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Clinical trial: the microbiological and immunological effects of synbiotic consumption – a randomized double-blind placebo-controlled study in active Crohn's disease

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SUMMARY

Background
Crohn's disease is an inflammatory illness in which the immune response against gut microorganisms is believed to drive an abnormal immune response. Consequently, modification of mucosal bacterial communities, and the immune effects they elicit, might be used to modify the disease state.

Aim
To investigate the effects of synbiotic consumption on disease processes in patients with Crohn's disease.

Methods
A randomized, double-blind placebo-controlled trial was conducted involving 35 patients with active Crohn's disease, using a synbiotic comprising *Bifidobacterium longum* and Synergy 1. Clinical status was scored and rectal biopsies were collected at the start, and at 3- and 6-month intervals. Transcription levels of immune markers and mucosal bacterial 16S rRNA gene copy numbers were quantified using real-time PCR.

Results
Significant improvements in clinical outcomes occurred with synbiotic consumption, with reductions in both Crohn's disease activity indices ($P = 0.020$) and histological scores ($P = 0.018$). The synbiotic had little effect on mucosal IL-18, INF-γ and IL-1β; however, significant reductions occurred in TNF-α expression in synbiotic patients at 3 months ($P = 0.041$), although not at 6 months. Mucosal bifidobacteria proliferated in synbiotic patients.

Conclusion
Synbiotic consumption was effective in improving clinical symptoms in patients with active Crohn's disease.

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INTRODUCTION

Crohn's disease (CD) is a debilitating inflammatory illness of uncertain aetiology. However, in genetically susceptible individuals, an environmentally initiated immune response against microbial antigens may be involved in driving inflammatory processes associated with the disease. The Th1-mediated inflammatory response in CD is characterized by increased IL-18, INF-γ and TNF-α, formed by infiltrating mononuclear cells in the lamina propria. The incidence of CD is increasing in the UK and is approximately 5–10 per 100 000 p.a., with a prevalence of 50–100 per 100 000, although this is believed to be an underestimate.[1] CD is incurable, but typical maintenance treatments involve the use of elemental diets, anti-inflammatory drugs, steroids and surgery. Newer therapies involving the use of monoclonal antibodies (MAB) against TNF-α have been found to be effective in inducing remission in CD. However, they are expensive and are given systemically, which have raised concerns about increasing susceptibility to infection, as well as the body mounting an immune response to the MAB, resulting in declining effectiveness.

Increasing interest is being shown in the use of alternative therapies to treat inflammatory bowel diseases (IBD), particularly through the use of probiotics, prebiotics and synbiotics.[2–4] Probiotics are live microbial food supplements that benefit health through, for example, stimulation of immune function. The principal probiotics used in humans are bifidobacteria and lactobacilli. Prebiotics are usually non-digestible food ingredients, e.g. inulins and fructo-oligosaccharides (FOS) that selectively stimulate the growth and/or activities of bifidobacteria or lactobacilli in the colon, thereby improving health. A synbiotic is a synergistic combination of a probiotic and prebiotic.[5] The aim is to support the probiotic by providing a preferred carbon and energy source to promote its growth, as well as other beneficial organisms that are indigenous to the gut. The use of well-designed and tested probiotics and prebiotics to treat CD and other forms of IBD offers several potential advantages in that they are inexpensive, easy to administer, demonstrably safe and have few side-effects.

A few trials have been conducted using probiotics in CD patients, with varying degrees of success. One of the main reasons for this has been the use of inappropriate or poorly characterized organisms with unknown immunomodulatory properties. *Lactobacillus rhamnosus* GG has been well studied, and it was used in a randomized placebo-controlled study aimed at reducing the rate and/or severity of CD recurrence after surgery.[6] Results showed that recurrence rates were not significantly different between the probiotic and placebo groups. Similarly, *Lactobacillus johnsonii* LA1 was also found to be ineffective in preventing endoscopic recurrence of CD. In a 6-month trial, 64% of patients in the placebo group had recurrence of symptoms, compared to 49% in the probiotic group.[7] The yeast *Saccharomyces boulardii* has also been used to prevent relapse in CD.[8] After 6 months, clinical relapses occurred in 38% of patients receiving mesalazine (mesalamine), and in 6% of those receiving mesalazine and the probiotic. Non-pathogenic *Escherichia coli* Nissle 1917 was used to maintain remission in colonic CD,[9] but no differences in probiotic and placebo rates of remission were observed. However, patients receiving the probiotic entered remission sooner than the placebos. *In vitro* studies with different lactobacilli showed that the probiotics reduced TNF-α production in inflamed ileal tissue taken from CD patients, but had no effect on cytokine formation in normal mucosa.[10] To date, there have been few trials on the use of prebiotics to treat CD.

Two small open-label studies with prebiotics have shown significant improvements in clinical scores in children and adults with CD.[11, 12] In one small open-label study, high-dose probiotic and prebiotic therapy (cotherapy) was shown to induce remission in patients with active CD; however, four of the 10 patients involved were unable to tolerate the prebiotic and the daily intake was uncontrolled.[13]

We have previously shown in ulcerative colitis (UC) patients that short-term therapy with a synbiotic combination of *Bifidobacterium longum* isolated from healthy rectal epithelium, and the prebiotic Synergy 1, a preferential inulin/oligofructose growth substrate for the probiotic strain, resulted in increased levels of mucosal bifidobacteria and reduced levels of IL-1α and TNF-α in mucosal tissue, which were associated with a range of clinical benefits.[14] TNF-α is also an important inflammatory mediator in CD, and the aims of the present investigation were to assess the medium to long-term effects of synbiotic feeding on mucosal bacterial populations, TNF-α and other inflammatory mediators associated with active CD, to determine whether they could be translated into clinical improvements in the disease state.

METHODS

Patients

Consecutive patients with active CD, attending the Gastroenterology Outpatients Clinic, Ninewells Hospital,

Dundee, gave written consent to participate in this investigation. Each patient was assessed in the IBD research clinic using the Crohn's disease activity index (CDAI).[15] Individuals with a CDAI score between 150 and 450 aged between 18 and 79 years were eligible for admission to the trial. Patients were not admitted to the study for reasons such as (i) pregnancy, (ii) alterations to medication within the last 3 months, (iii) antibiotic treatment within the last 3 months, (iv) indeterminate colitis, (v) UC, (vi) short gut syndrome and (vii) use of commercially available prebiotic or probiotic preparations within the past 3 months. The trial protocol was assessed and approved by the Tayside Committee on Medical Research Ethics, Dundee (study number 05/S1401/111). Clinical trials registration: ClinicalTrials.gov NCT00305409.

Randomization, blinding and treatment

Thirty-five study numbers were assigned (CRH01 to 35) and randomized using a table of random digits[16] by an independent clinician who was not part of the investigation. All study personnel and participants were blinded to treatment assignment for the duration of the trial, and this was not divulged to the clinician, patient or in-house researchers who carried out the experimental measurements. To ensure success, the appearance of the synbiotic and placebo were identical, and were distributed to the clinician in charge by an independent administrator, who was not part of the study. Patient's treatment assignment, synbiotic or placebo, was the first free treatment (not yet allocated) on the randomization sheet. Patients were also requested to continue on stable doses of conventional CD medication they were receiving at initiation of the trial, and to complete an IBD lifestyle questionnaire at baseline, after 3 months and at 6 months, and to keep a daily bowel habit diary.[17] Patients were enrolled by the study clinician, and the CDAI was assessed at baseline, after 3 months and at 6 months. Flexisigmoidoscopy or colonoscopy was performed at all three time points, and areas of macroscopic inflammation and non-inflamed tissues were sampled from various regions of the large bowel. Patients were examined after a phosphate enema, to limit any disruption to colonic mucosal microbiota populations, while allowing adequate viewing of the colonic mucosa. Venous blood samples were taken for measurement of C-reactive protein, full blood counts, plasma viscosity and liver function tests (Department of Biochemical Medicine, Ninewells Hospital). Biopsies were also taken for histological scoring by the Department of Pathology (Ninewells Hospital). Additional biopsies were used for in-house assessment of mucosal inflammatory mediators and bacteriology.

Patients were given 2×10^{11} freeze-dried viable *B. longum* in a gelatin capsule, and a sachet containing 6 g of Synergy I (Orafti, Tienen, Belgium), twice daily for 6 months, or a placebo which was prepared as described previously.[14] The synbiotic/placebo was taken immediately after breakfast, and following the evening meal to minimize anti-bacterial effects of gastric acid on the probiotic.

Primary objective

The main outcome of the study was a reduction in mucosal TNF-α in patients receiving the synbiotic. Secondary outcomes were numbers of patients in remission in each group, as assessed by changes in CDAI to <150, or those with a drop in CDAI of >75 from baseline in the synbiotic vs. the control group. Failure end-points were an increase in CDAI by 100 points or a score >450, and clinical relapse requiring steroid prescription, hospital admission or surgery. Patients were withdrawn if they required antibiotics for any reason, or for non-compliance. Any adverse events were reported to the ethics committee that approved the study. Patients were also allowed to withdraw without giving a reason in accordance with the ethics committee guidelines.

Manufacture of the probiotic

The probiotic preparation was prepared and packaged at the microbiology laboratory in Ninewells Hospital, as described previously.[14] All microbial standardization, quantification and purity tests were checked by two independent microbiologists.

RNA, cDNA and DNA preparation

mRNA, cDNA and DNA were prepared and purified from biopsy materials using methods described previously, and stored for subsequent analysis at −80 °C.[14, 18]

Quantitative PCR

This was performed using an iCycler and the iQ SYBR Green Supermix (BioRad, Hercules, CA, USA).[14] Test samples were added in triplicate at 2 μL/well in a 20 μL total reaction volume. The appropriate plasmid preparation was diluted to give a standard curve of 10^6–10^1 molecules/μL for all assays, except for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which had a standard curve of 10^8–10^1 molecules/μL.

Transcription levels of pro-inflammatory cytokines in mucosal tissue

Cytokines that are usually elevated in CD, TNF-α, IL-18 and INF-γ, together with IL-1β were measured using qPCR, using appropriate primer sets as described previously.[14, 19, 20]

Microbiological analysis of tissue biopsies

Total eubacteria, the probiotic and other bifidobacterial species, and other mucosal organisms (enterobacteria, enterococci, bacteroides) were quantified from both inflamed and non-inflamed regions of the large bowel by qPCR, using previously validated PCR primer sets targeting specific regions of the 16S rRNA gene.[21–24]

Histopathology

Rectal biopsies were fixed, processed to paraffin, then stained with haematoxylin and eosin and visualized using standard methods. All biopsies were reported and scored by a single histopathologist, who was blinded to the treatment group, using a global histological disease activity scoring system. This system, which has been developed and used in previous trials, features acute and chronic architectural changes, epithelial damage and the presence or absence of inflammation, erosions, granulomas and ulcers.[25, 26] Additionally, a purely inflammatory activity score was calculated based on the number of inflammatory cells in the epithelium and lamina propria, and was used as a sub-score of the global histological disease activity score.

Statistical analysis

A power calculation was done based on experimental data obtained in measurements of mucosal TNF-α in IBD patients and healthy controls. To reduce the number of TNF-α molecules in the mucosa to normal levels, it was calculated that 19 patients would be needed in the test and control groups (38 volunteers in total) to give 90% power to detect the difference (one-sided) at a significance level of 0.05%. The statistical package GRAPHPAD PRISM Version 4 (La Jolla, CA, USA) was used for analysis. Significant differences between presynbiotic and postsynbiotic therapy groups and between the postsynbiotic and postplacebo groups were assessed for CDAI and histology using the Student's *t*-test (two-tailed), as the data were normally distributed. Cytokine and microbiological results were analysed using the Mann–Whitney test (two-tailed) for nonparametric analysis, because the data were not normally distributed. Analysis of patient ratings of the amount of stool passed over a 2-week period at baseline, 3 and 6 months was performed using the repeated measures ANOVA test. Differences were considered statistically significant at $P < 0.05$.

Chemicals

Unless stated otherwise, all chemicals were obtained from Sigma (Poole, Dorset, UK). Bacteriological culture media were purchased from Oxoid (Basingstoke, Hamps, UK).

RESULTS

Clinical outcomes

Patients were recruited from January 2006 until December 2008. A description of patient involvement in the study is given in Figure 1. Eighty-two patients volunteered to take part in the investigation, and of these, 35 were candidates for inclusion in the trial. One patient died during the investigation, and review of cause of death showed that it was due to a stroke. The study was not un-blinded at this time, as it was determined that death could not have been caused by the synbiotic (the patient was subsequently found to be in the placebo group at the end of the study). Six patients in the synbiotic group and five patients in the placebo group were lost to the 3-month follow up, with one patient in each group having worsening of disease symptoms, and two patients unable to tolerate the synbiotic. The patients who withdrew at 6 months did not supply a reason for withdrawal, had been on the synbiotic or placebo for over 3 months and reported no side or adverse effects. The dropouts and reasons for withdrawal from the study are supplied in Figure 1. Consequently, before breaking the randomization code, 24 patients were determined to be included in the final analysis; this resulted in 13 patients in the synbiotic group and 11 in the placebo group. Their characteristics are given in Table 1. Comparison of the baseline characteristics of the two groups showed that only the difference in weight was significant (Table 1); however, the placebo group had a higher CDAI ($P = 0.35$), which may have been due to their lower weight ($P = 0.03$) and higher CRP ($P = 0.12$). Results are expressed as differences in values recorded at baseline, and at months 3 and 6. With respect to CDAI, there were significant improvements in mean scores in the synbiotic group (start 219 ± 74.6, finish 147 ± 74, $P = 0.020$), but not in the placebo group (start 249 ± 79.4, finish 233 ± 155, $P = 0.810$), as shown in Figure 2. Nine of 13 patients receiving the synbiotic had reduced CDAI scores, with five patients having

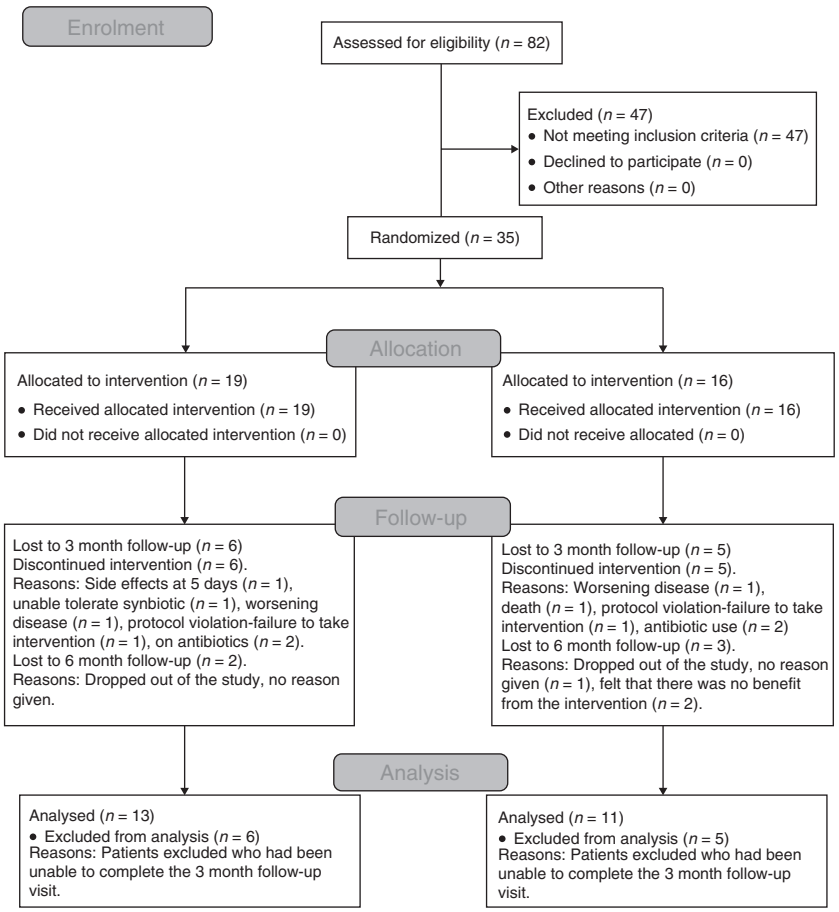

Figure 1 | Enrolment of patients included in the study.

reductions in CDAI of >100. In the analysed group, 62% of the synbiotics were in remission at the end of the study (eight of 13) and 45% of the placebos (five of 11). Histological analysis used data from areas with evidence of inflammation at the initial biopsy, and this zone was sampled consecutively. If no inflammation was found at endoscopy, all samples were averaged to give a mean result. Figure 3 shows that there were significant improvements in mean histological scores in the synbiotic group (start 6 ± 5, finish 3 ± 4, $P = 0.018$), though not in the in the placebos (start 6 ± 5, finish 5 ± 6, $P = 0.750$). Analysis of bowel habit diaries indicated that there were no significant improvements in the synbiotic or placebo groups during the trial ($P = 0.130$ and $P = 0.700$ respectively). There were no significant changes in any venous blood parameters. Similarly, there were no significant improvements in the IBD questionnaire in either group ($P = 0.230$ and $P = 0.300$ respectively). Analysis of patient ratings of the amount of stool passed over a 2-week period at baseline, and at 3 and 6 months, using the repeated measures ANOVA test, showed no differences in the subjective amount in either group. Similarly, analysis of stool consistency in a subjective manner indicated no changes in either patient cohort.

Inflammatory markers in inflamed and non-Inflamed tissues

All results shown for cytokines are normalized for epithelial cell numbers, as determined by levels of the housekeeping gene GAPDH. Figure 4 shows that there were significant differences in mRNA expression in inflamed tissue compared to macroscopically non-inflamed tissue in all of the patients who commenced the study at baseline. TNF-α was significantly increased in inflamed tissue ($P = 0.025$), while IL-18 was significantly increased in non-inflamed tissue ($P = 0.0002$). No significant differences were detected in the expression of INF-γ or IL-1β.

Effect of synbiotic consumption on inflammatory markers

Figure 5 shows inflammatory markers in patients with inflamed tissue at the start of the study who were

Table 1 | Baseline clinical characteristics of the patients included in the analysis data set

	Synbiotic (n = 13)	Placebo (n = 11)
Gender: Male/Female	7/6	6/5
Age (years)*	46.3 (33–71)	49 (20–78)
Smoker	2	2
Small bowel involvement	5	6
Weight (kg)*	84 (72–96)†	67 (58–77)
Initial Crohn's Disease Activity Index‡	219 ± 74.6	249 ± 79.4
Haemoglobin (g/dL)‡	13.4 (12.2–14.6)	13.1 (11.3–14.8)
Haematocrit‡	0.41 (0.38–0.44)	0.39 (0.34–0.43)
White cell count‡	7 (6–8)	6 (6–10)
Albumin‡	46 (44–48)	43 (40–46)
CRP (mg/L)‡	8 (3–13)	22 (3–42)
Plasma viscosity‡	1.71 (1.68–1.74)	1.67 (1.57–1.76)
Current medication		
Steroid	5	4
5-aminosalicylic acid	9	5
Azathioprine	5	1
Mercaptopurine	0	1
Elemental	1	0
PPI	1	0

* Mean (range)

† Statistically significant difference (P < .05) compared with the placebo group.

‡ Mean (95% confidence interval).

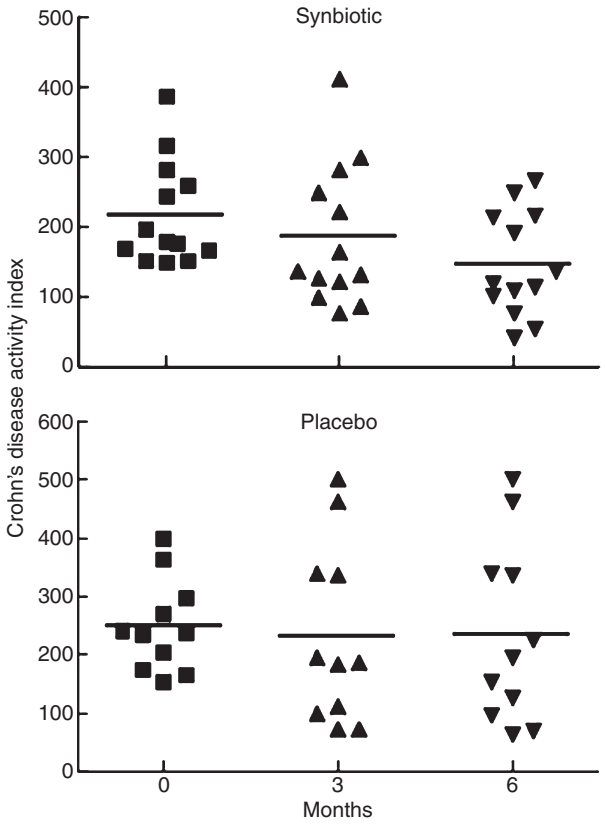

Figure 2 | Crohn's disease activity index (CDAI) values in synbiotic patients and placebos over the study period. Bars represent mean values. Values for patients who discontinued the treatment after 3 months of the study were maintained at 6 months for complete analysis of the treatment period. The significance on comparison of the CDAI values for pre- and postsynbiotic treatments at 6 months was P = 0.0200, compared with no significant change in the placebos (P = 0.810).

included in the final analysis. A significant reduction occurred in TNF-α expression in the synbiotic group at 3 months (P = 0.041), but the reduction was not significant at 6 months (P = 0.330). No significant differences were observed in the placebo group. No differences were seen in TNF-α in non-inflamed tissues at baseline in either group (data not shown), while no significant differences were observed in either group regarding expression of INF-γ, IL-18 and IL-1β in either inflamed or non-inflamed tissues.

Bacteriological analysis of mucosal tissue

Extensive bacterial colonization of mucosal tissues occurred in CD patients (Figure S1). While there was an apparent reduction in total bacterial numbers during the study period, this was not significant. Mucosal biopsies from both non-inflamed regions and inflamed regions of the gut were analysed to determine if there were differences in colonization that could be associated with inflammation at baseline. Figure 6 shows bacterial numbers from selected microbial groups that have been associated with inflammatory processes in IBD, expressed as percentages of total eubacterial counts. Results are shown as combined values from both inflamed and non-inflamed areas. Absolute numbers of bifidobacteria in both patient cohorts ranged between 10^5 and 10^6 per biopsy (results not shown). Significant increases in bifidobacteria were recorded in the synbiotic group, but not in the placebos. At the start of the study, bifidobacteria in the synbiotic group had a mean percentage of the total bacterial count of 1.1 ± 1.5, which increased to 5.5 ± 10.6 (P = 0.0475) and 6.5 ± 11.42 (P = 0.0259) at

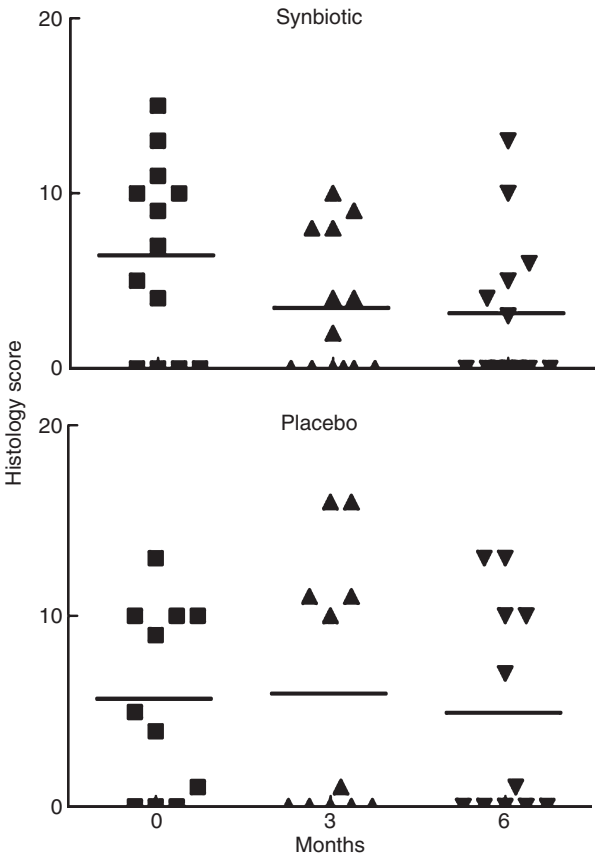

Figure 3 | Histology values in synbiotic patients and placebos over the study period. Bars represent mean values. Values for patients who discontinued the treatment after 3 months of the study were maintained at 6 months for complete analysis of the treatment period. Significance on comparison of the histology scores for pre- and postsynbiotic group at 6 months was $P = 0.018$, compared with no significant change in the placebos ($P = 0.750$).

3 and 6 months respectively. However, bifidobacteria predominated on the mucosa in some individuals. Similar increases were observed in non-inflamed tissues in the synbiotic group (Figure 6), which was approaching significance at 3 and 6 months ($P = 0.0725$ and $P = 0.054$ respectively), but not in the inflamed tissues. In the placebo group, there were significant reductions in bifidobacteria at 6 months ($P = 0.0302$), with a starting mean of $\log_{10}$ 2.5 $\pm$ 2.8 at baseline, which decreased to 2.2 $\pm$ 3.7 and 1.9 $\pm$ 2.6 at 3 and 6 months respectively.

Quantification of *Bifidobacterium longum* on the gut mucosa

The number of patients in which *B. longum* was detected in mucosal tissues at zero time was insufficient to allow statistical analysis to be performed. The organism was detected in only two patients at baseline in the synbiotic group; however, its prevalence increased with synbiotic consumption, being detected in eight patients at 3 months, and in 11 patients at 6 months (Figure 6). Naturally occurring strains of *B. longum* were detected in five patients in the placebo group at baseline, in two patients at 3 months, but in none at the end of the study.

Mucosal colonization by other bacteria

Bacterial prevalence on mucosal tissue by other organisms that have previously been linked to CD aetiology is shown in Figure 6. No significant differences were detected in mucosal bacteroides or enterobacteria, in either the synbiotic or placebo groups during the study. However, enterococcal numbers increased at 3 and 6 months in the synbiotic group, with a significant increase at 6 months ($P = 0.0368$). No differences were observed in the placebos or between inflamed and non-inflamed tissues (Figure 6).

Effect of synbiotic consumption on mucosal bifidobacteria

Figure 7 shows bifidobacterial numbers in mucosal tissue in individual patients in the synbiotic group who completed the study. Inflamed and non-inflamed tissues from the patients at baseline were compared in the same spatial sampling points in the gut during the course of the study. At 3 months, increased bifidobacterial colonization was found in six of eight patients who had inflamed tissue at zero time. In three of these individuals, bacterial numbers further increased at the 6-month time point. At 3 months, bifidobacteria also increased in non-inflamed tissues in these patients, in seven of the nine sampling zones, with numbers still increasing in three individuals up to the end of the study.

DISCUSSION

To our knowledge, this is the first DBRCT to show that synbiotic treatment can be of benefit to patients with CD. We had previously shown in short-term investigation lasting 4 weeks that the synbiotic used in the present work had therapeutic effects in patients with active UC.[14] Despite its successful outcome, results from that study suggested that 4 weeks of synbiotic consumption was not long enough for marked improvements in clinical outcomes to be manifested. The main objective of this investigation was to determine whether the same synbiotic combination could similarly benefit patients

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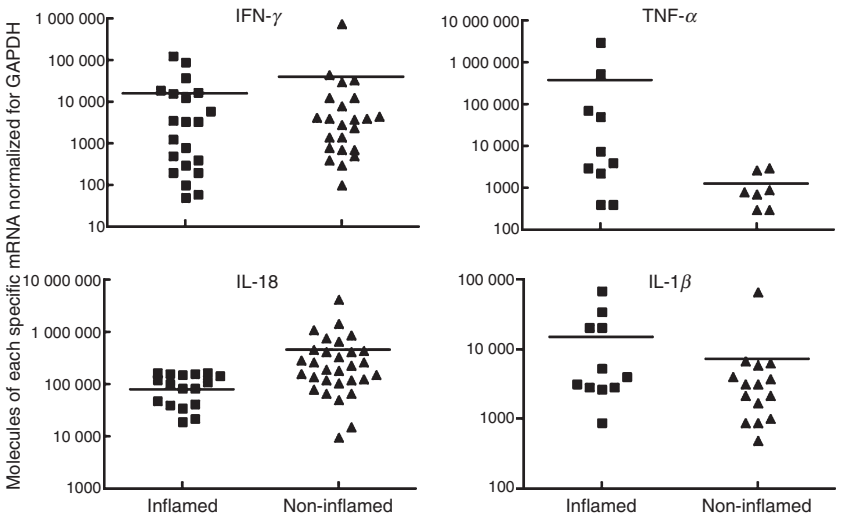

Figure 4 | IFN-γ, TNF-α, IL-18 and IL-1β mRNA in inflamed and non-inflamed biopsies from both patient groups at the start of the study. Results are normalized for total epithelial cells per biopsy, through expression levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Bars represent mean values. Significant differences were found in TNF-α (P = 0.025) and IL-18 (P = 0.0002).

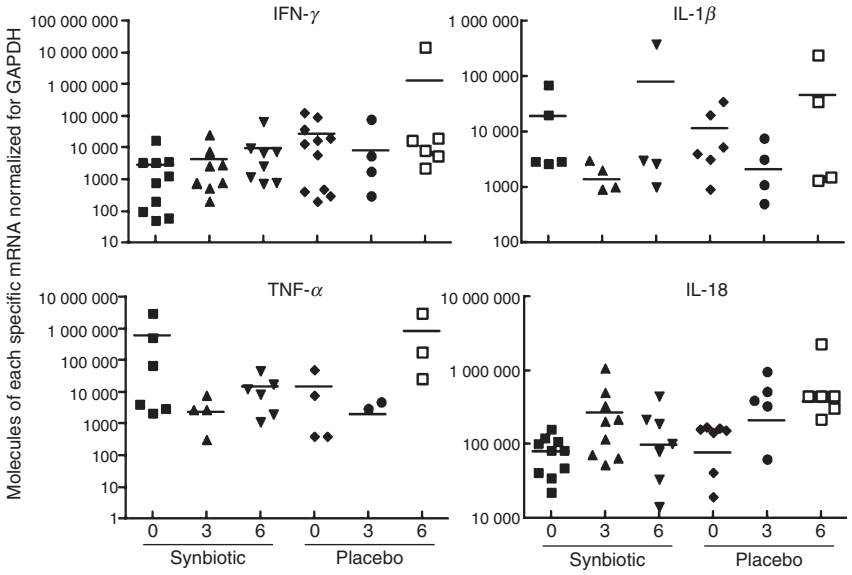

Figure 5 | IFN-γ, TNF-α, IL-18 and IL-1β mRNA in inflamed mucosal tissues at the start of the study, and after 3 and 6 months consumption of the synbiotic or placebo. Bars represent mean values. Results are normalized for total epithelial cells per biopsy through expression levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). TNF-α (P = 0.025) and IL-18 (P = 0.0002) were significantly different in the synbiotic group.

with active CD over a longer timeframe. Daily synbiotic consumption over the 6-month experimental period resulted in significant improvements in clinical symptoms and in histological scores in patients receiving the therapy. However, in contrast to the UC study, this was not associated with marked improvements in the way patients felt the disease impacted on their way of life, despite the fact that many of the synbiotic patients went into clinical remission. It was notable that despite significant improvements in clinical scores, four patients in the synbiotic cohort continued to have active disease and did not go into full remission (CDAI <100). This may be a

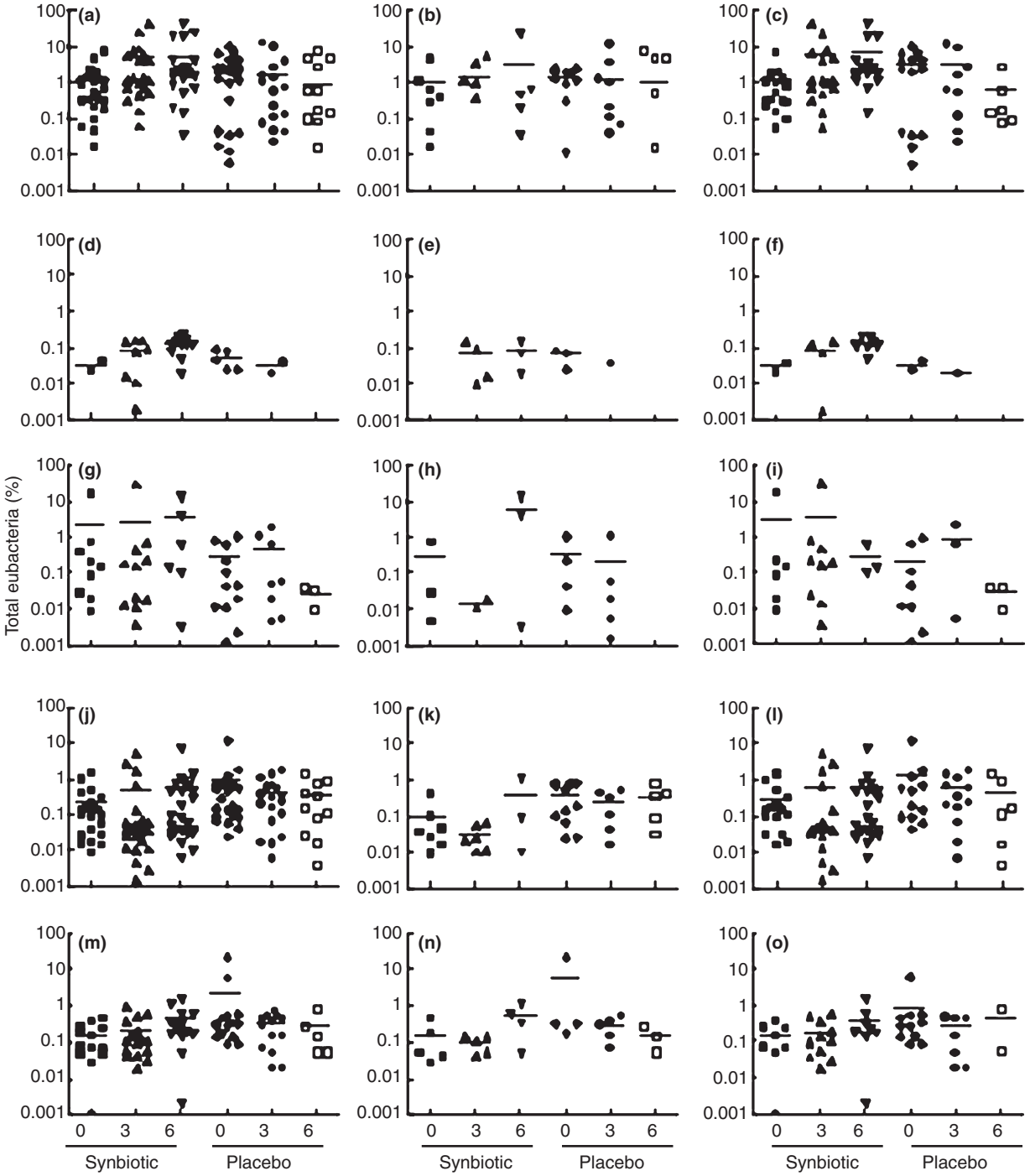

Figure 6 | Bacterial colonization of mucosal tissues at zero time, and after 3 and 6 months' consumption of the synbiotic or placebo. Bars represent mean values. Results are expressed as percentages of total eubacteria per biopsy. Total bifidobacteria (a–c), *Bifidobacterium longum* (d–f), total bacteroides (g–i), total enterobacteria (j–l) and total enterococci (m–o) were measured consecutively in the same region of the gut. Panels on the left show combined values for tissues that were both inflamed and non-inflamed at the start of the study. The middle panels and panels on the right show values from inflamed and non-inflamed tissues respectively. In the synbiotic group, significant increases were found in total bifidobacteria at 3 (P = 0.0475) and 6 (P = 0.0259) months, and in total enterococci at 6 months (P = 0.0368).

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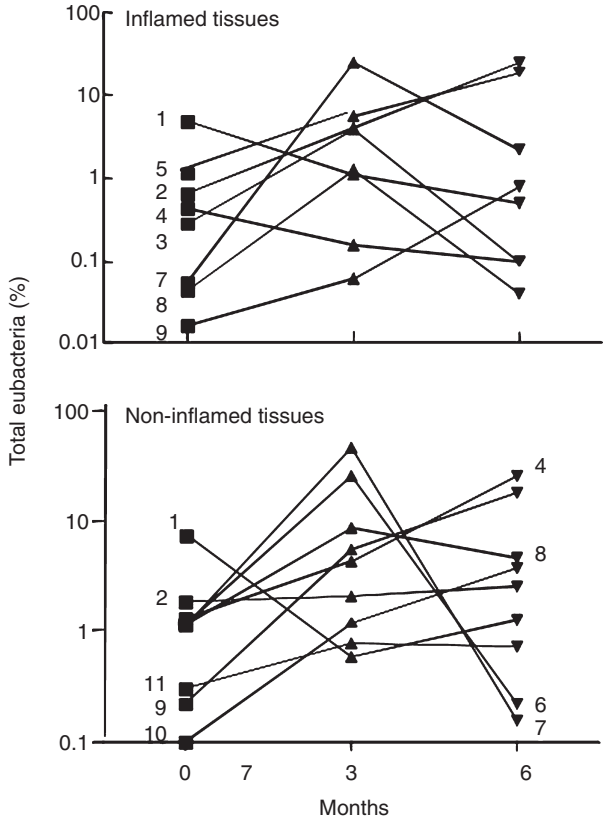

Figure 7 | Patterns of bifidobacterial colonization in mucosal tissue in the synbiotic group. Results are expressed as a percentage of total eubacteria per biopsy. The same region of the gut was sampled consecutively in individual patients. Data are from tissues that were inflamed at the start of the study and from non-inflamed tissues. Individual patients are labelled 1–1.

reflection of the numbers of patients with concomitant small bowel disease, which were included in the trial (four patients; two having CDAI>150 at the end of the study), and suggests that as with UC, the synbiotic may be more beneficial in Crohn's patients in which there is primarily colonic involvement. All patients included in the study had a CDAI of 150–450 and had active CD, and there was no significant difference in baseline CDAI between the synbiotic and placebo groups. Remission is usually taken as <150, mild disease as 150–220, moderate 220–450 and >450 severe disease. Clinical trials in CD that focus on therapies such as anti-TNFα therapy may have significant side-effects, thus are more appropriate for second or third line treatments, or in patients with moderate-to-severe disease. Probiotics and prebiotics are generally regarded as safe. The synbiotic used in the study had been used safely with no side-effects in a clinical trial in patients with active ulcerative colitis and therefore would be suitable as a first line treatment for patients with all grades of disease activity.

Currently, CDAI is the gold-standard for trials aimed at assessing clinical activity and it is based on eight criteria, including wellbeing, abdominal pain, stool habit, extraintestinal manifestations, bodyweight, haematocrit, the presence of an inflammatory mass and the use of codeine. Therefore, as found in this trial, patients with a high CDAI may not have macroscopic areas of inflamed tissue at endoscopy. Previous studies have shown that although segments of the gastrointestinal tract in Crohn's patients appear to be macroscopically un-inflamed, they can still have elevated levels of pro-inflammatory cytokines.[27] IL-6 and IL-1β have been shown to occur in higher concentrations in apparently non-inflamed tissues in CD,[27] and IL-18 has been found to be only activated in inflamed tissue in a minority of patients with the disease.[28] Differences occurred in pro-inflammatory cytokine expression in inflamed and non-inflamed segments of the gut in the Crohn's patients in this study. Our results indicated that TNF-α mRNA was significantly higher in inflamed tissues, and that IL-18 was more expressed in non-inflamed biopsy materials, with no differences being seen with IL-1β. Therefore, measurements of pro-inflammatory cytokines in inflamed and non-inflamed tissues would seem to be necessary to reveal the true patterns of inflammatory disease and the global effect of therapeutic treatment for CD.

This trial also demonstrated that the synbiotic reduced TNF-α in mucosal tissues to a significant degree. Expression of this cytokine was reduced in inflamed mucosa at 3 months, but not in non-inflamed tissues in the synbiotic patients. No distinctions were evident in the placebo group. One of the characteristics that determined our choice of probiotic was the ability of *B. longum* to reduce TNF-α *in vivo*, and *in vitro* in human intestinal cell lines, and in our previous UC study, the synbiotic significantly reduced levels of mucosal TNF-α.[14] Only one other investigation has shown that increases in numbers and metabolic activities of indigenous mucosal immunomodulatory bifidobacteria in healthy people can also be induced by prebiotic consumption,[29] and in one open-label trial involving 10 patients with active CD, FOS was shown to reduce the Harvey–Bradshaw index, increase faecal bifidobacteria and increase mucosal bifidobacteria in patients who went into remission.[12] Therefore, the two components of the synbiotic used in this study may have been acting in synergy to alleviate inflammation in the CD patients. This notion is supported by the fact

that there were massive increases in bifidobacterial species other than *B. longum* in patients receiving the synbiotic.

Although the aetiology of CD remains unclear, evidence suggests involvement of intestinal bacteria, and studies have shown that CD patients have higher concentrations of bacteroides, fusobacteria, enterococci, *E. coli* and lower numbers of bifidobacteria, lactobacilli, eubacteria, *Clostridium coccoides*, *Clostridium leptum* and *Faecalibacterium prausnitzii* than healthy people, and that on remission, faecal bacteroides populations are altered.[30–35]

The current investigation demonstrated that synbiotic consumption was effective in introducing beneficial bacteria into the gastrointestinal tract in Crohn's patients, thereby modulating the species composition of the mucosal biofilm in the large bowel. Although it was not possible to distinguish the probiotic strain from other *B. longum* strains detected on the gut mucosa, there was increased colonization with *B. longum*, and other bifidobacterial species were able to colonize intestinal mucosal surfaces in a majority of patients receiving the synbiotic. Bifidobacteria increased significantly in proportion to other mucosal species, reaching levels as high as 20% of the total eubacterial count in three individuals, while in one patient, bifidobacteria comprised 45% of the mucosal microbiota after 3 months of synbiotic consumption. Enterococcal numbers were also shown to increase significantly at 6 months in the synbiotic group, which may be partly due to the lower levels of these organisms found at baseline compared with the placebos. Although only a few selected groups of bacteria that might play a role in CD aetiology were examined in the study, the increased presence of the probiotic and other bifidobacteria could be out-competing other pathogenic species involved in the disease, down-regulating pro-inflammatory cytokine pathways and stimulating a more immunomodulatory and tolerant immune response. Therefore, manipulation of the microbiota to increase the numbers of beneficial organisms, and reduce putatively harmful bacteria, might reduce the inflammatory trigger.

In conclusion, this long-term placebo-controlled investigation has provided evidence that synbiotics have the potential to be developed into acceptable therapies for acute CD. Further studies are now needed to determine whether the synbiotic modulates other anti-inflammatory components of the mucosal microbiota, such as *F. prausnitzii,* which has recently been shown to be stimulated by the prebiotic inulin,[36, 37]a component of the synbiotic, or whether other synbiotic combinations can be as effective, or more effective in IBD therapy.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article;

Figure S1. Total eubacterial counts in inflamed and non-inflamed mucosal tissue before and at 3- and 6-month consumption of the synbiotic or placebo.

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