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Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* on azoxymethane-induced colon carcinogenesis in rats

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Prebiotics such as fructans, and probiotics such as Lactobacilli or Bifidobacteria, or a combination of prebiotics and probiotics (synbiotics) are thought to be protective against colon cancer. Therefore, we studied whether the prebiotic inulin enriched with oligofructose (Raftilose-Synergy1®, briefly, Synergy1, 10% of the diet), probiotics [*Bifidobacterium lactis* (Bb12) and *Lactobacillus rhamnosus* (LGG), each at 5×10^8 c.f.u./g diet] or synbiotics (a combination of the two) protect rats against azoxymethane (AOM)-induced colon cancer. Male F344 rats were divided into: Controls; PRE, which were fed a diet containing Synergy1; PRO, fed a diet containing LGG and Bb12; PREPRO, fed a diet containing Synergy1, LGG and BB12. Ten days after beginning the diets, rats were treated with AOM (15 mg/kg s.c. two times); dietary treatments were continued for the entire experiment. Thirty-one weeks after AOM, rats treated with Synergy1 (PRE and PREPRO groups) had a significantly lower ($P < 0.001$) number of tumours (adenomas and cancers) than rats without Synergy1 (colorectal tumours/rat were 1.9 ± 1.7, 1.1 ± 1.1, 2.2 ± 1.4 and 0.9 ± 1.2 in Controls, PRE, PRO and PREPRO groups, respectively, means ± SD). A slight, not significant effect of probiotics in reducing malignant tumours was also observed ($P = 0.079$). Caecal short-chain fatty acids (SCFA) were higher ($P < 0.001$) in the groups treated with Synergy1. Apoptosis was increased in the normal mucosa of the PRO group, while no variation was observed in the tumours. Colonic proliferation was lower in the PRE group as compared with Controls. Glutathione *S*-transferase placental enzyme pi type expression, and to a lesser extent, inducible NO synthase were depressed in the tumours from rats in the PRE and PREPRO groups. Cycloxygenase-2 expression was increased in the tumours of control rats but not in those from PRE, PRO or PREPRO rats. In conclusion, prebiotic administration in the diet decreases AOM-induced carcinogenesis in rats.

Abbreviations: ACF, aberrant crypt foci; AI, apoptotic index; AOM, azoxymethane; Bb12, *Bifidobacterium lactis* Bb12; COX-2, cyclooxygenase-2; GST-P, GST placental enzyme pi type; HF, high fat; LGG, *Lactobacillus rhamnosus*; PCNA, proliferating cell nuclear antigen; SCFA, short-chain fatty acids.

Introduction

Dietary habits have been associated with variations in the risk of colon cancer, either its increase or decrease (1). Among potentially protective foods, growing attention has been dedicated to prebiotics such as fructo-oligosaccharides or fructans, and probiotics, such as Lactobacilli or Bifidobacteria, as some studies suggest that their consumption may decrease experimentally induced colon cancer in animals (2–7). Prebiotics are defined as 'non-digestibile food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon' (8). Non-digestible carbohydrates in general—and chicory-derived β(2-1)-fructans in particular, like oligofructose and inulin—occur in significant amounts in many fruits and vegetables, and stimulate the growth of endogenous *Bifidobacteria*, which, after a short feeding period, become predominant in human faeces (9). Reddy *et al*. (6) have demonstrated that dietary oligofructose, a short-chain fructo-oligosaccharide, and other fructans such as inulin, reduce the number of aberrant crypt foci (ACF), purported preneoplastic lesions in the colon of rats. Short-chain fructo-oligosaccharides have also been reported to reduce colon tumours in Min mice, a genetic model mimicking human colon cancer (2).

Probiotics are viable microbial food ingredients supposed to be beneficial through their effect in the intestinal tract (8); they also have anticarcinogenic-antimutagenic effects *in vivo* (7). In fact, *Bifidobacterium longum* supplementation reduces colon and liver carcinogenesis by 2-amino-3-methylimidazo[4,5-f]quinoline as well as azoxymethane (AOM)-induced colon cancer in rats (10,11). Dietary supplements of Lactobacilli also increase the latency of induction of experimental colon cancer in rats (12), suggesting that Lactobacilli and Bifidobacteria may inhibit precancerous lesions and tumour development in animal models (13).

Recently, it has been suggested that a combination of prebiotics and probiotics, the so-called synbiotics, might be more active than the individual components on the colon (14). Accordingly, Rowland *et al.* (3) showed that concomitant administration of inulin and Bifidobacteria to rats resulted in a more potent inhibition of AOM-induced ACF than the administration of the two separately. While these results were obtained using short-term carcinogenesis tests, no data are available on the effect of synbiotics in long-term carcinogenesis.

Given these considerations and the fact that prebiotics, probiotics or synbiotics, if active, might represent a feasible chemoprevention of colon cancer also in humans, we decided to study the effect of prebiotics, probiotics and synbiotics in long-term carcinogenesis in which rats were treated with AOM.

We studied the prebiotic Raftilose Synergy 1® (briefly, Synergy1), an oligofructose enriched inulin. Synergy1 is a 1/1 mixture of long chain and short chain fractions of inulin, a β(2-1)-fructan extracted from chicory roots (*Cichorium intybus*). Inulin is made by a set of linear chains of fructose molecules, with a degree of polymerization (DP) ranging

between 3 and 65. It can be fractionated into a slowly fermentable long-chain fraction (DP ranging from 10 to 65, average 25) or in a rapidly fermentable fraction made of oligofructose (DP ranging from 3 to 8, average 4). Synergy1 is a mixture of both fractions, and has a higher amount of long chains relative to the native product. No data were available on the effect of Synergy1 in long-term carcinogenesis.

For probiotics, it has been suggested that a mixture of probiotic strains may be more effective on the intestine than the individual strains (15,16). Therefore, as probiotic supplementation, we used two strains, namely *Lactobacillus* GG, *Lactobacillus delbrueckii* subsp. *rhamnosus* (LGG) and *Bifidobacterium lactis* Bb12 (Bb12).

Prebiotic, probiotics and synbiotics were administered during all phases of cancer induction, meaning that rats were fed experimental diets starting 10 days before AOM until death, which was performed 31 weeks after AOM.

To understand the possible mechanisms of actions, we also measured biomarkers, which have been associated with colon carcinogenesis, like the proliferative activity in the colonic mucosa, apoptosis in tumours or in the mucosa and short-chain fatty acids (SCFA) in the caecum, factors that can influence carcinogenesis (17–22).

We were also interested in studying whether prebiotics, probiotics and synbiotics might affect the expression of genes codifying enzymes involved in the colon carcinogenesis process. In humans, colon tumours express high levels of glutathione *S*-transferase (GST), and especially GST placental enzyme pi type (GST-P); this characteristic is associated with failure of cancer chemotherapy and poor patient survival (23). Cyclooxygenase-2 (COX-2) is also up regulated in cancers and COX-2 inhibitors have chemopreventive activity (24). Inducible nitric oxide synthase (iNOS) also plays an important role in colon tumour growth and progression (25,26).

Materials and methods

Materials

AOM was purchased from Sigma (Milan, Italy). Dietary components were purchased from Piccioni (Gessate, Milan, Italy). Raftilose Synergy1® was provided by ORAFTI (Tienen, Belgium).

Animals and treatments

We used 4–5-week-old male F344 rats (Nossan, Correzzana, Milan, Italy). The animals were housed in plastic cages with wire tops and maintained at a temperature of 22°C, with a 12/12-h light/dark cycle, according to the European Union Regulations on the Care and Use of Laboratory Animals (30). The experimental protocol was approved by the Commission for Animal Experimentation of the Ministry of Health, Rome, Italy. After their arrival from the supplier, animals ($n = 129$) were quarantined for 1 week, during which they were fed a standard lab chow. Rats were then randomly allocated to the following experimental groups: Controls ($n = 32$): were fed a high-fat diet (HF) based on the AIN76 diet (20), with a higher amount of fat (230 g/kg corn oil w/w) and a lower level of cellulose (20 g/kg w/w) as the diet of some western populations at risk for colon cancer; the source of carbohydrates in this diet was sucrose (360 g/kg w/w) and maltodextrins (100 g/kg w/w). The PRE group ($n = 33$): rats were fed the same HF diet as controls in which maltodextrins were replaced by 100 g/kg w/w of Synergy1. The PRO group ($n = 32$): rats were fed the same HF diet as controls but supplemented with LGG and Bb12 to provide ~5×10^8 c.f.u. of each strain/g diet. The PREPRO group ($n = 32$): rats were fed the same diet as the PRE group supplemented with LGG and Bb12 to provide ~5×10^8 c.f.u. of each strain/g diet as in the PRO group. Diets were prepared every 2 weeks, divided into aliquots frozen at –20°C. Aliquots were left at room temperature for 2 h and fed to rats every day. Samples of different batches of diets were taken to estimate the viability of bacteria at different times after preparation of the diets. Ten days after beginning feeding of the experimental diets, rats were administered AOM (15 mg/kg two times s.c., 1 week apart); in each dietary group some animals were treated with saline instead of AOM and served as control for the carcinogen treatment (five in the PRE, PRO and PREPRO groups and four in the Control group).

Bacterial strains

Lactobacillus GG, *L.delbrueckii* subsp. *rhamnosus* (LGG) and *B.lactis* Bb12 (Bb12) were provided by Valio (Helsinki, Finland) and purchased from Chr. Hansen (Horsholm, Denmark), respectively. They were supplied as a freeze-dried powder in sealed sachets which contained ~4×10^{11} c.f.u./g in the case of LGG, while the sachets with Bb12 contained ~3×10^{10} c.f.u./g. The bacteria were kept at –20°C until used. The concentration of LGG and Bb12 in the diet was evaluated by dissolving 1 g of diet in 100 ml of half-strength Peptone water (Oxoid, Basingstoke, UK) containing 0.5 g/l cysteine HCl (Sigma-Aldrich, Milano, Italy). Serial dilutions were prepared using the same diluent and plating them in Wilkins-Chalgren anaerobe agar (Oxoid, Basingstoke, UK). The dishes were then incubated at 37°C in an anaerobic jar (Anaerobic System, Oxoid, Basingstoke, UK) with anaerobic atmosphere generated by Anaero*Gen*™ (Anaero*Gen*™, Oxoid, Basingstoke, UK). Plates were incubated for 2 days for LGG, whereas Bb12 was incubated for 3 days. When the two strains were incubated together they were easily recognizable as LGG form large colonies, whereas those of Bb12 are pinpoint in size.

Batch fermentation studies using faecal slurry from healthy volunteers has demonstrated that both Bb12 and LGG can use Synergy1 as a carbon source for their growth. Furthermore, using a three-stage gut model, it could be demonstrated that both probiotic strains, in each other's presence and with Synergy1 as the sole carbon source, survived and grew well in this *in vitro* model mimicking the human colon (G.Gibson, University of Reading, UK, personal communication).

Spontaneously occurring rifampicin-resistant mutants (50 μg/ml) were selected from Bb12 and LGG and referred to as Bb12 rif^R and LGG rif^R, respectively. These strains were used to test viability of probiotics in the gastrointestinal tract of the rats. Previous experience in humans and rodents demonstrated in fact that such rif^R strains were not present in stool samples, and that rifampicin resistance is a suitable marker to allow selective isolation and enumeration of introduced probiotic strain from faeces (27,28). For both rif^R strains we prepared freeze-dried cultures grown in modified MRS broth [yeast extract (Merck, Darmstadt, Germany) 0.4%, glucose 2%, K_2HPO_4 0.2%, Na-acetate 0.5%, $(NH_4)_3$ citrate 0.2%, $MgSO_4$ 0.02%, $MnSO_4$ 0.0046% and peptone 1.8%] for LGG rif^R or modified MRS broth containing 0.05% cysteine (Sigma, Dublin, Ireland) for Bb12 rif^R strain. Viable bacteria per gram of freeze-dried product were enumerated as described before and found to be 5.9 $\times10^{10}$ c.f.u./g in the case of Bb12 rif^R strain and 4.7×10^{10} c.f.u./g in the case of LGG rif^R strain. The freeze-dried rif^R strains replaced the wild-type strains in the diet of PRO and PREPRO groups for 7 days. This feeding experiment was performed only in the rats that were treated with saline (four and five rats in the PRO and PREPRO groups, respectively). Faecal samples were freshly collected from individual rats prior to and following 7 days feeding with rif^R strains. Faecal samples were stored at –80°C in PBS/10% glycerol until analysis. Faecal samples were then thawed at room temperature and serially diluted in sterile PBS with 0.05% cysteine. The relevant dilutions were plated in triplicate, in the presence of rifampicin (50 μg/ml), on MRS agar in the case of LGG rif^R strain and on modified Raffinose-Bifidobacterium agar (29) in the case of Bb12 rif^R strain. Plates were incubated anaerobically for 48–72 h and LGG rif^R and Bb12 rif^R colonies identified by criteria outlined above were counted.

Determination of caecal SCFAs

At death, caecal content was collected on ice into a vial, and frozen at –80°C until analysis of SCFAs using gas chromatography and flame ionization detection (GC VEGA 6000, Carlo Elba, Milano, Italy). In brief, the caecal sample was weighed and an internal standard solution (2-methyl valeric acid, 0.01%) was added. The solution was acidified with 0.5 ml H_2SO_4 ($pH < 2$), and SCFAs were extracted by shaking with 2.0 ml diethylether and subsequent centrifugation (10 min at 2000 r.p.m.). The ether phase was injected directly onto a column (BT21-FFAPP 25 m×0.53 mm×0.5 mm, Achrom NV/SA, Belgium) at 150°C, using N_2 as the carrier gas; detection temperature was set at 230°C. Peaks were integrated using the Atlas software (ThermoLab Systems, The Netherlands).

Histopathological evaluation of the tumours

At death all the organs were macroscopically examined for the presence of tumours or other pathological lesions. Tissues showing a deviation from normal morphology were fixed in 10% buffered formalin and embedded in paraffin blocks. Samples of tumour tissue and of apparently normal mucosa were also harvested and stored at –80°C until analysis for RT–PCR (see below). Paraffin blocks were sectioned and stained with haematoxylin–eosin to confirm the presence and type of tumours by histopathological examination, which was performed by a pathologist unaware of the codes of the specimens. Before being fixed in formalin, suspected macroscopic lesions were measured

with a caliper and their dimensions were calculated by multiplying the two main diameters of each lesion. Cancer histological types were evaluated on the basis of the histotype, grading and pattern of growth; adenomas were classified according to Morson *et al*. (31).

Proliferative activity in the colonic mucosa

Proliferative activity in the colonic mucosa was evaluated by determining proliferating cell nuclear antigen (PCNA) immunoreactivity in slides from normal colon. Sections (3 μm) from paraffin-embedded colon were mounted on electrostatic-treated slides (Superfrost® Plus, Medite, Italy). Sections were then deparaffinized with xylene, hydrated through a graded series of ethanol until distilled water. Endogenous peroxidase activity was blocked by 1.5% H_2O_2 for 20 min with three subsequent washings in distilled water. To unmask PCNA protein, tissue sections were immersed in citric acid buffer (pH 6.0) and microwaved for 20 min. The slides were then allowed to cool for 30 min while immersed in citrate buffer, washed twice in distilled water and then immersed in PBS containing 0.05% Tween 20. Immunohistochemical staining was performed with the streptavidin–biotin immunoenzymatic antigen detection system (Ultravision Large Volume Detection System Anti-Polivalent, HRP; LAB Vision, CA). To block non-specific background staining the slides were incubated with serum Ultra V Block of the antigen detection system (LAB Vision) in a humid chamber for 5 min. The slides were then incubated in a humid chamber with the primary antibody (mouse monoclonal PC-10; Santa Cruz, CA) diluted 1:200 in PBS for 60 min at room temperature. The slides were rinsed twice in PBS, covered with biotinylated goat anti-polyvalent as the secondary antibody (LAB Vision). Immunohistochemical staining was performed using the streptavidin–biotin immunoenzymatic antigen detection system (Ultravision Large Volume Detection System Anti-Polivalent, HRP) and incubated in a humid chamber for 15 min. After this period slides were rinsed in PBS twice, covered with streptavidin peroxidase (LAB Vision) for 15 min in a humid chamber, and, after rinsing twice in PBS, were finally reacted with 3,3′-diaminobenzidine (Liquid DAB Substrate Pack, concentrated; Biogenex, CA) for 5–10 min. The slides were weakly counterstained with Harris' haematoxylin. Proliferative activity was evaluated for each rat by counting PCNA-positive nuclei in at least 10 full longitudinal crypt sections (i.e. from the base to the bottom of the crypt) under a light microscope at 400× magnification. Only dark brown stained nuclei were considered PCNA-positive. For each crypt we recorded the number of cells per crypt column (i.e. number of cells from the bottom to the top of the half crypt appearing in the section) and the number and position of the labelled cells (LC) along the crypt, dividing each crypt into three equal parts: lower, mid and upper compartments. The microscopic slides were coded and read independently by two observers. The correlation coefficient between these two observers on a set of 30 LC/crypt scores was 0.821 ($P < 0.001$).

Apoptosis in the colonic mucosa and in the tumours

Apoptosis evaluation was carried out in paraffin-embedded sections of normal colonic mucosa and tumours stained with haematoxylin–eosin, as recommended by Samaha *et al*. (19). At least 20 full longitudinal crypt sections of normal mucosa/rat were scored at the microscope, determining the presence of cells in each crypt with the following characteristics of apoptosis: cell shrinkage, loss of normal contact with the adjacent cells of the crypt, chromatin condensation or formation of round or oval nuclear fragments ('apoptotic bodies'). When clusters of more than one apoptotic body were seen within the diameter of one cell, these bodies were considered as fragments of one apoptotic cell. Tumour apoptosis was determined by scoring at least 1000 cells/rat for the presence of apoptotic cells that were coded as described above. In tumours and colon mucosa, apoptosis was scored by a single observer on coded samples and quantified as apoptotic index (AI = number of apoptotic cells/cells scored ×100).

RT–PCR

Total RNA was isolated from 30 colorectal tumours (15–25 mg of tissue) and from the matched normal mucosa using the Trizol protocol as suggested by the supplier (Life Technologies, Milano, Italy). We analysed 10 tumours harvested from the Control group, eight harvested from PRO group and six tumours from PRE and PREPRO groups.

For first strand cDNA synthesis, 200 ng of RNA from each sample were reverse transcribed using 100 U RT superscript II (Life Technologies, Milano, Italy) and 1× random examers (Roche, Mannheim, Germany). To amplify each gene, primers were designed on the basis of the sequences reported by the UNIGENE database for rat, as already described (32). Each gene was co-amplified together with β-actin as a control. For each gene the PCR reaction was carried out on aliquots of the cDNA preparation, in a 25 μl volume containing 1× PCR buffer, 2 mM $MgCl_2$, 0.5 mM dNTPs, 8 ng/μl of each target gene primer, 0.2 ng/μl of the β-actin primers and 1.25 U *Taq* polymerase (Advanced Biotechnologies, UK). The PCR conditions were the same for the three genes: 95°C for 7 min and then 30 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 55 s and a final extension at 72°C for 5 min. The PCR products were separated on 1.6% agarose gel. The amplified products were photographed with a digital camera and the intensity of the bands was analysed with Quantity-One software (Bio-Rad, Segrate, Italy). For each target gene, we calculated the relative amount of mRNA in the samples using β-actin co-amplified as internal standard as already described (32).

Statistical evaluation of the data

Data obtained from individual rats in the different experimental groups were summarized for quantitative continuous responses by calculating group means and standard deviations, using the Statgraphics Statistical Package (Statistical Graphic Corporation, Rockville, MD). The experimental scheme adopted was a two-by-two factorial design (both PRE and PREPRO groups contained the prebiotic Synergy 1, and both PRO and PREPRO groups contained probiotics). Therefore, the groups were compared two-by-two (i.e. the effect of Synergy1 was evaluated comparing Controls and PRO versus PRE and PREPRO, while the effect of probiotics was evaluated comparing Controls and PRE versus PRO and PREPRO). The systematic sources of variability which accounted for the group differential response were analysed by specifying analysis of variance for quantitative continuous response models with main effects and with/without appropriate interaction terms (*P*-level fixed was at 0.05, two-sided) or, when the response variable considered was the occurrence of tumours, by fitting Poisson regression models with main effects and with/without appropriate interaction terms. We tested the presence of overdispersion by maximum likelihood, using the negative binomial model to take multiple occurrences per rat into account (20,33). We were also interested in testing whether prebiotics and probiotics, if effective, interact in an additive or in a synergistic manner: in the first case their combined effect would be just the sum of the effects of the two separate components, while in the second case (synergistic effect) the combined effect would be higher than their sum. This possibility was tested by the *F*-test of interaction terms in the factorial analysis-of-variance models, and by the likelihood ratio test of interaction terms in the Poisson regression models. In this latter case, we used both additive and multiplicative link function.

Results

Microbiological studies

The mean concentration of the two bacterial strains in the diet was $5.0 \pm 1.3 \times 10^8$ c.f.u./g of diet for LGG, and $5.5 \pm 2.4 \times 10^8$ c.f.u./ g for Bb12 (mean ± SD, $n = 6$). The viability of the bacteria in the diet kept frozen at –20°C for 2 weeks was good and similar in the two groups (mean ± SD: $5.9 \pm 3.1 \times 10^8$ c.f.u./g).

To study the ability of the probiotic strains to transit the rat gastrointestinal tract, antibiotic-resistant mutants of each probiotic strain, isolated as described in the Materials and methods, were also fed to rats in the PRO and PREPRO groups, replacing the wild-type strains in the diet at the same concentration. The numbers of rif^R bacteria in the faeces of these rats were determined prior to the beginning of feeding with the rif^R strains and after 7 days feeding with these strains. Prior to feeding no rif^R bacteria were present in the faeces. Following 7 days feeding, counts of LGG rif^R were $4.8 \pm 3.4 \times 10^5$ c.f.u./g of faeces (mean ± SD, $n = 4$) and $21.1 \pm 18 \times 10^5$ c.f.u./g of faeces (mean ± SD, $n = 5$), in the PRO and PREPRO groups, respectively; counts of rif^R Bb12 were $6.1 \pm 8.1 \times 10^5$ c.f.u./g of faeces and $8.4 \pm 12 \times 10^5$ c.f.u./g of faeces in the PRO and PREPRO groups, respectively. The good recovery of the probiotics shows the ability of the rif^R strains to survive and transit the rat gastrointestinal tract.

Carcinogenesis

The mean weight of the rats at the beginning of the experiment was 108.8 ± 12.2 g (mean ± SD; $n = 129$). At death, 31 weeks after AOM, the mean weight was similar among dietary groups (mean value ± SD, 479.5 ± 34). All rats were killed 31 weeks after the first AOM injection.

As the experimental scheme adopted is a two-by-two factorial design (both PRE and PREPRO groups contained the prebiotic

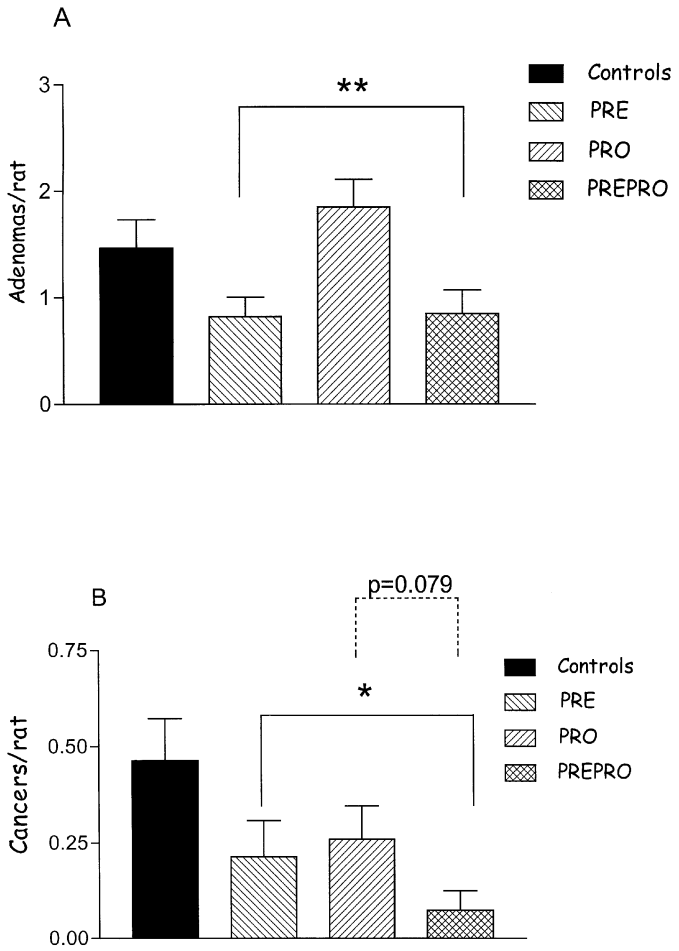

Fig. 1. (**A**) Bars represent the mean number (+SE) of adenomas/rat (considering all the rats in the group) in the colorectum in Controls ($n = 28$), PRE ($n = 28$), PRO ($n = 27$) and PREPRO ($n = 27$) groups. **Synergy1-treated groups (PRE and PREPRO) are significantly different ($P < 0.001$) from groups not treated with Synergy1 (Controls and PRO). (**B**) Bars represent the mean number (+SE) of cancers/rat in the colorectum in Controls ($n = 28$), PRE ($n = 28$), PRO ($n = 27$) and PREPRO ($n = 27$) groups. *Synergy1-treated groups (PRE and PREPRO) are significantly different ($P < 0.05$) from groups not treated with Synergy1 (Controls and PRO). $P = 0.079$ refers to the level of statistical significance of the effect of probiotics (PRO and PREPRO groups) when compared with groups not treated with probiotics (Controls and PRE).

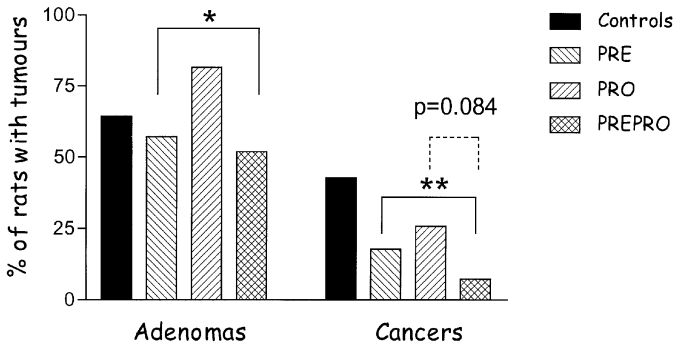

Fig. 2. Bars represent the incidence of adenomas or cancers (number of rats with tumours/number of rats in that group ×100) in the different groups. *,**Synergy1-treated groups (PRE and PREPRO) are significantly different ($P < 0.05$, $P < 0.01$, for adenomas and cancers, respectively) from groups not treated with Synergy1 (Controls and PRO). $P = 0.084$ refers to the level of statistical significance of the effect of probiotics (PRO and PREPRO groups) when compared with groups not treated with probiotics (Controls and PRE).

Synergy 1, and both PRO and PREPRO groups contained probiotics), the groups were compared two-by-two. Therefore, the effect of Synergy1 was evaluated comparing rats not-treated (Control and PRO groups) versus rats treated with Synergy1 (PRE and PREPRO groups), while the effect of probiotics was evaluated comparing rats not-treated (Control and PRE groups) versus rats treated with probiotics (PRO and PREPRO groups).

No tumours were found in the rats treated with saline. In the AOM-treated rats the majority of tumours were found in the colon and rectum. However, we found some tumours also in the small intestine and the ear. These latter types of tumours were squamous papillomas and their number did not differ among the experimental groups (data not shown).

All the tumours found in the colon, rectum and small intestine were classified as adenomas or cancers according to Morson *et al*. (31). The results relative to the number of tumours per rat (considering all the rats in the group) in the colorectum (Figure 1) clearly show that the rats treated with Synergy1 (groups PRE and PREPRO) had a lower number of tumours (both adenomas and cancers) when compared with groups without Synergy1; in particular (Figure 1A), we observed that the number of colorectal adenomas in PRE and PREPRO groups was markedly lower ($P < 0.001$) than in the rats not treated with the prebiotic Synergy1, while the two strains of probiotics were ineffective. The data relative to cancers in the colorectum (Figure 1B) showed a similar protective effect of the prebiotic Synergy1 ($P < 0.05$); the cancers were fewer also in the probiotic treated groups (PRO and PREPRO groups) but this effect did not attain statistical significance ($P = 0.079$). However, analysing the proportion of cancers in relation to the total number of tumours, in the groups treated with probiotics we found that the proportion of cancers was lower ($P = 0.04$) than in the groups not treated with probiotics: PRO and PREPRO groups had in fact nine cancers over 84 tumours (11%), while Controls and PRE groups had 19 cancers over 83 tumours (23%). On the contrary, the treatment with prebiotics did not change the proportion of malignant tumours.

The number of tumours (both adenomas or cancers) per rat in the small intestine was similar among the different groups (means values ± SD, 0.18 ± 0.41, $n = 110$).

We were also interested in testing whether the effects of the prebiotic and probiotics in the PREPRO group would interact in an additive or in a synergistic manner: in the first case their combined effect would be just the sum of the effects of the two components, while in the second case (synergistic effect) their combined effect would be higher than the sum of the two. The statistical analysis (see Materials and methods) suggests that the prebiotic and probiotics interacted in an additive manner in the PREPRO group.

In the groups treated with the prebiotic Synergy1 (PRE and PREPRO groups) we also found a lower incidence of colorectal adenomas or cancers (Figure 2); the incidence of cancers was slightly, but not significantly, lower in the groups treated with the probiotics LGG and Bb12 ($P = 0.084$).

We also determined the dimensions of the tumours, dysplasia in the adenomas and cancer invasiveness; all these parameters were similar among the different groups (data not shown).

SCFA determination in the caecum

SCFA in the caecum of the groups treated with prebiotic Synergy1 (PRE and PREPRO groups) were significantly higher (Figure 3) compared with the groups without Synergy1

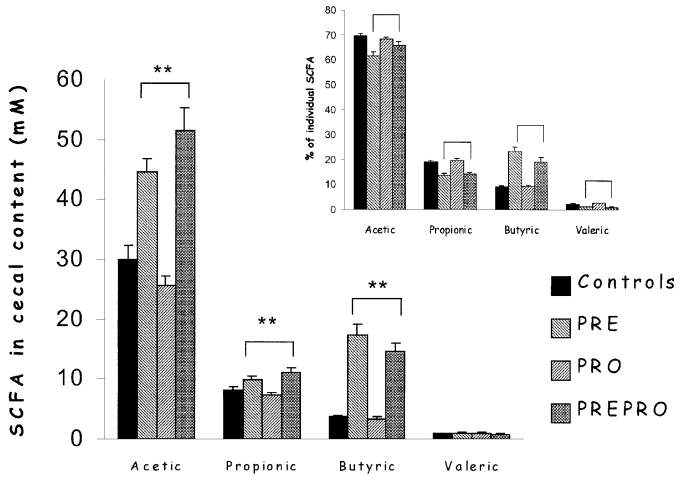

Fig. 3. SCFA concentration in the caecal content of rats fed the experimental diets. Bars are means ± SE. **Synergy1-treated groups (PRE and PREPRO) are significantly different ($P < 0.001$) from groups not treated with Synergy1 (Controls and PRO). (Insert) Per cent of individual SCFA/total SCFA in the caecum. Means ± SE. **Synergy1-treated groups (PRE and PREPRO) are significantly different ($P < 0.001$) from groups not treated with Synergy1 (Controls and PRO).

($P < 0.001$). The analysis of the percent distribution of individual SCFA/total SCFA showed (Figure 3, insert) that the groups treated with the prebiotic Synergy1 (PRE and PREPRO groups) had relatively less acetic, propionic and valeric acids but more butyric acid ($P < 0.001$) than rats not treated with Synergy1. Probiotics had no significant effects on caecal SCFA.

Apoptosis in the normal mucosa and in the tumours

Apoptosis in the normal mucosa was significantly increased in the PRO group as compared with Controls (Figure 4A), but no changes were found in the other groups. The analysis of the distribution of apoptosis along the crypt showed that the increase in apoptosis seen in the PRO group was due to an increase in apoptotic index (AI) in the lower third of the crypt (AI: 0.33 ± 0.36, 0.35 ± 0.34, 0.68 ± 0.35 and 0.35 ± 0.37 in the Controls, PRE, PRO and PREPRO groups, respectively, means ± SD). The results also showed that the apoptotic index in the upper compartment of the crypt tended to be higher in the groups treated with PRE, PRO and PREPRO but this difference did not attain statistical significance (AI in the upper third of the crypt: 0.22 ± 0.25, 0.35 ± 0.27, 0.33 ± 0.25 and 0.36 ± 0.25 in the Controls, PRE, PRO and PREPRO groups, respectively, means ± SD). Apoptosis in the tumours was higher than in the normal mucosa, but the values were not varied in the different groups (mean value of AI in all the tumours was 3.0 ± 1.7, means ± SD, $n = 97$).

Proliferative activity in the colonic mucosa

A significantly lower number of labeled cells/crypt was measured in the PRE group as compared with controls (Figure 4B); we also observed that PRO and PREPRO groups had slightly lower proliferation, although this effect was not statistically significant. The distribution of the proliferative activity along the crypt was similar among the different groups (data not shown).

RT–PCR experiments

The expression of i-NOS, GST-P and COX2 was evaluated in the tumours and in the matched normal mucosa of the same animal in all experimental groups. The results relative to i-NOS expression (Figure 5A) indicate that the tumours in all the experimental groups had a significantly higher expression

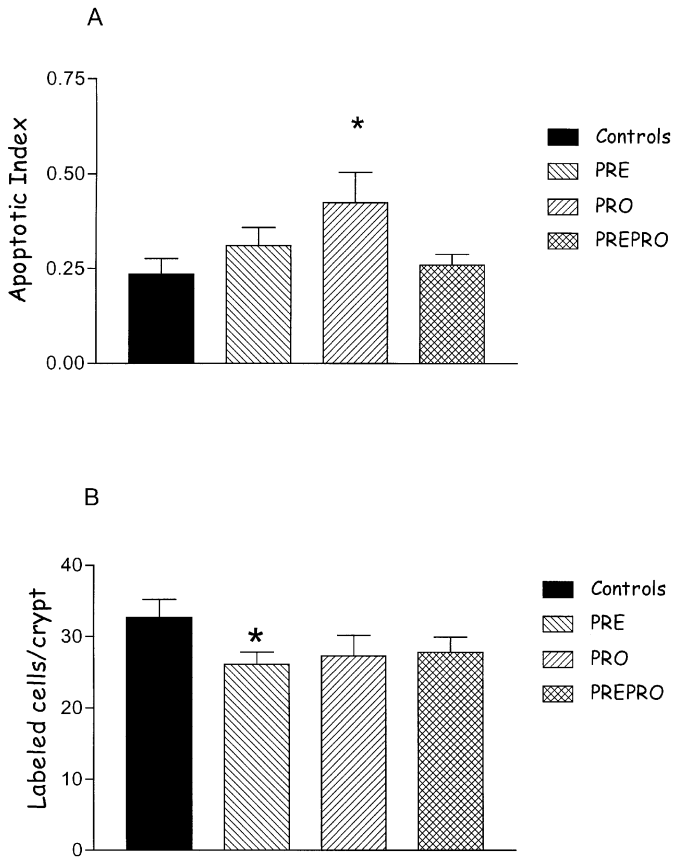

Fig. 4. (**A**) AI in the colonic mucosa of rats in Controls ($n = 13$), PRE ($n = 15$), PRO ($n = 10$) and PREPRO ($n = 17$) groups. *Significantly different from Controls ($P < 0.05$; F-test for interaction: $P < 0.05$). (**B**) Number of PCNA LC/crypt in the colonic mucosa of rats in Controls ($n = 10$), PRE ($n = 11$), PRO ($n = 9$) and PREPRO ($n = 13$) groups. *Significantly different when compared to Controls ($P < 0.05$; F-test for interaction: $P < 0.05$).

than their normal matched mucosa, in which expression was similar among different groups. However, when considering the tumours, we observed that the expression of i-NOS tended to be lower in the groups treated with the prebiotics ($P = 0.06$); probiotics were not effective. The data relative to GST-P (Figure 5B) indicated that the expression of this gene was significantly higher in the tumours of the Controls and PRE group as compared with their normal matched mucosa ($P < 0.05$). Moreover, considering only the normal mucosa, we observed that the rats treated with prebiotics (PRE and PREPRO groups) had a lower expression ($P < 0.05$) of GST-P when compared with the mucosa of rats not-treated with prebiotics (Controls and PRO). A similar effect was observed in the tumours, where the GST-P expression was lower ($P < 0.05$) in the groups treated with prebiotics. Probiotics were ineffective. The data relative to COX-2 expression (Figure 5C) showed a significant increase ($P < 0.001$) in the tumours of Control rats when compared with their normal matched mucosa; a slight COX2 increase in the tumours was also observed in the other experimental groups (PRE, PRO and PREPRO), but in this case the differences between tumours and normal matched mucosa did not attain statistical significance. When considering only tumours, or normal mucosa, there were no differences among the different groups.

Discussion

The main finding of this study is that rats treated with the prebiotic Synergy1 (groups PRE and PREPRO) have a lower number of AOM-induced colorectal adenomas and cancers when compared with untreated rats. The magnitude of this effect is similar to that of purported chemopreventive substances, like some non-steroidal antinflammatory drugs (aspirin or ibuprofen) or phytochemicals like morin, which halve the multiplicity of AOM-induced colorectal tumours (34).

With the exception of one study on the effect of short-chain fructo-oligosaccharides on Min mice tumours (2), the chemopreventive activity of fructans such as inulin and fructo-oligosaccharides, had been demonstrated only on the induction of aberrant crypt foci (ACF) (3,5,6). Moreover, although short-chain fructo-oligosaccharides have been reported to reduce colon tumours in Min mice (2), Mutanen *et al.*, using the same mouse model, found that inulin promote intestinal tumours (35).

In the present study we evaluated the activity of two strains of probiotics, namely LGG and Bb12. The data relative to malignant tumours show a borderline significant effect of the treatment with LGG and Bb12 ($P = 0.079$). The PRO and PREPRO groups had in fact a lower number of cancers than rats untreated with probiotics. Indeed, in the groups treated with probiotics, the proportion of cancers relative to the total number of tumours in the same groups was significantly lower ($P = 0.04$) than in not-treated rats suggesting that the possible protective effect of probiotics is restricted to malignant tumours.

Some results with Lactobacilli and Bifidobacteria strains suggest that probiotics protect against experimentally induced colon cancer (10–13,36); however, this effect is not completely consistent and may depend on the experimental conditions (36–40). For instance, in the study by Gallaher *et al.* (37), using Bifidobacteria and Lactobacilli, a high variability was found as a function of the weight of the rats at DMH administration.

The viability study we performed with the rif^R strains demonstrated a good recovery of the probiotics in the faeces, thus indicating that LGG rif^R and Bb12 rif^R survive and transit in the rat gastrointestinal tract.

We administered probiotics throughout all phases of carcinogenesis, starting 10 days before AOM, which consisted of only two injections. Previous studies (36) showed that while LGG reduces colon cancer when given 3 weeks before multiple carcinogen administration (16 weekly DMH injections), it is not active if given after the ninth week of DMH treatment. This result suggests that probiotics are active during initiation and that to effectively prevent colon cancer they must be administered long before exposure to the carcinogen and not shortly before, as in our study.

It is also possible that the small effect of probiotics might be explained by the composition of the diet. In fact, the HF diet used should render rats more sensitive to the protective action of probiotics (38); however, the carbohydrates fed to Control and PRO groups were sucrose and maltodextrins, a poor substrate for fermentation (as suggested by low caecal SCFA). Accordingly, in some studies showing a protective

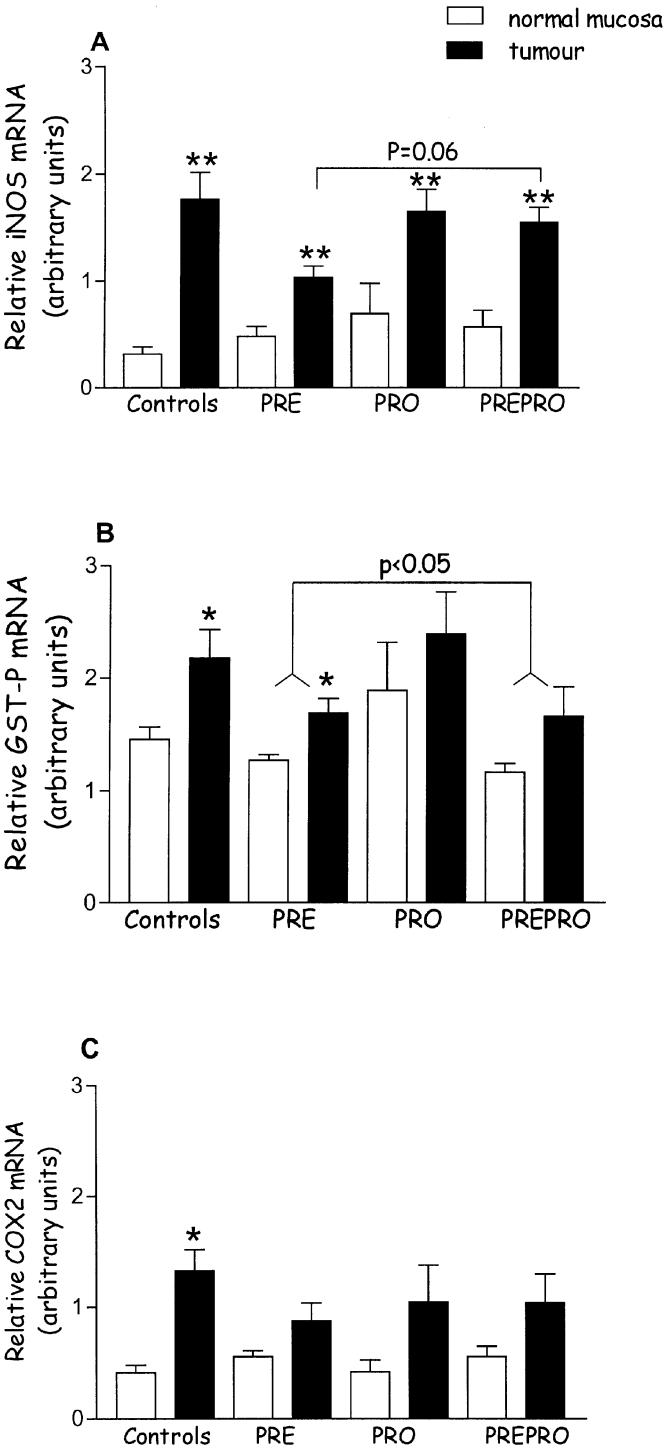

Fig. 5. (**A**) Relative expression of iNOS in the normal mucosa (white bars) and in tumours (black bars) in Controls, PRE, PRO, PREPRO groups, as shown. Values are means ± SE (n = 10, 8, 6 and 6 for controls, PRE, PRO and PREPRO, respectively); $^{**}P < 0.01$ by ANOVA, compared with normal matched mucosa; $P = 0.06$ over the bar refers to the level of statistical significance of the effect of Synergy1 (PRE and PREPRO groups) compared with groups not treated with Synergy1 (Controls and PRO). (**B**) Relative GST-P expression in the normal mucosa (white bars) and tumours (black bars) in Controls, PRE, PRO, PREPRO groups, as shown. Values are means ± SE (n = 10, 8, 6 and 6 for Controls, PRE, PRO and PREPRO, respectively); $^{*}P < 0.05$ by ANOVA, compared with normal matched mucosa; $P = 0.05$ over the bar refers to the level of statistical significance of the effect of Synergy1, obtained by comparing tumours or normal mucosa in the rats treated with Synergy1 (PRE and PREPRO groups) with tumours and normal mucosa from groups not treated with Synergy1 (Controls and PRO). (**C**) Relative expression of COX-2 in the normal mucosa (white bars) and in tumours (black bars) in Controls, PRE, PRO, PREPRO groups, as shown. Values are means ± SE (n = 10, 8, 6 and 6 for Controls, PRE, PRO and PREPRO, respectively); $^{**}P < 0.01$ by ANOVA, compared with normal matched mucosa.

effect of probiotics, the diet contained at least 30% of corn starch (10,11), a good substrate for bacterial fermentation (41). We used sucrose and maltodextrins in the Control and PRO diets, as the effect of the prebiotic Synergy1 could not have been detected if partially undigested carbohydrates were present in the Control diet.

We were also interested in studying whether prebiotics and probiotics, if effective, would interact in an additive or synergistic manner. We found that a combined administration of prebiotic and probiotics was able to further reduce the occurrence of malignant tumours; the results obtained suggest that the two components may interact in an additive and not in a synergistic manner. Previous studies by Rowland *et al.* (3) also suggested a beneficial effect in reducing preneoplastic lesions with the contemporary administration of prebiotics and probiotics. Similar results, although less marked and consistent, were obtained by Gallaher and Khil (42). All these results taken together (3,42), suggest that the combined administration of prebiotics and probiotics is beneficial.

To evaluate possible mechanisms of action of prebiotics and probiotics we did additional exploratory analyses on some parameters associated with colon cancer. These studies were performed only in subgroups of rats randomly drawn from each experimental group; therefore, we did not perform a full inferential analysis, but we used the data to generate hypotheses about possible mechanisms of action.

SCFA production in the caecum showed, as expected, that rats in PRE and PREPRO groups had a higher SCFA concentration than Control or PRO groups, a result explained by the fact that Synergy1 is not totally absorbed in the small intestine but it is fermented in the colon. Moreover, when considering the relative amount of individual SCFA over total SCFA, the treatment with Synergy1 caused a relative increase in butyrate, a compound, which has been demonstrated to have antitumour effects *in vitro* (22). However, recent results obtained *in vivo* do not confirm this chemopreventive effect and suggest that butyrate is not the only link between fermentation and colon cancer (43). Apart from the direct biological effects of SCFA and of butyrate in particular, it is also possible that the decrease in pH due to these organic acids may be beneficial for the intestine since high colonic pH has been shown to promote colon cancer (44).

Previous studies have suggested that variations in apoptosis in the normal colon mucosa may be correlated with carcinogenesis (45), higher apoptosis being associated with a lower risk of developing cancer. In the present paper apoptosis was higher in the normal mucosa of PRO group than in Controls. This effect was mainly due to increased apoptosis in the first compartment of the crypt, a phenomenon, which could be beneficial if mutated stem cells—lying in this compartment—were removed in this way. Surprisingly, apoptosis was not increased in the PREPRO group where the probiotics were administered with Synergy1, an effect we cannot explain. Moreover, although it has been reported that prebiotics like oligofructose or inulin increase apoptosis in the colon (46) in our study Synergy1 did not affect apoptosis. Therefore, in the present study apoptosis in the normal mucosa and carcinogenesis were not correlated. It has to be noticed, however, that some of the studies linking apoptosis to cancer risk have been obtained in rats killed shortly after carcinogen treatment; several months later, like in our study, apoptosis levels may follow a different pattern.

We also found that apoptosis in tumours was not varied among the different groups, a result at variance with previous studies in which chemopreventive activity was linked to a high apoptotic index in the tumours (20).

The results relative to the proliferative activity indicate a lower number of labeled cells/crypt in the PRE group; in the PRO and PREPRO groups we observed a borderline reduction in proliferative activity but the effect was not significant. As high proliferative activity in the colon mucosa has been associated with an increased risk of colon cancer (17,18), the data suggest that, although not as a principal mechanism, prebiotics and to a lesser extent, probiotics might act also by lowering proliferation. Accordingly, dietary components have been reported to influence colon carcinogenesis through variation in the colonic proliferative activity (17,18).

Finally, we were interested in studying the mRNA expression of genes codifying enzymes possibly involved in colon carcinogenesis. GST-P is a member of the glutathione *S*-transferase (GST) superfamily, a group of enzymes detoxifying xenobiotics. However, GST-P overexpression has also been demonstrated in many types of cancers, including colon (47). It has been suggested that up-regulation of GST-P is associated with poor prognosis in humans (23) and is induced by K-Ras mutation during human colon carcinogenesis (48). COX-2 and i-NOS are also up-regulated in human and experimental colon cancers (24,25), a phenomenon which has been linked to resistance to apoptosis, DNA damage, mutation, increased proliferation, oxidative stress and increase in tumour vascularity and metastatic potential (48). It has also been demonstrated that inhibition of COX-2 and i-NOS activities decrease AOM-induced carcinogensis (26,49,50).

We observed that the prebiotic Synergy1, which inhibits intestinal carcinogenesis, depresses GST-P overexpression in both colon tumours and normal mucosa. We also found that i-NOS expression was slightly lower in the tumours of rats treated with prebiotics. COX-2 expression tended to be higher in all the tumours studied compared with their normal matched mucosa, but this effect attained statistical significance only in Controls and not in the PRE, PRO or PREPRO groups. Up-regulation of these enzymes has been reported in colonic tumours; therefore, it is possible that some of the results observed could be a secondary effect of the decreased tumourigenesis in the treated groups. However, the fact that some variations (e.g. on GST-P expression) were observed also in the normal mucosa and not only in the tumours, suggests that diet influences gene-expression.

In conclusion, while a possible protective effect of probiotics needs further confirmation, the results indicate that prebiotics decrease AOM-induced carcinogenesis. The mechanisms by which they act are less clear, but the data presented suggest that they may act through a combination of mechanisms involving an increase in SCFA production, lower proliferative activity and a variation in the expression of some enzymes involved in the pathogenesis of colon cancer. How these variations are causally related is not yet clear and further studies will be necessary to understand the role of diet in colon carcinogenesis and in gene-expression in particular.

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