

# Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients<sup>1–4</sup>

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## ABSTRACT

**Background:** Animal studies suggest that prebiotics and probiotics exert protective effects against tumor development in the colon, but human data supporting this suggestion are weak.

**Objective:** The objective was to verify whether the prebiotic concept (selective interaction with colonic flora of nondigested carbohydrates) as induced by a synbiotic preparation—oligofructose-enriched inulin (SYN1) + *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium lactis* Bb12 (BB12)—is able to reduce the risk of colon cancer in humans.

**Design:** The 12-wk randomized, double-blind, placebo-controlled trial of a synbiotic food composed of the prebiotic SYN1 and probiotics LGG and BB12 was conducted in 37 colon cancer patients and 43 polypectomized patients. Fecal and blood samples were obtained before, during, and after the intervention, and colorectal biopsy samples were obtained before and after the intervention. The effect of synbiotic consumption on a battery of intermediate biomarkers for colon cancer was examined.

**Results:** Synbiotic intervention resulted in significant changes in fecal flora: *Bifidobacterium* and *Lactobacillus* increased and *Clostridium perfringens* decreased. The intervention significantly reduced colorectal proliferation and the capacity of fecal water to induce necrosis in colonic cells and improve epithelial barrier function in polypectomized patients. Genotoxicity assays of colonic biopsy samples indicated a decreased exposure to genotoxins in polypectomized patients at the end of the intervention period. Synbiotic consumption prevented an increased secretion of interleukin 2 by peripheral blood mononuclear cells in the polypectomized patients and increased the production of interferon  $\gamma$  in the cancer patients.

**Conclusions:** Several colorectal cancer biomarkers can be altered favorably by synbiotic intervention. *Am J Clin Nutr* 2007;85:488–96.

**KEY WORDS** Prebiotic, probiotic, synbiotic, cancer, biomarkers, genotoxicity

## INTRODUCTION

Mortality from colorectal cancer is second only to that from lung cancer in men and breast cancer in women and has shown little sign of decreasing in the past 20–30 y (1, 2). Diet makes an important contribution to colorectal cancer risk (3), which implies that risks of colorectal cancer are potentially reducible.

Evidence from a wide range of sources supports the view that the colonic microflora are involved in the etiology of colorectal cancer (4, 5). For example, intestinal bacteria can produce substances from dietary components that have genotoxic, carcinogenic, and tumor-promoting activities, and human feces have been shown to be genotoxic and cytotoxic to colon cells. Furthermore, studies in germ-free rats have shown lower concentrations of DNA adducts and a lower incidence of chemically induced tumors than in rats with an intact microflora. Although the precise bacterial types associated with colorectal cancer risk have not been elucidated, it is clear that some bacterial groups (eg, lactobacilli and bifidobacteria) have much lower activities of enzymes that can generate carcinogens than do other gut microflora components such as clostridia and *Bacteroides* (6, 7). This suggests that the balance of microbial types in the gut is important in terms of colorectal cancer risk and has led to the research of dietary factors that can modulate beneficially the intestinal microflora. Studies have shown that prebiotics (nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth or activity of one or a limited number of resident colonic bacteria; 8) and probiotics (live microbial food

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ingredients that may be beneficial to health, such as lactobacilli or bifidobacteria) increase the proportion of bifidobacteria, lactobacilli, or both in the gut and modulate microflora metabolism (9, 10). For a comprehensive review of the existing evidence (considerably stronger from animal than from human studies) of the protective effects of these factors on colon cancer, see the review articles of Rafter (11) and Pool-Zobel (12). In summary, it has been shown that strains of lactic acid-producing bacteria and certain prebiotics inhibit carcinogen-induced DNA damage in the rat colon (13, 14) and that both probiotics and prebiotics have been shown to suppress preneoplastic lesions and tumors in the colons of rats treated with chemical carcinogens (15–17). Furthermore, evidence suggests that synbiotics (combinations of probiotics and prebiotics) are more effective than are either probiotics or prebiotics alone (18) and that a mixture of probiotic strains may be more effective than the individual strains (19). These findings justified a phase 2 anticancer study with a synbiotic food supplement containing the prebiotic SYN1 (oligofructose-enriched inulin) and the probiotics *Bifidobacterium lactis* Bb12 and *Lactobacillus rhamnosus* GG. We describe this first randomized, double-blind, placebo-controlled trial in polypectomized and colon cancer patients. A comprehensive battery of state-of-the-art biomarkers for colon cancer was used, which included genotoxic, cell kinetic, inflammation, and immunologic markers.

It was hoped that this trial would provide much-needed information on the potential antitumorigenic effects of synbiotics in humans and, through the use of a large number of biomarkers, clues to the underlying mechanisms.

## SUBJECTS AND METHODS

### Patients and study design

A 12-wk randomized, double-blind, placebo-controlled trial of a synbiotic for reduction in cancer risk biomarkers was carried out at the Mercy University Hospital, Cork, Ireland. This study was evaluated and approved by the Cork University Hospitals Ethics Committee.

Subjects who met the inclusion and exclusion criteria of the study were identified from the patient lists of consultant gastroenterologists. To be eligible for inclusion, the subjects had to have had biopsy and histologically confirmed adenomatous polyps or to have undergone resection for histologically confirmed colon cancer within the past 5 y; both retrospective and prospective polypectomy and colon carcinoma-resected subjects were eligible. To observe change (improvement or worsening of the anticancer markers) resulting from the dietary treatment over time, it was decided to evaluate the anticancer potential of synbiotics in 2 groups of subjects: colon cancer patients and polypectomized patients who were at increased risk of colon cancer. It was not the goal of the study to evaluate whether both types of subjects would interact differently or not on the food ingredients. Patients were excluded from the study if they were aged >75 y, were pregnant or had a desire to become pregnant during the study period, had a known lactose intolerance, had clinically significant immunodeficiency, were considered to be poor clinic attendees, were unlikely for any reason to be able to comply with the trial, were concomitant drug users (eg, COX-2 inhibitors or other selected nonsteroidal anti-inflammatory drugs), had been taking antibiotics within the previous month or were likely to

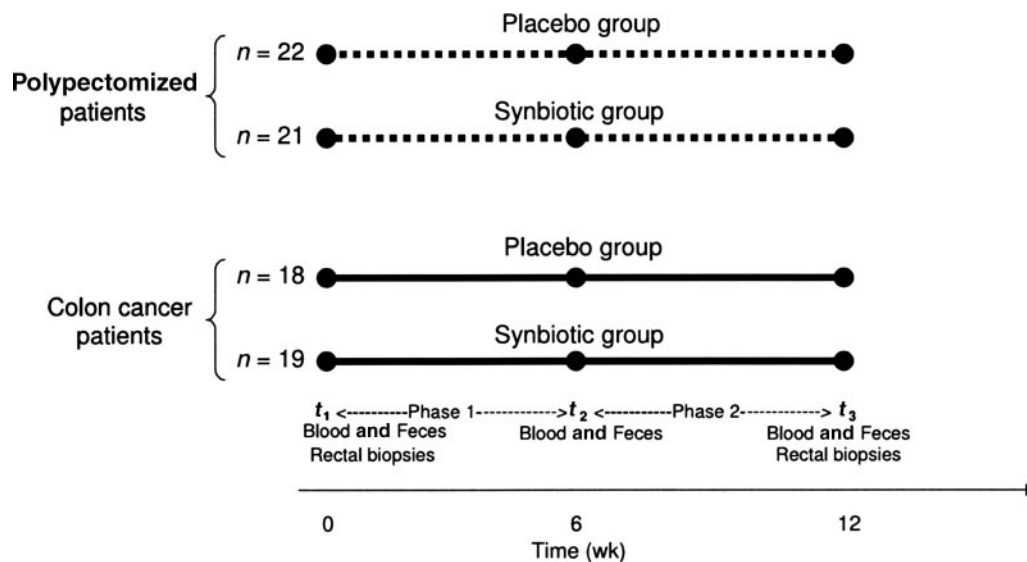
require antibiotics during the trial period, had other gastrointestinal disorders (eg, Crohn disease or ulcerative colitis), or had a malignancy or any concomitant end-stage organ disease. Randomization was performed by an individual unconnected with the study using a random number table to generate the randomization sequence.

The subjects attended appointments with the study nurse in her consulting room on day 0, before the intervention began ( $t_1$ ); after 6 wk of the intervention ( $t_2$ ); and after 12 wk of the intervention ( $t_3$ ). Blood and fecal samples were obtained at  $t_1$ ,  $t_2$ , and  $t_3$ . Colorectal biopsy samples were obtained from normal appearing mucosa at  $\approx 15$  cm from the anal verge by sigmoidoscopy at  $t_1$  and  $t_3$ , without bowel preparation. The subjects were assigned randomly to a synbiotic group or to a placebo group, and they kept a 6-wk diary for each phase of the intervention. At  $t_1$  and  $t_2$ , the subjects received a numbered box containing sufficient product for 6 wk. The subjects were interviewed at  $t_2$  and  $t_3$ , and any reactions to the product or to the medications taken and any adverse events that had occurred in each 6-wk period were recorded. The amount of product returned was recorded to confirm compliance.

A total of 80 subjects were recruited to the intervention: 37 cancer patients (6 Dukes A, 17 Dukes B, and 14 Dukes C) and 43 polypectomized patients. The inclusion number (goal:  $n = 20$ ) was not based on a power calculation, because this was the first study of its kind and the size of the effect on the various biomarkers was not known. The decision was based on consensus. The subjects were recruited in a staggered way, starting in December 2001. The last subject finalized the 3-mo intervention in January 2003. Of the cancer subjects, 34 completed the trial: 13 were women and 21 were men with a mean ( $\pm$ SD) age of  $60.1 \pm 5.8$  and  $62.1 \pm 5.3$  y, respectively. Of this group, 15 subjects received placebo and 19 subjects received synbiotic treatment. Of the polypectomized subjects, 40 completed the trial: 18 were women and 22 were men with a mean ( $\pm$ SD) age of  $56.0 \pm 9.8$  and  $58.0 \pm 9.7$  y, respectively. Of this group, 21 subjects received placebo and 19 subjects received synbiotic treatment. Six subjects (3 cancer patients and 3 polypectomized patients) withdrew from the study: 3 cancer patients and 1 polypectomized patient from the placebo group and 2 polypectomized patients from the synbiotic group. No adverse effects of the intervention were reported.

### Products

Treatment consisted of a synbiotic preparation that contained the prebiotic BeneoSynergy1 (ORAFIT, Tienen, Belgium) and the probiotic strains *Bifidobacterium lactis* Bb12 and *Lactobacillus delbreuckii* subspecies rhamnosus strain GG. BeneoSynergy1 (SYN1) is an oligofructose-enriched inulin preparation. Inulin is a natural food ingredient that is extracted from the chicory root with hot water. It is a linear  $\beta(2-1)$ -linked fructan with a degree of polymerization (DP) ranging from 3 to 65. Inulin chains with a DP of 2–8 (average DP: 4) are oligofructoses, which are highly soluble in water (>80%, by wt) and are rapidly fermented. The chains with a DP > 12 (average DP: 25) are hardly soluble in water (<5% in water at room temperature) and are fermented slowly. Both fractions are produced on a commercial scale as food ingredients worldwide. It was shown that a mixture of the 2 fractions is physiologically more efficacious than are the individual compounds (20).



**FIGURE 1.** Overview of the tests carried out on the colonic biopsy samples and in the blood, feces, and fecal water from the polypectomized and colon cancer patients in the dietary intervention trial with synbiotics.

BeneoSynergy1 is a commercialized food ingredient composed of a mixture of long-chain inulin and short-chain oligofructose. The product contains 95% fructan chains and 5% monosaccharides and disaccharides (fructose, glucose, and sucrose). Besides carbohydrates, the product contains 5% humidity.

As was the case with the prebiotics, it was decided to take a cocktail of probiotics to maximize the probability of efficacy. The probiotics taken up in the cocktail were selected on the basis of published evidence on anticancer properties in experimental models, on availability, and on shelf-life stability. In the SYNCAN project, it was not foreseen to produce certain strains or to develop the technology to produce them in sufficient quantities in a stabilized highly viable dry form. We screened for commercial strains that were frequently mentioned in papers on anticancer properties of probiotics. We selected *Bifidobacterium lactis* Bb12 (BB12) from Christian Hansen labs in Denmark and *Lactobacillus delbreuckii* subsp. rhamnosus strain GG (LGG) from Valio in Finland. They were both available as lyophilized highly viable powders ( $> \log_{10} \text{CFU/g}$  product). It was observed that the shelf-life of the *Lactobacillus* strain decreased up to 2 log units/wk when mixed with the prebiotic powder. Presumably, the low moisture content was sufficient to start the metabolism of the bacteria but was not sufficient to keep the bacteria viable. It therefore was decided to administer  $\log_{10}$  colony forming units (CFU) of both probiotics in a capsugel, which had to be taken together with the prebiotic to recompose the synbiotic in situ.

As a control, the capsugels were filled with maltodextrins (Glucidex IT38; Roquette Freres, Lestrem, France). Hydroxypropyl methylcellulose capsules were soaked in Eudragit L30 D-55 (an aqueous dispersion of a copolymer consisting of methylacrylic acid and methacrylates; Unigene Laboratories Inc, Fairfield, NJ), which forms an acid-resistant coating that is only soluble in an aqueous environment at a  $\text{pH} > 5.5$ . This prevented the bacteria from being liberated in the acidic environment of the stomach. Thus, the probiotics were given maximal chance for survival during their passage through the upper intestinal tract. The subjects were administered a daily sachet of 12 g SYN1 together with the probiotic capsule (synbiotic).

### Healthy Human Transit Study

To test the probiotic recovery and survival after transit through the intestine, the Healthy Human Transit Study was performed. Spontaneously occurring rifampicin-resistant ( $\text{rif}^{\text{R}}$ ) mutants ( $50 \mu\text{g/mL}$ ) were selected from Bb12 and LGG, freeze-dried, encapsulated, and administered ( $10^{10}$  CFU) to 3 subjects together with a 12 g sachet of SYN1 for 7 d. The number of  $\text{rif}^{\text{R}}$  bacteria present in the feces before and after 7 d of feeding was determined by using the standard plate count technique (21).

### Biomarkers and testing methodologies

An overview of the tests carried out in the colonic biopsy samples, blood, feces, and fecal water from the subjects in the dietary intervention trial with synbiotics is presented in **Figure 1**.

### Fecal flora composition

The total amount of stool from one passage was collected in a plastic container, and a fecal flora analysis was performed by using the standard plate count techniques as previously described (21).

### Fecal calprotectin

Mucosal inflammation and increased concentrations of fecal calprotectin is evident in patients with inflammatory bowel disease or cancer of the large bowel (22). Calprotectin was extracted from total feces according to a generally accepted procedure (23). Briefly, 100 mg human feces was vortex mixed and homogenized in extraction buffer at a weight-volume ratio of 1:50 and then centrifuged. The calprotectin concentrations in the fecal supernatant fluid were assessed by using a calprotectin enzyme-linked immunosorbent assay (Calpro AS, Oslo, Norway). The calprotectin concentrations in the fecal samples were calculated from the standard curve obtained with the standards. The concentrations of extracted calprotectin were expressed as  $\mu\text{g/g}$  stool.



## Toxicity of fecal water

### Fecal water preparation

Fecal water was prepared from the total feces of one passage, as previously described by Osswald (24). Feces were collected, stored cool until preparation, and then mixed in a 1:1 (by wt) dilution with ice-cold DMEM to increase volume and total yield of fecal water (25). The resulting suspensions were homogenized twice for 3 min, and the diluted homogenates were centrifuged at  $60\,000 \times g$  for 2 h at 4 °C. Supernatant fluid was collected and filtered through a 0.2- $\mu\text{m}$  pore size filter (Nalgene; Nalge Europe Ltd, Neerijse, Belgium), and aliquots (1 mL) were stored at  $-80\text{ }^{\circ}\text{C}$  until used.

### Induction of DNA damage by fecal water

The DNA-damaging capacity of fecal water was analyzed by using HT29 clone 19a cells (26). The cells were harvested and adjusted to a concentration of  $2.5 \times 10^6$  cells/mL. They were pelleted by centrifugation (8 min,  $400 \times g$ , room temperature), and cell pellets were then suspended in equal volumes of fecal water and prepared as described above. DMEM was used as the negative control, and 75  $\mu\text{mol/L}$   $\text{H}_2\text{O}_2$  was used as the positive control. The tubes were incubated for 30 min at 37 °C with shaking. Cell numbers and viabilities were determined with trypan blue in 12- $\mu\text{L}$  aliquots of the suspensions.

### Effects of fecal water

Effects of fecal water samples on tight junction permeability was monitored *in vitro* by measuring changes in the transepithelial resistance (TER) over 48 h and of mannitol flux in Caco-2 cells in monolayer as described previously (27). The capacity of fecal water samples to alter the invasive potential of HT115 human fibrosarcoma colorectal cancer cells was determined as described previously (27). HCT116 cells were exposed to fecal water (1:10 dilution, by vol) for 2 h. Cells were labeled with annexin V-FITC and propidium iodide according to the manufacturer's instructions (Caltag Laboratories, Burlingame, CA). Ten thousand cells were analyzed by flow cytometry in a FACscan instrument (Becton Dickinson, Mountain View, CA). The quadrants were set with untreated cells, and the same settings were used for all experiments (28).

## DNA damage in colorectal biopsy samples

Cells were isolated by first mincing the biopsy samples with fine scissors and incubating the resulting suspension with 6 mg proteinase K and 3 mg collagenase in 3 mL Hank's Balanced Salt Solution (HBSS) for 30 min in a shaking water bath at 37 °C (29, 30). The suspensions were then diluted with HBSS to a volume of 15 mL and centrifuged for 6 min at  $139 \times g$ . The pellets were resuspended in 6 mL HBSS for further processing. Viability and cell yield were determined with trypan blue, and cell number was adjusted to  $0.2 \times 10^6$  cells/mL before the analysis of DNA damage with the comet assay. For the comet assay, the cell pellets were mixed with agarose and spread on glass microscope slides. Single-cell gel electrophoresis (alkaline version of the comet assay) was carried out as described previously (30). DNA migration (tail length) was used as the basis for evaluation and was quantified by using the image analysis system of Perceptive Instruments (Halstead, United Kingdom); 50 DNA spots per slide were evaluated. Each data point consisted of 6 (colon cells

from biopsy samples) or 3 (HT29 clone cells treated with fecal water) parallel slides in one assay (*see above*).

## Proliferative activity in colorectal biopsy samples

Proliferative activity was assessed in polyp patients at  $t_1$  and  $t_3$  by *in vitro* [ $^3\text{H}$ ]thymidine incorporation and autoradiography in  $\geq 2$  colorectal biopsy samples as described previously (31). Only full longitudinal crypt sections (ie, from the base to the bottom of the crypt) were scored. For each crypt, we recorded the number of cells per crypt column (ie, the number of cells from the bottom to the top of the half crypt appearing in the section) and the number and position of the labeled cells along the crypt, dividing each crypt into 3 equal compartments: lower, middle, and upper. For each subject, proliferative activity was expressed as the Labeling Index (LI; the number of labeled cells counted in all the crypts of the same subject/number of cells in all the crypt sections of the same subject  $\times 100$ ). The distribution of proliferative activity along the crypt was calculated for each patient by dividing the total number of labeled cells in each compartment (lower, middle, and upper) of all the crypts scored by the total number of labeled cells scored in the crypts and multiplying this value by 100 (%LI in each compartment of the crypt). The microscopic slides were coded and read independently by 2 observers.

## Measurement of immunologic functions in peripheral blood mononuclear cells and in fecal water

Standard immunologic assays were applied to assess immune functions of peripheral blood mononuclear cells (PBMCs) and to quantitate prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ) and transforming growth factor  $\beta$  ( $\text{TGF-}\beta$ ) concentrations in fecal water (32–34).

## Statistical analysis

Data obtained from individual patients in the different intervention groups were summarized for quantitative continuous responses by calculating group means and SDs. Generalized linear modeling (GLM) was used for all biomarkers (Tables 1 and 2). Statistical calculations were done with the PROC-MIXED module in the SAS software package (version 9.1; SAS Institute Inc, Cary, NC).

Because homogeneity was a slight problem with some of the biomarkers and we opted to use the same model for all markers, we chose to use an unstructured correlation matrix to capture all possible deviations and keep the structure as flexible as possible.

For the mean structure we also used the most flexible version of a full interaction model incorporating time and treatment in the model. The conclusions with respect to possible treatment effects can then be discussed taking into account  $P$  values for the treatment effect and for the interaction effect between treatment and time. The treatment effect intuitively indicated whether the 2 treatment groups were already different at baseline or at  $t_1$  in this study. A  $P$  value  $<0.05$  was significant, and a  $P$  value  $<0.1$  was considered a trend and of possible interest.

## RESULTS

### Healthy Human Transit Study

Average counts of  $4.17 \times 10^6$  CFU/g feces and  $2.65 \times 10^7$  CFU/g feces of  $\text{rif}^{\text{R}}$  lactobacilli and  $\text{rif}^{\text{R}}$  bifidobacteria, respectively, were recovered from the feces after 7 d of synbiotic feeding. There were no  $\text{rif}^{\text{R}}$  bacteria present in the feces before feeding. If we assume a 10% survival of the probiotic strains during

**TABLE 1**  
Effect of synbiotic treatment on fecal flora in the 2 groups of subjects<sup>1</sup>

Fecal bacteria	Placebo group			Synbiotic group			<i>P</i> <sup>2</sup>
	Day 0	6 wk	12 wk	Day 0	6 wk	12 wk	
Polypectomized patients							
<i>Lactobacillus</i>	7.39 ± 0.89 (18)	7.46 ± 1.11 (18)	7.31 ± 1.21 (18)	6.52 ± 2.64 (18)	7.35 ± 1.41 (18)	7.74 ± 1.38 (18)	0.021
<i>Bifidobacterium</i>	7.67 ± 0.93 (18)	7.76 ± 0.73 (18)	7.75 ± 0.93 (18)	6.84 ± 1.26 (19)	7.55 ± 1.27 (19)	8.08 ± 0.96 (19)	0.008
<i>Enterococcus</i>	6.74 ± 1.07 (21)	6.27 ± 1.06 (21)	6.40 ± 1.32 (21)	6.04 ± 1.07 (19)	6.00 ± 1.36 (19)	6.11 ± 1.23 (19)	0.461
<i>Clostridium perfringens</i>	3.90 ± 2.49 (20)	3.90 ± 1.75 (20)	4.18 ± 1.35 (20)	4.41 ± 1.30 (17)	4.32 ± 1.85 (17)	3.03 ± 2.30 (17)	0.022
Coliforms	6.08 ± 1.19 (20)	6.01 ± 1.24 (20)	6.22 ± 1.01 (20)	6.16 ± 1.02 (19)	5.70 ± 1.27 (19)	6.10 ± 1.00 (19)	0.582
<i>Bacteroides</i>	7.47 ± 1.10 (21)	7.46 ± 1.08 (21)	7.93 ± 0.98 (21)	7.67 ± 1.42 (18)	7.96 ± 0.97 (18)	7.92 ± 1.32 (18)	0.401
Cancer patients							
<i>Lactobacillus</i>	5.68 ± 1.51 (11)	5.75 ± 1.52 (11)	5.73 ± 1.37 (11)	6.28 ± 1.25 (19)	6.92 ± 1.33 (19)	6.79 ± 1.39 (19)	0.570
<i>Bifidobacterium</i>	7.52 ± 1.58 (14)	7.19 ± 1.59 (14)	7.20 ± 1.49 (14)	7.79 ± 1.24 (18)	8.44 ± 1.16 (18)	8.76 ± 0.90 (18)	0.001
<i>Enterococcus</i>	5.26 ± 1.02 (14)	5.47 ± 1.21 (14)	5.79 ± 1.11 (14)	5.84 ± 1.15 (18)	6.42 ± 1.48 (18)	6.44 ± 1.13 (18)	0.623
<i>Clostridium perfringens</i>	4.01 ± 2.22 (7)	4.33 ± 1.97 (7)	4.13 ± 2.07 (7)	4.84 ± 1.29 (14)	4.10 ± 2.44 (14)	3.79 ± 2.69 (14)	0.609
Coliforms	5.33 ± 1.37 (14)	5.46 ± 1.56 (14)	5.47 ± 1.52 (14)	6.56 ± 1.24 (18)	6.00 ± 1.35 (18)	5.63 ± 1.35 (18)	0.150
<i>Bacteroides</i>	7.12 ± 1.10 (13)	7.29 ± 1.46 (13)	7.56 ± 1.40 (13)	7.31 ± 1.10 (13)	7.41 ± 1.15 (19)	7.24 ± 1.34 (19)	0.596

<sup>1</sup> All values are  $\bar{x} \pm SD$ ; *n* in parentheses.

<sup>2</sup> *P* values represent the combined treatment  $\times$  time effects calculated with the generalized linear modeling technique (PROC MIXED; SAS Institute Inc, Cary, NC) and reflect the differences in evolution of the bacteria over the 12-wk study period between the synbiotic and placebo groups. The analyses were done separately for the group of polypectomized patients and cancer patients.

the gastric duodenal transit, these results indicate full recovery of the strains in feces and possibly even some growth, especially in the case of the bifidobacteria.

#### Effect of synbiotic intervention on the composition of fecal flora

The effects of the synbiotic intervention on the fecal flora of polyp and cancer patients are presented in Table 1. Some groups of bacteria (*Bifidobacterium* in both groups and *Lactobacillus* in polyp patients) increased in numbers, whereas other groups of bacteria (*Bacteroides* and *Enterococcus*) were not affected. The number of *Clostridium perfringens* in polyp patients decreased significantly. These results correlate with other studies, which reported increases in lactobacilli and bifidobacteria (35, 36) and decreases in clostridia and coliforms (37) after prebiotic consumption.

#### Effect of synbiotic intervention on DNA damage

DNA damage in the colonic epithelium and the DNA-damaging capacity of fecal water (free water in stool) were analyzed to assess the effect of the intervention on exposure to genotoxins, a variable that has been shown to be associated with an enhancement of the neoplastic transformation process (38). Fecal water has been shown to mediate many of the effects of diet on tumorigenesis in the colon and is the fecal fraction that is in most intimate contact with the epithelium, which makes it an interesting target in biomarker studies (39). Smokers were omitted from the evaluation of this biomarker because smoking is a particularly strong confounding factor for this biomarker (40). The synbiotic intervention resulted in a significantly decreased level of DNA damage in the colonic mucosa in polyp patients at the end of the intervention period (Table 2).

#### Effect of synbiotic intervention on colorectal mucosal proliferative activity in polyp patients

Colorectal proliferation is a surrogate biomarker widely used to measure colon cancer risk in chemoprevention trials (41).

Synbiotic intervention tended to reduce colorectal proliferation in polyp patients (Table 2). There was no time  $\times$  treatment effect observed on the number of cells per crypt ( $62.6 \pm 7.2$ ) or on the distribution of proliferative activity along the crypt (%LI: lower compartment,  $60 \pm 12$ ; middle compartment,  $32.7 \pm 7.5$ ; and upper compartment:  $7.25 \pm 5.9$ ). Proliferative activity was not assessed in cancer patients.

#### Effect of synbiotic intervention on epithelial barrier function and tumor cell invasion

Common effects of tumor promoters are increased tight junction permeability and reduced epithelial barrier function (27). Thus, we examined the effect of the synbiotic intervention on the capacity of fecal water to modify these variables in colonic cells. Fecal water from the polyp patients during the synbiotic intervention significantly improved barrier function of the Caco-2 monolayer, as assessed by TER (Table 2). No significant effects of synbiotic treatment were apparent in the cancer patients. The effect of the synbiotic intervention on the capacity of fecal water to modify the invasive potential of colonic tumor cells was examined by using the Matrigel invasion assay. No effect of the intervention on the ability of fecal water to modify HT115 cell invasion was observed in the cancer or polyp patients.

#### Effect of synbiotic intervention on markers of cell toxicity and inflammation

Increased cytotoxicity of luminal contents and mucosal inflammation have been suggested to be associated with an increased risk of colonic cancer (42, 43). In the polyp patients, the synbiotic intervention significantly decreased the capacity of the fecal water samples to induce necrosis in HCT116 cells compared with the placebo group (Table 2). No effect of the synbiotic intervention on the capacity of the fecal water samples to influence early apoptosis ( $12.0 \pm 8.9\%$ ) in the colonic cells or fecal concentrations of the protein calprotectin (marker of colonic inflammation;  $57.0 \pm 58.8 \mu\text{g/g}$  feces) was observed in the colorectal cancer or polyp patients.

**TABLE 2**  
Effect of synbiotic treatment on various colon cancer–related biomarkers in the 2 groups of subjects<sup>1</sup>

Biomarkers	Polypectomized patients										<i>P</i> <sup>2</sup>		
	Placebo group					Synbiotic group							
	Day 0	6 wk	12 wk	Day 0	6 wk	12 wk	Day 0	6 wk	12 wk	Day 0		6 wk	12 wk
Genotoxicity (tail lengths) <sup>3</sup>	48.30 ± 16.78 (13 nonsmokers)	—	39.51 ± 8.76 (13 nonsmokers)	51.46 ± 12.62 (15 nonsmokers)	—	38.98 ± 8.84 (15 nonsmokers)	49.26 ± 13.53 (10 nonsmokers)	—	59.18 ± 15.94 (10 non-smokers)	53.57 ± 13.83 (15 non-smokers)	—	55.84 ± 21.21 (15 non-smokers)	0.98711
Proliferative activity in biopsy samples	—	—	—	—	—	—	—	—	—	—	—	—	—
Labeling index (% labeled cells/total crypt cells) <sup>4</sup>	5.7 ± 2.2 (16)	—	6.1 ± 2.1 (16)	5.9 ± 1.8 (16)	—	5.2 ± 1.4 (16)	—	—	—	—	—	—	—
Labeled cells/crypt <sup>5</sup>	7.12 ± 2.94 (15)	—	7.62 ± 2.50 (16)	7.16 ± 2.22 (16)	—	6.19 ± 1.64 (15)	—	—	—	—	—	—	—
Epithelial barrier function and tumor cell invasion in fecal water	132.6 ± 3.4 (20)	126.1 ± 4.9 (20)	124.2 ± 4.6 (20)	126.1 ± 4.9 (20)	131.2 ± 4.5 (20)	128.4 ± 4.5 (20)	103.2 ± 4.8 (18)	104.8 ± 5.3 (18)	101.9 ± 6.6 (18)	109.6 ± 44.9 (15)	107.0 ± 7.5 (15)	104.9 ± 6.2 (15)	0.65479
Trans epithelial resistance (%)	—	—	—	—	—	—	—	—	—	—	—	—	—
Cell toxicity and inflammation in fecal water	7.56 ± 8.0 (22)	7.99 ± 7.5 (19)	11.8 ± 11.2 (20)	17.59 ± 13.9 (19)	13.2 (19)	11.90 ± 9.8 (17)	8.35 ± 9.0 (18)	8.00 ± 9.2 (15)	5.52 ± 3.6 (14)	11.41 ± 15.0 (18)	13.65 ± 12.4 (18)	10.20 ± 12.4 (17)	0.70079
Necrosis (%)	—	—	—	—	—	—	—	—	—	—	—	—	—
Cytokine production by mitogen-activated PBMCs in blood	168 ± 174 (20)	153 ± 174 (21)	353 ± 276 (21)	153 ± 116 (20)	190 ± 170 (20)	171 ± 160 (20)	260 ± 259 (15)	149 ± 130 (15)	132 ± 181 (15)	235 ± 279 (19)	115 ± 79 (19)	221 ± 252 (19)	0.24075
IL-2	850 ± 866 (20)	1014 ± 1009 (21)	1317 ± 857 (21)	928 ± 991 (19)	1013 ± 989 (19)	826 ± 809 (19)	826 ± 871 (15)	741 ± 710 (15)	712 ± 784 (15)	642 ± 767 (19)	504 ± 541 (19)	1071 ± 819 (19)	0.05871

<sup>1</sup> All values are  $\bar{x} \pm$  SD; *n* in parentheses. PBMCs, peripheral blood mononuclear cells; IL-2, interleukin 2; IFN, interferon.

<sup>2</sup> *P* values represent the combined treatment × time effects calculated with the generalized linear modeling technique (PROC MIXED; SAS Institute Inc, Cary, NC) and reflect differences in evolution of the biomarker over the 12-wk study period between the synbiotic and placebo groups. The analyses were done separately for the group of polypectomized patients and cancer patients.

<sup>3</sup> Defined as DNA damage in biopsy samples, determined with the comet assay.

<sup>4</sup> Total number of labeled cells in crypt/total number of labeled cells in crypt × 100.

<sup>5</sup> Total number of labeled cells in crypt of the same subject/total number of cells in all crypts of the same subject.

### Effect of synbiotic intervention on immunologic functions of PBMCs and fecal water

In view of the fact that the immune system has been shown to be a target for probiotic effects in several systems (44), the effect of synbiotic intervention on several immunologic markers was examined (Table 2). Synbiotic consumption prevented an increased secretion of interleukin (IL) 2 by PBMCs in the polyp group ( $t_3$ ) but showed no effect in the cancer group. In contrast, the synbiotic increased the production of interferon  $\gamma$  ( $t_3$ ) in the cancer group but not in the polyp group (Table 2).

No time  $\times$  treatment effect was observed in natural killer cell cytotoxicity ( $17 \pm 8\%$  lysed target cells), phagocytic activity of blood granulocytes ( $42 \pm 19\%$  active cells) or monocytes ( $18 \pm 9\%$  active cells), or respiratory burst activity of blood granulocytes ( $89 \pm 6\%$  active cells). No influence on secretion of IL-10 ( $130 \pm 110$  pg/mL), IL-12 ( $663 \pm 607$  pg/mL), or TNF- $\alpha$  ( $7720 \pm 10915$  pg/mL) by PBMCs was observed. No effect on PGE<sub>2</sub> ( $18 \pm 22$  ng/mL) or TGF- $\beta$  ( $1736 \pm 1803$  pg/mL) in fecal water was observed.

### DISCUSSION

The present study was inspired by the abundant data from experimental models indicating the consistent anticancer efficacy of prebiotic chicory fructans and of probiotics (11, 12). Thus, numerous studies have shown that inulin-type fructans prevent chemically induced preneoplastic lesions, aberrant crypt foci (ACF), and tumors in the colon of rats and mice (16, 18, 45, 46). The type of fructan used in the present study—a mixture of short-chain and long-chain inulin (SYN1)—has been shown to be particularly chemopreventive in this ACF model (35) and to prevent the development of tumors in the Apc Min mice model. Probiotics on their own have also been shown to prevent ACF (18, 47) as well as DNA damage in the colon of rats (48). Some of these activities may be due to the scavenging of carcinogenic intermediates by probiotics, which may result in a reduced carcinogenic exposure of colonocytes, and this type of antigenotoxic effect may consequently decrease the likelihood of cancer initiation and progression (49, 50). Also, the chemopreventive effects of prebiotics have been shown to be associated with an enhanced production of butyrate, which inhibits the survival of colon cancer cells, serves as a survival factor in normal colon cells, and enhances the expression of phase II detoxifying enzymes in both normal and transformed cells and protects them from genotoxic substances (51–55). Despite these and numerous other potential mechanisms by probiotics, prebiotics, and synbiotics that have been disclosed via the use of experimental systems, the mechanisms by which prebiotics and probiotics exert their protective effects in humans are not known.

The SYNCAN project (DG Research, QLK1-CT99-00346; Internet: [www.syncan.be](http://www.syncan.be)), supported by the European Community, integrated a long-term rat carcinogenesis experimental model and the present human dietary intervention study to test the efficacy of the same synbiotic preparation to reduce colon cancer risk. Tumor-suppressing activity was shown in the SYNCAN experimental model for the prebiotic SYN1 and for the synbiotic combination (SYN1 + LGG + Bb12) administered in the present human intervention trial (35).

At present, polyp recurrence is the strongest surrogate marker of colon cancer in dietary intervention studies. However, studies

exploiting this marker are lengthy and expensive. Thus, present strategies include preliminary trials aimed at evaluating the effects on biomarkers of colon cancer risk in healthy subjects, polypectomized persons, and cancer patients with the aim to generate data that will be used to design further studies. Thus, in the present phase 2 study, we exploited a wide range of colon cancer-related biomarkers in blood (immunologic markers), stool (fecal water markers and flora), and biopsy samples (DNA damage and proliferation). For a comprehensive review and a justification for the use of these biomarkers, *see* a recent review by Rafter (56). Both previous colon cancer patients and polypectomized patients were used because they both have shown to be at high risk of the disease and are thus an ideal study group for a relatively small trial.

To show that any observed effect was due to the synbiotic intervention, prebiotic efficacy (significant and selective effects on the colonic bacterial population) was shown and confirmed during the intervention trial in the polyp patients and in the cancer patients (Table 1). Probiotic survival (viability of administered bacteria in stool) was shown and confirmed separately in healthy subjects by means of rif<sup>R</sup> isolates from LGG and BB12 (21). The results of our study clearly show that the synbiotic intervention exerted marked effects on several of the studied markers and no effects on other markers.

Also of interest was the observation that both the polyp patients and cancer patients appeared to respond differently to the synbiotic, as evidenced by the different biomarkers being affected to a different extent (Table 2). This may have been due to the fact that the intestinal microflora was more refractory to changes induced by the synbiotic in the cancer patients than in the polyp patients. A significant synbiotic-related reduction in colorectal proliferation in the polyp group was shown (Table 2). The degree of this effect was similar to that reported by Rozen et al (57) with calcium supplementation, although greater effects of calcium supplementation have also been reported (58). A significant reduction in proliferative activity (20% decrease compared with control subjects) has also been reported in intervention trials with putative chemopreventive compounds, such as resistant starch (59) or folate (60). A similar reduction in proliferative activity was observed in the SYNCAN experimental model for both the synbiotic and the prebiotic alone, but not for the probiotic (35). Given the correlation between colorectal proliferative activity and colon cancer risk (61), these results suggest that synbiotics might be beneficial for patients with an increased risk of colon cancer. The synbiotic intervention significantly decreased the capacity of the fecal water to induce necrosis in colonic cells in the polyp patients but not in the cancer patients (Table 2). Cytotoxicity of fecal water has been reported to be diet-dependent and correlated with colonic cell proliferation (43). This observation suggests that the synbiotic decreased the ability of the fecal water to cause cell death in the colonic epithelium, which may, in turn, have contributed to the decreased proliferative activity in the epithelium of the polyp patients.

The synbiotic intervention resulted in a decrease in DNA damage in the colon cells obtained from the biopsy samples from the polyp patients. This result possibly provides indirect evidence that genotoxin exposure in the colon decreased at the end of the intervention period (Table 2). In the SYNCAN experimental model, an inverse relation of fecal water genotoxicity and tumor risk was shown (25).



No effect of the synbiotic intervention on markers of apoptosis, colonic inflammation, or tumor cell invasion was observed. However, in the polyp patients, the synbiotic intervention was associated with a significant improvement in barrier function as assessed by TER of Caco-2 cell monolayers after exposure to fecal water samples. This antipromotion effect may reflect changes to the balance of short-chain fatty acids and secondary bile acids in the samples because these gut microbial metabolites have been shown to influence TER, beneficially and adversely respectively, in this system (62).

The synbiotic supplement had no adverse effect on the systemic immune system of the 2 patient groups (Table 2). However, in contrast with the results of the SYNCAN experimental study (63), results from the present human trial do not support a strong systemic immunomodulatory effect of the synbiotic. In addition, in the experimental study, the major immunologic effects were observed at the level of the gut-associated lymphoid tissue. Because of limitations in the availability of biopsy samples in the present human trial, it was not possible to study the presumably more affected gut-associated lymphoid tissue-related markers. However, indirect markers of intestinal immunity, such as the concentration of PGE<sub>2</sub> and TGF- $\beta$  in fecal water, were not affected.

In conclusion, the synbiotic intervention resulted in significant alterations in the composition of the colonic bacterial ecosystem, which presumably have consequences for the metabolic activity of this organ. Our results also provide indirect evidence that some of the consequences of the synbiotic intervention might be decreased exposure of the epithelium to cytotoxic and genotoxic agents, decreased colonic cell proliferation, and improved mucosa structure. Future research should focus on the effects of prebiotics and probiotics separately. Our results offer valuable corroboration of the wealth of animal data in the field and suggest that synbiotics of the type studied in the present study may represent a feasible means of chemoprevention of colon cancer in humans. Finally, it is hoped that our results will aid in the design of future clinical studies in this challenging field.

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JVL, JR, GC, JKC, BP-Z, GR, IR, and BW contributed to the development of the idea and design of the study and were involved in the data analysis and the writing of the manuscript. JR had overall responsibility for the final manuscript. JKC, YC, GCO, MB, and MO were responsible for the Healthy Human Transit Study, patient recruitment, the dietary intervention study, blood collection, biopsy and fecal collection, and preparation, distribution, and analysis of fecal flora. BP-Z and AK performed the comet assay. GC and MS determined the colorectal mucosa proliferative activity. IR and RH were responsible for determining tight junction integrity, epithelial barrier function, and tumor cell invasion. JR and PCK performed the assays for apoptosis, necrosis, and fecal calprotectin. BW and MR were responsible for measuring the immunologic markers in PBMCs and in fecal water. JVL is an ORAFTI employee who functioned as coordinator of the European Union-sponsored SYNCAN project; he did not interfere with the execution of the study protocol, analyses, or interpretation of the data. None of the other authors had any conflicts of interest to declare. The study sponsor had no role in the study design; the collection, analysis, or interpretation of the data; the writing of the manuscript; or the decision to submit the paper for publication.

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