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In vitro screening of probiotics and synbiotics according to anti-inflammatory and anti-proliferative effects

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ABSTRACT

There is emerging evidence of the efficiency of probiotic, prebiotic and synbiotic treatments in inflammatory bowel diseases (IBDs) and one of their long-term complications, colorectal cancer (CRC). In this study, various strains of probiotic lactic acid bacteria, prebiotic glucooligosaccharides (GOS) or a synbiotic combination of the two were screened for anti-inflammatory and anti-proliferative effects in different *in vitro* models in the context of such diseases. To mimic IBD response to Gram negative bacteria, HT-29 cells were sensitised to inflammatory response to lipopolysaccharide (LPS) by IFNγ which increased expression of TLR4, the LPS biosensor, and were then treated by probiotics, prebiotics and synbiotics. Secreted IL-8 and activated NF-κB were monitored as inflammation biomarkers. A selection of active strains were then subjected to a second inflammatory cell culture model consisting of inflammatory activated transgenic Caco-2 cells transfected by a reporter gene under the control of NF-κB inducible promoter. Quantification of reporter gene expression allowed us to demonstrate some probiotic inhibitory properties or to confirm such characteristics in two different models. Proliferation of cancerous HT-29 cells was monitored by XTT assay. Only three probiotic strains induced a proliferation decrease, but with a lack of reproducibility. Binary or ternary probiotic associations, complemented or not by prebiotic GOS, significantly decreased proliferation, especially with a synbiotic association of *Bifidobacterium breve*, *Lactococcus lactis* and oligoalternan, a GOS. This combination was selected for the following experiments. We showed the involvement of both bacterial and carbohydrate compounds of this synbiotic in the observed effect by dose range tests. We demonstrated that this decrease in proliferation may be due to an induction of a differentiated phenotype, as shown by the up-regulation of intestinal alkaline phosphatase, a biomarker of differentiation, monitored by real-time RT-PCR in HT-29 cells treated by the selected synbiotics. Thus, this study demonstrates the ability of probiotics to exert anti-inflammatory effects and shows some anti-proliferative characteristics for a specific synbiotics. These products should be further evaluated in animal models to confirm the *in vitro* results.

1. Introduction

Inflammatory bowel diseases (IBDs) are mainly represented by ulcerative colitis and Crohn's disease and have had a high and increasing prevalence in western countries during recent decades (Karlinger et al., 2000; Lakatos, 2006). These pathologies are characterized by an excessive and prolonged inflammatory response (Laroux et al., 2001) and, despite their unknown aetiology, genetic and environmental risk factors seem to be involved in IBD pathogenesis (Bosani et al., 2009). Some genetic alterations in IBDs concern microbial Pattern Recognition Receptors (PRRs), such as NODs or TLRs, which influence NF-κB activation and lead to a deregulated inflammation of the intestinal mucosa (Yamamoto-Furusho, 2007). Environmental risk factors are related to smoking, appendectomy or the diet of western countries (Loftus, 2004). The key role of microbiota imbalance has been highlighted in IBDs in several studies, with a loss of diversity and an increase in Gram negative bacteria (Baumgart et al., 2007; Gradel et al., 2009; Wohlgemuth et al., 2009). Lipopolysaccharide (LPS) of Gram negative bacteria is

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recognised by PRRs and triggers an inflammatory response through the NF-κB pathway and the subsequent overexpression of pro-inflammatory cytokines like IL-8 (Riedel et al., 2006). The major involvement of gut microbiota imbalance in IBD pathogenesis suggests that modulating this part of the intestinal ecosystem could help to prevent such diseases. Probiotics, prebiotics and synbiotics are new concepts recently tried in IBD prevention and/or treatment. Some probiotics, which are defined as "live microorganisms which beneficially affect the health of the host" (FAO/WHO, 2002), belonging to lactic acid bacteria and especially the genera *Bifidobacterium* and *Lactobacillus*, have recently been demonstrated to have therapeutic properties in IBD (Isaacs and Herfarth, 2008; Martins et al., 2009; Vanderpool et al., 2008). Probiotics could also prevent IBD crises by inhibiting pathogenic bacteria (Servin, 2004) or modulating intestinal mucosa immunity (Delcenserie et al., 2008). The revised definition of a prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health." (Roberfroid, 2007). Most of the prebiotics studied have been carbohydrate polymers and they have been shown to be effective in attenuating inflammation. Prebiotics exert their anti-inflammatory effects through different pathways such as fermentation into short chain fatty acids (SCFA) by colic microflora (Lara-Villoslada et al., 2006) or the specific stimulation of beneficial gut microflora (Hoentjen et al., 2005). This observation has led to the development of the concept of synbiotics as a synergistic combination of probiotics and prebiotics (Roberfroid, 1998). They have been demonstrated to be efficient in preventing IBD symptoms and improving patients' quality of life in clinical trials (Fujimori et al., 2009; Furrie et al., 2005).

Chronic inflammation occurring in IBDs induces persistent damage along the digestive tract and plays a role in the long-term development of colorectal cancer (CRC) (McConnell and Yang, 2009). As for classical chronic inflammatory symptoms, some data suggest that probiotics could be useful to prevent such pathologies. *Lactobacillus salivarius* UCC118 was shown to reduce both inflammatory response and gastrointestinal tumour development in IL-10 knockout mice (O'Mahony et al., 2001). *Lactobacillus* peptidoglycan is able to induce Th1 response and thus activate peritoneal macrophages which exert effects against cancer CT26 cell development in mice (Sun et al., 2005). Prebiotics have also been evaluated for their antitumorigenic effects. Germinated barley foodstuff treatments showed some significant protective effects against chemically induced CRC in rat models (Kanauchi et al., 2008). Furthermore, resistant starch induced apoptosis of cancerous cells in another CRC rat model (Bauer-Marinovic et al., 2006). These results could be explained by the selective induction of growth of beneficial bacteria by prebotiotics and their fermentation leading to antitumorigenic compounds. These results obtained with probiotics and prebiotics against CRC led to the assaying of synbiotic anticancer properties in an attempt to increase the effects one of these compounds in comparison with when the components were used alone. In a CRC rat model, *Bifidobacterium longum* and inulin showed a synergistic reduction of colon carcinogenesis (Rowland et al., 1998) and *B. lactis* and resistant starch induced cancerous cell apoptosis (Le Leu et al., 2005).

These results suggest that probiotics, prebiotics and synbiotics could be useful in IBD and/or CRC prevention or treatments. In the present study, we first aimed to evaluate the anti-inflammatory characteristics of products in *in vitro* models by monitoring inflammation biomarkers. For a second aim, we tested them for their anti-proliferative properties on cancer cell lines. All assays were conducted with probiotics and/or with glucooligosaccharides (GOS), which are prebiotic carbohydrates and had been previously demonstrated to support growth of some of the tested probiotic strains (Grimoud et al., 2010)*. These compounds were tested in an attempt to provide probiotics, prebiotics or synbiotics efficient for inhibiting the mentioned phenomenon *in vitro* as a first step to selecting them for *in vivo* studies.

2. Materials and methods

2.1. Probiotic strains and growth conditions

The probiotic lactic acid bacteria tested are listed in Table 1. *Bifidobacterium bifidum* 02, 20 and *Bifidobacterium pseudocatenulatum* 14 had previously been described newborn faeces clinical isolates (Vaugien et al., 2002) and were chosen on the basis of their high adherence to human colonic epithelial cells and their immunomodulatory activities on macrophage cell lines (Vaugien et al., 2005). *Lactobacillus farciminis* CIP 103136 was obtained from Institut Pasteur Collection (Paris, France) and had previously been demonstrated to have anti-inflammatory and anti-nociceptive effects in TNBS rat model (Ait-Belgnaoui et al., 2009; Lamine et al., 2004a,b). Other strains were obtained from the Lallemand Company collection and were selected for their industrial characteristics. The strains were stored at −80 °C in Eugon broth (AES Chemunex, Bruz, France) with 20% (v/v) glycerol (Fluka, Butch, Switzerland) and reactivated at 37 °C under anaerobic conditions on agar plates as indicated: (a) for the bifidobacteria on Reinforcement Clostridial Medium (Oxoid, Cambridge, UK), (b) for the lactobacilli, *Lactococcus lactis* and *Pediococcus acidilactici*, on MRS (AES Chemunex, Bruz, France) and (c) for *Streptococcus thermophilus*, on M17 (AES Chemunex, Bruz, France). Before the experiments, overnight cultures were prepared in MRS broth (AES Chemunex, Bruz, France) at 37 °C under anaerobic conditions.

2.2. Glucooligosaccharides

The glucooligosaccharides tested (CRITT-Bio Industries, Toulouse, France) were: oligoalternan (OA) synthesised by maltose acceptor reaction catalyzed by *Leuconostoc mesenteroides* NRRL B-1355 alternansucrase (Lopezmunguia et al., 1993) and oligodextran (OD) synthesised by maltose acceptor reaction catalyzed by *L. mesenteroides* NRRL B-512F dextransucrase (Remaud et al., 1991).

2.3. Cell culture

HT-29 cells were obtained from the European Collection of Cell Cultures (ECACC), stored at −190 °C and were cultured in a 75 cm^2 flask (Becton Dickinson, Franklin Lake, USA) with McCoy's 5A medium (Lonza, Basel, Switzerland) supplemented with 10% (v/v) heat-inactivated foetal bovine serum, non-essential amino acids (Lonza, Basel, Switzerland) 1% (w/v), and the antibiotics penicillin and streptomycin (Lonza, Basel, Switzerland) 1% (w/v). Cells were cultured at 37 °C under an atmosphere of 5% CO_2. The medium was changed every 48 h and cultures were maintained subconfluent.

Table 1
Probiotic strains tested.

Probiotic species	Strains	Origins
Bifidobacterium bifidum	02	Clinical
Bifidobacterium bifidum	20	Clinical
Bifidobacterium breve	R0070	Lallemand
Bifidobacterium longum	R0175	Lallemand
Bifidobacterium pseudocatenulatum	14	Clinical
Lactobacillus acidophilus	R0240	Lallemand
Lactobacillus buchneri	R1102	Lallemand
Lactobacillus farciminis	CIP103136	Institut Pasteur Collection
Lactobacillus helveticus	R0052	Lallemand
Lactobacillus plantarum	R1012	Lallemand
Lactobacillus rhamnosus	R1102	Lallemand
Lactococcus lactis	R1058	Lallemand
Pediococcus acidilactici	R1001	Lallemand
Streptococcus thermophilus	R0083	Lallemand

2.4. NF-κB and IL-8 Elisa quantification

HT-29 cells were seeded at a concentration of 2×10^5 cells/mL in 24-well plates and cultured for 7 days at 37 °C under an atmosphere of 5% CO_2 to reach confluence. Overnight cultures of microorganisms were collected by centrifugation (5 min at 3600 g) and resuspended in fresh McCoy's 5A medium without antibiotic. HT-29 monolayers were washed twice with PBS (Lonza, Basel, Switzerland) and 1 mL of fresh McCoy's 5A medium without antibiotic was added to each well. Monolayers were treated by LPS (Sigma, Saint Louis, USA) at 100 ng/mL and IFNγ (MilleGen, Toulouse, France) at 200 ng/mL and then i) inoculated with probiotics at a multiplicity of infection (MOI) of 100, ii) supplemented with sterile-filtered prebiotic GOS at a final concentration of 10 g/L or iii) inoculated with a combination of probiotics and GOS. Non-activated HT-29 cells were also treated with bacteria or GOS alone to evaluate the absence of pro-inflammatory properties of our products. Plates were incubated for 6 h for IL-8 quantification and 16 h for activated NF-κB quantification at 37 °C under 5% CO_2. These incubation times were chosen for an optimal detection previously evaluated considering the assay conditions. At the end of the incubation times, cell viability was checked by trypan blue (Sigma, Saint Louis, USA), supernatants were collected by aspiration and cell debris were removed by centrifugation (5 min at 14,000 g). Nuclear extracts were harvested using a commercially available kit (Activemotif, Rixensart, Belgium) according to the manufacturer's instructions. Supernatants and nuclear extracts were stored at −80 °C before being assayed. Secreted IL-8 was quantified in the supernatants by a classical ELISA test (Diaclone, Besançon, France). Activated NF-κB was monitored on the nuclear extracts with an ELISA based TransAM NF-κB kit (Activemotif, Rixensart, Belgium) consisting of a 96-well plate with immobilized oligonucleotides containing a sequence that allowed specific binding of activated NF-κB. Quantifications were performed according to the manufacturer's instructions. The percentage of variation in IL-8 secretion or NF-κB activation was calculated as follows:

$$\frac{value_{assay}-value_{negative\ \ control}}{value_{positive\ \ control}-value_{negative\ \ control}}\times 100 \quad (1)$$

Positive controls were HT-29 cells treated with LPS (100 ng/mL) and IFNγ (200 ng/mL) and negative controls were untreated HT-29 cells. For each condition, bacterial quantification was performed at 6 h and 16 h by numeration on agar media. Separate experiments were performed three times.

2.5. NF-κB quantification in transgenic cell models — AGIR™ test

Assays were performed by the Libragen Company (Toulouse, France) on cytokine activated Caco-2 transgenic cells transfected by alkaline phosphatase gene under control of an NF-κB inducible promoter. Caco-2 transgenic cells were seeded at a concentration of 5.10^4 cells/well in 96-well plates and cultured for 48 h. Thus, after a cytokine-induced inflammatory stimulation and a probiotic treatment for 6 h, the monitored alkaline phosphatase activity was directly correlated with NF-κB activation (confidential protocol, to be published). The percentage of reporter gene induction was calculated following the equation presented above in NF-κB and IL-8 Elisa quantification.

2.6. Quantification of the proliferation of colic epithelial cancer cell line HT-29

The HT-29 cell proliferation was monitored by XTT assay. XTT reduction into formazan, a coloured compound, by mitochondrial dehydrogenases is considered to be proportional to cell number and thus related to cell proliferation after incubation time. HT-29 cells were seeded at a concentration of 2×10^5 cells/mL in 96-well plates and cultured for 24 h. The culture medium was removed and the cell monolayers were washed twice with PBS. 250 µl of fresh McCoy's 5A medium supplemented with non-essential amino acids 1% (w/v), glutamine 1% (w/v), and newborn calf serum 10% (v/v) were added to each well. Wells were then inoculated with i) probiotics, at a multiplicity of infection (MOI) of 100 alone (noted glucose, as this carbohydrate was the sole carbon source in the medium in this condition), ii) prebiotics, sterile-filtered and added at a final concentration of 10 g/L or iii) synbiotics, a combination of probiotics at a MOI of 100 and prebiotics at 10 g/L. Microplates were incubated at 37 °C under 5% CO_2. After 16 h, cell viability was checked by trypan blue, supernatants were removed and cell monolayers were washed twice in PBS. Then wells were filled with 100 µL of RMPI medium with XTT (sodium 3-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulphonic acid hydrate) at 5 mg/mL and coenzyme Q at 0.04 mg/mL and plates were incubated at 37 °C in an atmosphere containing 5% CO_2. After 3 h, 100 µL of a solution at 10% SDS were added to each well and formazan absorbance was assayed at λ = 450 nm. The percentage of proliferation was calculated as follows:

$$\frac{OD_{assay}-OD_{negative\ \ control}}{OD_{negative\ \ control}}\times 100 \quad (2)$$

where OD_{assay} represents optical density for HT-29 cells treated by probiotics, prebiotics or synbiotics and $OD_{negative\ control}$ the control carried out with HT-29 cells alone. Separate experiments were performed six times.

2.7. Differentiation of colic epithelial cancer cell line HT-29

HT-29 cells were cultivated for 24 h and then inoculated with the selected synbiotics or were cultivated for 14 days from post-confluence to obtain a differentiated phenotype as a control. After incubation, total RNA was extracted from HT-29 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The expression rate of the human intestinal alkaline phosphatase and β-actin were analysed by real-time Reverse Transcriptase-PCR using the IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, USA) with the SuperScript™ III Platinum® SYBER® Green One-Step qRT-PCR Kit (Invitrogen, San Diego, USA). The RT-PCR program included 1 cycle at 50 °C for 10 min, 1 cycle at 95 °C for 5 min and then 45 cycles at 95 °C for 15 s, 55 °C for 45 s and 72 °C for 45 s, followed by 1 cycle at 95 °C for 1 min and 55 °C for 1 min with a melting curve to check amplicon specificity. The sequences of oligonucleotide primers (Invitrogen, San Diego, USA) are listed in Table 2. Separate experiments were performed three times.

Table 2
Target genes and sequence of the primers used.

Gene	Primer	Sequence	Product size (bp)	Reference
Intestinal	hIAP-F	GCAACCCTGCAACCCACCCAAGGAG	503	Malo et al. (2006)
Alkaline phosphatase	hIAP-R	CCAGCATCCAGATGTCCCGGGAG		
β actin	hbACT-F	GGGTCTGGACCTGGCTGGCCGGGACCTG	277	Malo et al. (2006)
	hbACT-R	GGGCCGCCGATCCACACGGAGTACTTGC		

Table 3
IL-8 and NF-kB relative quantification in HT-29 cells inflammatory activated and challenged with probiotics. The results are expressed as ratio between assay and control realized with cells inflammatory stimulated and untreated with probiotics. The results are given as average values of three different experiments ± standard deviation.

Strains	% of induction	
	IL-8	NF-kB
B. bifidum 02	−112.4 ± 12.0*	−131.8 ± 37.7*
B. bifidum 20	−72.0 ± 13.1*	−124.9 ± 45.8*
B. breve R0070	−46.1 ± 26.8	−47.5 ± 37.7
*B. longum*R0175	−81.2 ± 34.5*	−20.2 ± 18.1
B. pseudocatenulatum 14	−61.6 ± 07.7*	−113.5 ± 47.6*
L. acidophilus R0240	−48.5 ± 51.2	−02.9 ± 36.7
L. buchneri R1102	−42.1 ± 08.2	19.2 ± 52.6
L. farciminis CIP103136	−82.5 ± 03.3*	−16.3 ± 43.0
L. helveticus R0052	−80.5 ± 11.8*	−93.4 ± 38.5*
L. plantarum R1012	−95.0 ± 07.0*	−25.0 ± 23.7
L. rhamnosus R0011	−92.8 ± 04.4*	06.5 ± 18.8
L. lactis R1058	−109.1 ± 08.1*	−80.4 ± 7.6*
P. acidilactici R1001	−90.6 ± 04.8*	08.1 ± 28.9
S. thermophilus R0083	−63.8 ± 11.4*	89.1 ± 64.8

* Different from untreated by probiotics control, $P<0.05$.

3. Results

3.1. NF-κB and IL-8 quantification in HT-29 cells

None of the tested strains or GOS alone induced secretion of IL-8 or NF-κB activation when they were co-cultured with HT-29 cells without any activation (data not shown). Table 3 shows the variation values of both secreted IL-8 and activated NF-κB in inflammatory stimulated HT-29 cells treated by probiotics in comparison with the untreated control. By considering the parameters monitored, different groups can be distinguished. Three of the five bifidobacteria (*B. bifidum* LMI 02 and LMI 20, *B. pseudocatenulatum* LMI 14), *Lactobacillus helveticus* R0052 and *L. lactis* R1058 induced a dramatic reduction of both secretion of IL-8 and activation of NF-κB with a more reproducible effect on IL-8 secretion. The second group including *Bifidobacterium* breve R0070, *B. longum* R0175, three lactobacilli (*L. farciminis* CIP 103136, *Lactobacillus plantarum* R1012, *Lactobacillus rhamnosus* R0011) and the other two genera (*P. acidilactici* R1001 and *S. thermophilus* R0083) showed a significant decrease of secreted IL-8 only in stimulated HT-29 cells. Surprisingly, *S. thermophilus* R0083 reduced secretion of IL-8 but increased NF-κB activation. *L. acidophilus* R0240 and *Lactobacillus buchneri* R1102 had no significant effect on either IL-8 secretion or NF-κB activation. Prebiotics alone had no effect and, when combined with probiotics, did not give any improvement of the anti-inflammatory effects observed with bacteria alone (data not shown). Bacterial numeration at 6 h and 16 h showed that GOS complementation of the McCoy's 5A medium did not improve probiotic development.

3.2. NF-κB quantification in transgenic Caco-2 cells (AGIR™ — Libragen)

Some strains selected in the previous *in vitro* anti-inflammatory screening both activated NF-κB and secreted IL-8. Additional industrial strains were also selected for their ease of cultivation and industrial production. In order to confirm the potential anti-inflammatory properties or to check this activity, these strains were subjected to another model provided by Libragen. Fig. 1 shows the variation of the expression of alkaline phosphatase in transgenic Caco-2 cells after a cytokine-induced inflammatory response and treatment by probiotics in comparison with untreated controls. Inhibition effects were confirmed for most of the selected strains, which inhibited NF-κB phosphorylation in the inflammatory induced HT-29 cell model (*B. bifidum* LMI 02, *B. pseudocatenulatum* LMI 14, *L. helveticus* R0052 and *L. lactis* R1058). *L. acidophilus* R0240 did not have any effect in this model, confirming the absence of anti-inflammatory characteristics for this strain. The lack of anti-inflammatory properties in the *in vitro* model of *B. longum* R0175 was confirmed in this model. *B. breve* R0070, *L. farciminis* CIP 103136 and *L.* rhamnosus R0011 induced a significant decrease in these transgenic Caco-2 cell lines whereas a poor or non-significant effect was observed in the HT-29 model.

3.3. Colic epithelial cancer cell line HT-29 proliferation

When tested alone, GOS did not induce any significant variation of proliferation. 20% of inhibition of proliferation was considered as the limit for the effects to be considered significant because of the poor

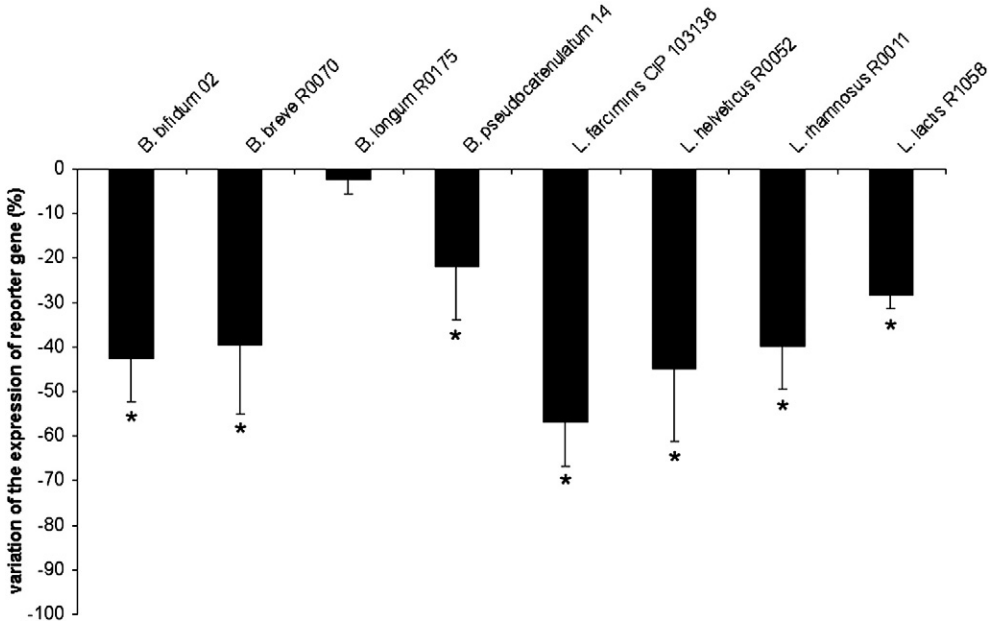

Fig. 1. Expression of a reporter gene under control of inducible NF-κB promoter in activated transgenic Caco-2 cells, inflammatory activated and treated by probiotics during 6 h. The results are expressed as the ratio between assay and control obtained with cells stimulated and untreated with probiotics. The results are given as average values of three different experiments ± standard deviation. *Different from control untreated by probiotics, $P<0.05$.

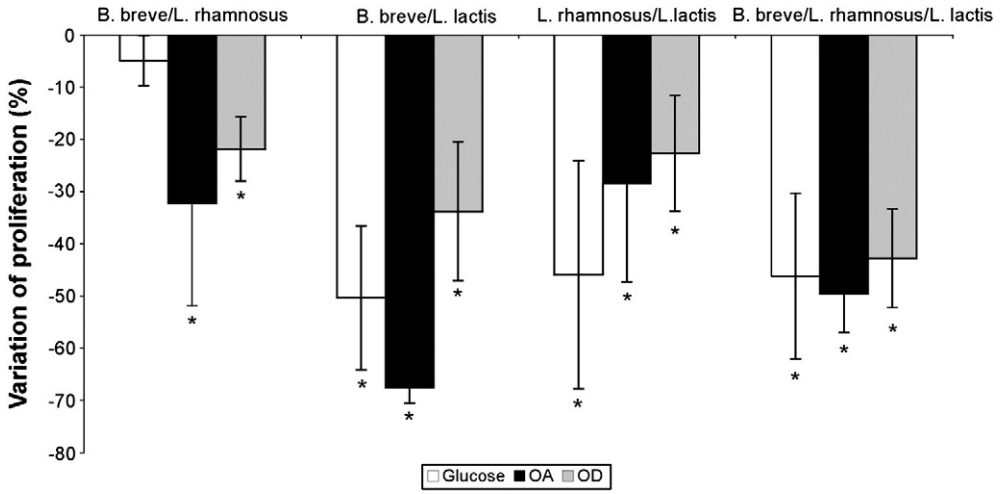

Fig. 2. Variations of HT-29 cell proliferation after a treatment by combined probiotics associated with GOS or glucose. The results are expressed as a ratio between assay and control performed with untreated cells. The results are given as average values of three different experiments ± standard deviation. *Different from negative control, $P<0.05$.

reproducibility under this limit. Only *B. breve* R0070, *L. rhamnosus* R0011 and *L. lactis* R1058 decreased proliferation by more than 20% in presence of glucose or GOS. Inhibition of proliferation observed with *L. rhamnosus* R0011 reached 50% in presence of glucose (data not shown).

To further improve these results and characterize synergistic activities, these three potentially active strains were monitored in association for anti-proliferative effects. Fig. 2 shows HT-29 cell proliferation variations with different combinations with glucose as a control and GOS. When associated probiotics were used with glucose, the inhibition values were between 45% and 50% except for *B. breve* R0070 and *L. rhamnosus* R0011, which did not inhibit HT-29 proliferation.

The strongest and most reproducible effect was observed with *B. breve* R0070 and *L. lactis* R1058 in presence of OA, with an inhibition value of 66.31% +/− 2.05%. For the other probiotic mixtures, there were no significant differences between OA and OD for a given probiotic mixture. All the other probiotics/glucose or synbiotic combinations exerted lower and less reproducible proliferation inhibitions. The combination of *B. breve* R0070, *L. rhamnosus* R0011, *L. lactis* R1058 had lower but still significant effects for any carbon source. *L. rhamnosus* R0011 and *L. lactis* R1058, when co-cultured with cells on glucose as the sole carbon source, reached the same level but with less reproducibility. *B. breve* R0070 and *L. rhamnosus* R0011 did not reduce HT-29 proliferation in presence of glucose and inhibited cell growth in the same way as *L. rhamnosus* R0011 and *L. lactis* R1058 with OA and OD. Moreover, OA inhibition rates were greater for all probiotic combinations compared to assays conducted with OD and for associations of three bacteria when compared to the results obtained in the presence of glucose. These data show the anticarcinogenic potential of this oligosaccharide when combined with probiotic(s).

For further experiments, we focused on the *B. breve*/*L. lactis*/OA association because of the proliferation inhibition values and specific GOS effect, which could lead to synbiotic applications. In an attempt to determine whether bacteria and/or OA were involved in the anti-proliferative effects of the *B. breve*/*L. lactis*/OA association, dose range assays were conducted. Fig. 3 gives the HT-29 cell proliferation variations at different multiplicities of infection (MOI) of the combined bacteria in the presence of OA at 10 g/L or at different OA concentrations in the presence of combined bacteria (MOI = 100). For a constant concentration of OA at 10 g/L, inhibition of proliferation was significantly reduced for MOI 1000 and 100 (at MOI 1000 the observed effect may have been due to cytotoxic effects induced by bacterial acidification of the medium). Proliferation inhibition decreased from MOI 1000 to MOI 10 and no influence on proliferation was observed for MOI 1 and MOI 0.1. When OA was added at a final concentration of 10 g/L, the anti-proliferative effects were correlated to the MOI value with a loss of cell growth reduction for MOI<10. For a constant MOI = 100 the anti-

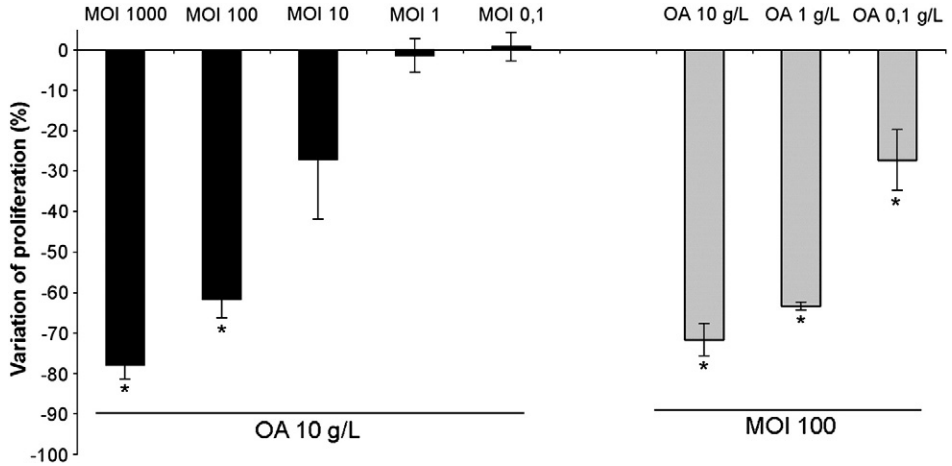

Fig. 3. Dose range effect of MOI of *B. breve* R0070 and *L. lactis* R1058 or OA concentration on HT-29 cell proliferation expressed as percentage of cell proliferation in comparison to untreated control. The results are given as average values of three different experiments ± standard deviation. *Different from negative control, $P<0.05$.

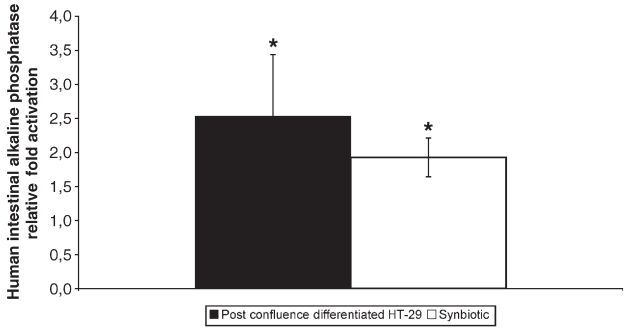

Fig. 4. Real time RT-PCR analyses of human intestinal alkaline phosphatase expression. The data are normalized to ß-actin expression and displayed relative to control. The results are given as average value of three different experiments ± standard deviation. *Different from negative control, $P<0.05$.

proliferative effects were equivalent for concentrations of OA at 10 g/L and 1 g/L but decreased at 0.1 g/L, although remaining significant. These observations suggest that both bacterial and GOS compounds are involved in the inhibition of proliferation.

3.4. HT-29 cell differentiation

We investigated the intestinal alkaline phosphatase (hIAP) expression as a biomarker of colic differentiation (Marvin-Guy et al., 2008). Fig. 4 shows the variation rates of hIAP mRNA levels in HT-29 cells treated by the synbiotic *B. breve* R0070/*L. lactis* R1058/OA during 16 h' contact time or in 14 days post-confluence differentiated HT-29 cells in comparison with untreated control.

Post-confluence differentiated cells displayed a 2.5-fold expression factor for hIAP mRNA levels and cells treated by the synbiotic expression a 2-fold activation factor. Statistical analyses showed that the induction exerted by the synbiotics was significantly equivalent to that of the post-confluence differentiated cells. Thus, differentiation may explain the limitation of proliferation observed with the *B. breve* R0070/*L. lactis* R1058/OA combination.

All the results from inflammation and proliferation *in vitro* models are summarised in Table 4.

4. Discussion

In the present study, we first aimed to select some potential anti-inflammatory probiotics *in vitro*. None of our tested strains or prebiotic GOS had any intrinsic pro-inflammatory characteristics, which suggests safe use for a possible human application. Through our first cell culture model, we demonstrated the anti-inflammatory properties of 11 strains, belonging to the different genera tested, in HT-29 cells during a pro-inflammatory treatment in a strain-dependent manner. 5 of these strains were able to dramatically inhibit both NF-κB activation and IL-8 secretion. The other 6 strains were only significantly active on IL-8 secretion and a lack of effects or poor reproducibility of some results concerning the NF-κB activation assays did not allow us to conclude on some possible anti-inflammatory effects. For example, *L. farciminis* CIP 103136, which has been reported to be effective for its anti-inflammatory properties (Lamine et al., 2004a,b), dramatically reduced secreted IL-8 but high standard deviation made quantification of the reduction of NF-κB activation non-significant. So, for a more efficient anti-inflammatory strain selection, we used a second model of transgenic Caco-2 cells transfected with a reporter gene under control of NF-κB inducible promoter. This model allowed us to confirm the results obtained with the HT-29 cell model or to highlight strong anti-NF-κB effects for some strains that had non-significant effects in the first model. In addition to the presence of the transfected plasmid in the transgenic cells, the observed discordances between the two cell culture models could be due to the differences of the genetics of the two cancer cell lines used and by the use of different pro-inflammatory stimuli which triggered NF-κB activation through different pathways.

The blocking of inflammatory response by probiotic bacteria after a sensitisation by IFNγ and activation by LPS suggests that the tested bacteria could interact with LPS, for example by inhibiting LPS/TLR4 interaction. These hypotheses are related to studies that have shown the role of LPS in gut inflammation (Selleri et al., 2008; Zoumpopoulou et al., 2009), especially to the observation of LPS/TLR4 signalling pathway inhibition by probiotics (Watanabe et al., 2009).

Because gut microbiota imbalance with a predominance of Gram negative bacteria and their LPS (Baumgart et al., 2009) plays a key role in IBD pathogenesis, our first inflammatory *in vitro* model used pro-inflammatory stimulation based on LPS of *E. coli* and IFNγ. IFNγ was

Table 4
Summarised results from the inflammation and proliferation models.

Probiotics, synbiotics tested	Anti-inflammatory effects			Anti-proliferative effects		
	HT-29 + LPS + IFNg		Caco-2 (cytokine)	HT-29 proliferation		
	Activated NF-κB	Secreted IL-8	Activated NF-κB	Glucose	OA	OD
Bifidobacterium bifidum LMI 02	+++	+++	++	NS	NS	NS
Bifidobacterium bifidum LMI 20	+++	+++	ND	NS	NS	NS
Bifidobacterium breve R0070	NS	++	++	+	NS	NS
Bifidobacterium longum R0175	NS	+++	NS	NS	NS	NS
Bifidobacterium pseudocatenulatum LMI 14	+++	+++	++	NS	NS	NS
Lactobacillus acidophilus R0240	NS	NS	ND	NS	NS	NS
Lactobacillus buchneri R1102	NS	++	ND	NS	NS	NS
Lactobacillus farciminis CIP103136	NS	+++	+++	NS	NS	NS
Lactobacillus helveticus R0052	+++	+++	++	NS	NS	NS
Lactobacillus plantarum R1012	NS	+++	ND	NS	NS	NS
Lactobacillus rhamnosus R1102	NS	+++	++	++	NS	+
Lactococcus lactis R1058	+++	+++	ND	NS	+	NS
Pediococcus acidilactici R1001	NS	+++	++	NS	NS	NS
Streptococcus thermophilus R0083	NS	+++	ND	NS	NS	NS
Bifidobacterium breve R0070 + *Lactobacillus rhamnosus* R1102				NS	++	++
Bifidobacterium breve R0070 + *Lactococcus lactis* R1058				++	+++	++
Lactobacillus rhamnosus R1102 + *Lactococcus lactis* R1058				++	++	++
Bifidobacterium breve R0070 + *Lactobacillus rhamnosus* R1102 + *Lactococcus lactis* R1058			++	++	++	

Legend: +++ inhibition of more than 50%, ++ inhibition of more than 20%, + inhibition reaching 20%, NS no significant result, ND not determined.

added to increase inflammatory response and induce an up-regulation of TLR4, the LPS biosensor (Pearl-Yafe et al., 2007), so as to mimic IBD conditions as it has been shown that TLR4 is over expressed in IBD patients, (Cario and Podolsky, 2000) making the mucosa most sensitive to Gram negative bacteria. To our knowledge, this is the first time that LPS/IFNγ have been used in combination to induce inflammation *in vitro* by pathways similar to IBD situations. We also evaluated inflammatory response, especially NF-κB activation, with LPS and IFNγ alone, LPS combined with soluble CD14, the TLR4 co-receptor, or with TNFα (data not shown) but none of these stimulators reached the same values as the LPS/IFNγ combination. We evaluated the probiotics' anti-inflammatory effects on HT-29 cells through the activation of NF-κB and secreted IL-8. NF-κB was chosen as a major effector of the inflammatory response, in particular in the IBDs, and for its implication in pro-inflammatory cytokine production (Rogler et al., 1998). We also used IL-8 as an inflammatory biomarker, since this cytokine has been demonstrated to be up regulated in epithelial cells from mucosa biopsies sampled specifically during crisis compared to quiescent state, in patients with the two main IBDs, Crohn's disease and ulcerative colitis (Banks et al., 2003).

The anti-inflammatory properties of probiotics have been demonstrated *in vitro* and also in animal models or even in clinical trials. Some bifidobacteria were characterized for their ability to inhibit NF-κB inducible reporter gene expression in transgenic Caco-2 cells and in IL-8 secretion by normal HT-29 treated with both LPS and soluble CD14, which improved inflammatory response (Riedel et al., 2006). A strain of *L. lactis subsp. cremoris* has been previously demonstrated to be efficient in decreasing IL-8 mRNA in Caco-2 cells and NF-κB nuclear translocation in RAW264.7 cells after LPS treatment. The *in vitro* anti-inflammatory properties of these strains were correlated to the decrease of pro-inflammatory cytokines and improvement of gut histological score using a dextran sulphate sodium mouse model (Nishitani et al., 2009).

Prebiotic GOS had no anti-inflammatory effects *in vitro,* demonstrating their absence of intrinsic anti-inflammatory properties. When combined with bacteria, the observed effects were no different from those noted with bacteria alone. Probiotic strain numeration was checked at 6 h and 16 h (data not shown). At 6 h, no significant growth was observed whatever the carbon source used. At 16 h there was no significant difference between medium with glucose alone or complemented with GOS and increase was under one log. Thus, the observed effects could not be explained by some variation of the MOI. Aerobic conditions required for cell culture and phosphate buffered medium are far from optimal culture conditions for lactic acid bacteria and could explain the lack of MOI variation in the assay conditions and the lack of effect in anti-inflammatory experiments performed with GOS. We designed experimental conditions to obtain optimised cell response to the pro-inflammatory stimuli while preventing cytotoxic effects due to the presence of bacteria in the culture medium. The incubation time of 6 h or 16 h and the MOI of 100 were chosen to take these two constraints into account. Although *B. breve* R0070 is able to use OA as a carbon source when cultured in microbiological culture medium (to be published), the lack of growth of probiotic strains in coculture with HT-29 cells suggests that the effects observed with OA should only be due to the intrinsic properties of this carbohydrate. Potential prebiotic characteristics of this oligosaccharide need to be demonstrated through assays on *in vivo* models, where OA may specifically support development of effective probiotics and thus could improve the results from *in vitro* screening.

Selected prebiotics associated with probiotic strains were screened for anticancer characteristics through their anti-proliferative effects on cancerous epithelial HT-29 cell lines, as anti-proliferative activity is a biomarker that is widely used to evaluate cancer chemoprevention (Einspahr et al., 1997). We selected strains that were able to inhibit at least 20% of the proliferation of HT-29 cells, the results obtained being inconsistent under this limit. Only *B. breve* R0070, *L. rhmanosus* R0011 and *L. lactis* R1058 reached this limit, whatever the carbon source used. In order to improve these results, we combined these strains as Rafter et al. (2007) had demonstrated a synergic alteration of cancer biomarkers in polypectomized and CRC patients after treatments by a three-component synbiotics composed of two probiotics associated with one prebiotic as in the present study. Rafter et al. used the association of *Bifidobacterium lactis* BB12 and *L. rhamnosus* GG with oligofructose-enriched inulin prebiotic to attenuate the proliferative activities of colorectal biopsy samples from polypectomized and CRC patients. Here, neither GOS alone nor most of the probiotics had significant effects on HT-29 cell proliferation. We needed to use binary or ternary associations of probiotic strains to obtain significant inhibition of proliferation. Combining these associations with prebiotic GOS led to an improvement of the observed effect, especially for the association of *B. breve* R0070, *L. lactis* R1058 and OA, where a very reproducible inhibition was obtained, which reached 70%. The mechanisms by which synbiotics exert their anti-proliferative effects remain largely unknown. However, dose range assays performed here clearly showed that both bacteria and OA were required for the effect to be observed in a dose dependent manner. Bacterial numeration showed that probiotics did not grow during the coincubation time with HT-29 cells. This lack of growth means that the anti-proliferative effects observed could not be due to OA metabolisation by bacteria but to the potential additional effects of the mixture of the probiotic strains and the intrinsic effect of prebiotics. Other prebiotics have been thought to exert their effects through their fermentation in SCFA, which are inhibitors of colonic cancer cells (Lan et al., 2007; Pool-Zobel, 2005). It would be interesting to further characterise some potent SCFA produced by OA fermentation since *B. breve* R0070 can use this GOS as a carbon source in a microbiological medium under anaerobic conditions.

The inhibition of proliferation of HT-29 cells by the combination of *B. breve* R0070/*L. lactis* R1058/OA in absence of cytotoxic effect (checked by trypan blue) could be explained by the induction of an undifferentiated phenotype to a more differentiated one as has been reported before for another synbiotic combination (Pool-Zobel, 2005).

Among the mechanisms proposed as being involved in probiotics, prebiotics and synbiotics anticarcinogenic properties is the induction of differentiation of cancer cells (Liong, 2008). We therefore investigated the expression of hIAP, considered as a biomarker of cell differentiation in human colonic epithelial cells (Marvin-Guy et al., 2008), in the subconfluent cancerous HT-29 cells treated by *B. breve* R0070, *L. lactis* R1058 and OA and in 14 days post-confluence differentiated HT-29 cells. The results showed that hIAP in cells treated by the synbiotic reached the same values as differentiated HT-29. Because of the short time of exposition (16 h), our results suggest that synbiotic treatment induced a dramatic differentiation pathway in HT-29 cells, similar to or higher than that of 14 days post-confluence differentiated HT-29 cells. The start of differentiation could explain the decrease of proliferation.

Considering the inhibition exerted on proliferation rates by *B. breve* R0070, *L. lactis* R1058 and OA and the induction of a differentiation pathway in one model of cancer cells in culture, this association should be further confirmed in a colorectal cancer animal model efficient to study synbiotic protective effects, such as azoxymethane-induced colon cancer (Le Leu et al., 2010) to confirm *in vitro* results before considering their interest in human gut colorectal cancer prevention.

We have demonstrated here the potential anti-inflammatory properties of different strains of lactic acid bacteria in two different *in vitro* models. The strains active in these models should now be subjected to *in vivo* models in order to confirm these *in vitro* results. In addition, *in vivo* assays with oligodextran and oligoalternan, which can support the growth of the tested strains (Grimoud et al., 2010), could help us to assess the potential anti-inflammatory properties of these two prebiotics. This study also led to the demonstration *in vitro* of the considerable interest of combining probiotics and prebiotics to

reach specific anti-proliferative and physiological properties on colonic cancer cells. These results have to be confirmed by *in vivo* assays.

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