-propanol, p-cresol, phenol, 1-butanol, dimethyl disulphide and fatty acid ethyl esters (Table 1). These are known to be toxic chemicals which we have shown not to be present in the stools of healthy volunteers. It seems possible that the production of such chemicals might be a factor initiating an immune attack on the microflora. This could lead to coating of microflora with immunoglobulin – a suggestion which has been supported by the significant reduction in bacterial coating seen after 2 weeks feeding with enteral feeds.

Similar toxic chemicals also appear in UC, but in contrast to CD, do not fall after enteral feeding, but only after successful treatment by immunosuppression with prednisolone. Although evidence on the role of diet in UC remains weak, this suggests that the microflora in UC differs from that in CD in that it derives its nutritional requirements, not from food residues, but from other substances present in the large
intestine – possibly mucus or intestinal secretions. It is therefore feasible, that the production of toxic chemicals resulting from abnormal bacterial metabolism, may be an important factor in the initiation of an immune attack on the microflora in inflammatory bowel disease.

Reference List


13. Hunter JO. Food Allergy – or entero metabolic disorder Lancet 1991 338 495-6

15. Harvey RF, Bradshaw JM, A simple index of Crohn’s disease activity Lancet 1980 i 514


20. Sartor RB, Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases; antibiotics, probiotics and prebiotics. Gastroenterology 2004 126 1620-33


Acknowledgements:

This work was supported by the Wellcome Trust (Grant no. WT080238 MA).

Conflicts of interest: Professor J O Hunter has received grants for research and honoraria for speaking from both Nutricia UK and Nestle UK.
Table 1 Changes in faecal chemicals before and after elemental feeding in patients with CD.

<table>
<thead>
<tr>
<th>Compound</th>
<th>VOC concentration (ng/l)</th>
<th>Median (lower quartile, upper quartile)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treatment</td>
<td>Post-treatment</td>
<td></td>
</tr>
<tr>
<td>acetone</td>
<td>57 (38, 128)</td>
<td>80 (50, 104)</td>
<td>0.435</td>
</tr>
<tr>
<td>propanoic acid</td>
<td>169 (0, 328)</td>
<td>12 (0, 84)</td>
<td>0.031*</td>
</tr>
<tr>
<td>butanoic acid</td>
<td>1110 (316, 1596)</td>
<td>24 (0, 104)</td>
<td>0.001*</td>
</tr>
<tr>
<td>1-propanol</td>
<td>229 (41, 892)</td>
<td>36 (0, 233)</td>
<td>0.025*</td>
</tr>
<tr>
<td>propanoic acid, ethyl ester</td>
<td>19 (0, 117)</td>
<td>0 (0, 15)</td>
<td>0.008*</td>
</tr>
<tr>
<td>butanoic acid, methyl ester</td>
<td>19 (7, 121)</td>
<td>0 (0, 1)</td>
<td>0.013*</td>
</tr>
<tr>
<td>butanoic acid, ethyl ester</td>
<td>46 (4, 255)</td>
<td>0 (0, 15)</td>
<td>0.008*</td>
</tr>
<tr>
<td>p-cresol</td>
<td>518 (118, 1160)</td>
<td>480 (144, 1051)</td>
<td>0.687</td>
</tr>
<tr>
<td>indole</td>
<td>118 (54, 146)</td>
<td>20 (0, 128)</td>
<td>0.125</td>
</tr>
<tr>
<td>dimethyl disulphide</td>
<td>83 (34, 683)</td>
<td>39 (0, 140)</td>
<td>0.113</td>
</tr>
<tr>
<td>1-butanol</td>
<td>99 (57, 256)</td>
<td>58 (0, 199)</td>
<td>0.030*</td>
</tr>
<tr>
<td>butanoic acid, 3-methyl</td>
<td>147 (48, 504)</td>
<td>0 (0, 45)</td>
<td>0.015*</td>
</tr>
<tr>
<td>phenol</td>
<td>64 (16, 102)</td>
<td>24 (10, 177)</td>
<td>0.332</td>
</tr>
</tbody>
</table>

Concentrations of volatile organic compounds (VOCs) in fecal headspace from healthy controls and volunteers diagnosed with Crohn’s disease (CD) before and after treatment. Median and upper and lower quartile values are shown. Differences were examined by Wilcoxon matched pairs test, p<0.05 (two-sided) being considered significant and indicated by an asterisk.
Copy of the stool colour chart provided to volunteers in study 1. The colours are numbered from brown (1) to increasing greenness (5) the blue is just for the slide background. This is not for publication but to be made available for any readers who may enquire about it.

SUPPORTING INFORMATION

Declaration of funding interests:
The work for this study was funded by the Wellcome Trust Grant No. 080238/Z/06/Z. Professor Hunter has acted as a consultant and received research grants from Nutricia (UK) Liverpool and from Nestle.

STROBE STATEMENT
All items on the strobe checklist have been checked and confirmed to be included in this paper.