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Interaction between probiotics and human immune cells

The prospective anti-inflammatory activity of *Bifidobacterium breve* BR03

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ABSTRACT: The human intestinal microflora may be considered as a post-natally acquired organ composed of a large diversity of bacteria with different functions on human health. Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. Probiotics are widely used to rebalance microbiota composition, thus improving gut functionality and immune system responses. However, only few data on the specific immunologic pathways are currently available. This study showed that *Bifidobacterium breve* BR03 (DSM 16604) is able to induce a relevant increase of helper T lymphocytes and the secretion of Th2 cytokines (IL-10 and IL-4). This strain demonstrates a strong anti-inflammatory property through the shift of Th1/Th2 balance towards a Th2 response and could therefore be beneficial in irritable bowel diseases.

Keywords: Probiotics, lymphocytes, cytokines, immunomodulation, Th1/Th2 balance.

INTRODUCTION

Probiotics have been defined as "a live microbial food supplement which beneficially affects the host by improving the intestinal microbial balance" and, more broadly, as "living micro-organisms, which when administered in adequate amounts, confers a health benefit on the host" (1). The criteria for a microorganism to be defined as probiotic include that the strain is of human origin, safe for human use, sufficiently stable during gastro-duodenal transit, and preferably able to adhere to the intestinal mucosa. Although there is a lot of information about the healthy properties of probiotics (2, 3), it is not always based on proven evidence and little is known about the precise mechanisms of action by which such bacteria may exert their beneficial effects (4). There is good evidence that certain strains of lactobacilli and bifidobacteria can influence immune system, particularly through regulation of the production of pro-inflammatory and anti-inflammatory cytokines (5) and the T helper 1 (Th1)/Th2 balance. The pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, and IL-12, are hallmarks of inflammatory responses in the intestine (6). The inhibition of pro-inflammatory cytokines and the supplementation of anti-inflammatory ones were able to reduce inflammation in animal studies and clinical trials. Certain probiotics were demonstrated to induce IL-12 production by macrophages and DCs, thus promoting IFN-γ secretion and inflammatory Th1 responses (7-9). On the other side, some reports have shown that probiotic-induced IL-10 levels play a main role in the restraint of Th1-mediated pro-inflammatory responses (10, 11).

Probiotic bacteria are shown to promote the endogenous host defence mechanisms. In addition to the effects of probiotics on non-immunologic gut defences, which are characterized by stabilization of the gut microflora, probiotic bacteria have been shown to enhance immune responses and thereby promote the intestine's immunologic barrier (12-14).

It is well known that infective bacterial, viral, and protozoan invasions are often accompanied by serious gastrointestinal inflammatory responses (15). Local inflammation has also been implicated in gastrointestinal diseases such as Crohn's disease (CD), ulcerative colitis (UC), food allergies and atopic dermatitis, though the etiology and pathogenesis of these chronic diseases have not been well established (6, 16-17). Intestinal inflammation is one potential target for probiotic therapy; clinical improvement and protective effects of probiotics have been demonstrated in food allergies and atopic dermatitis (18-20). Bifidobacteria are Gram-positive, anaerobic microorganisms that inhabit mainly the colon of healthy infants and adults. In breast-fed infants, soon after birth, up to 90 percent of all bacteria in faecal samples detected by fluorescence *in situ* hybridization are bifidobacteria and they still make up 3-5 percent of the adult microflora (21, 22). Thus, bifidobacteria undoubtedly constitute one of the predominant species of the human colonic microflora. Several beneficial health effects have been related to the presence of bifidobacteria in the colon (23-28). Based on these properties, bifidobacteria have become increasingly interesting for probiotic use, both in pharmaceutical application and dairy products.

In the light of the well-known immunomodulatory activity of Bifidobacteria, in this study we investigated the specific immune activation properties of the probiotic strain *Bifidobacterium breve* BR03 (DSM 16604) with the aim to determine the most useful criteria for its clinical use

One of these applications is their use in probiotic intervention in chronic intestinal inflammation. Probiotic products containing bifidobacteria have been shown to be effective in reducing the severity of inflammation in several rodent models and patients with an Irritable Bowel Disease (IBD) (25, 29-32).

Like other probiotic bacteria, it has been described that Bifidobacterium sp. may present distinct immunomodulatory effects both *in vitro* (4, 10, 33-38) and *in vivo* (3, 6-7, 39). However, there is a very limited number of studies on the immunological traits of different Bifidobacteria strains that could support a rationale selection of probiotic strains for

specific immunomodulatory benefits. In the light of the well-known immunomodulatory activity of Bifidobacteria, in this study we investigated the specific immune activation properties of the probiotic strain *Bifidobacterium breve* BR03 (DSM 16604) with the aim to determine the most useful criteria for its clinical use. For this reason, we analyzed proliferation and cytokines secretion of human peripheral blood mononuclear cells (PBMCs) after co-culture with BR03 strain, which is useful to assess the Th profile that could be promoted in healthy subjects by the probiotic strain.

Results further highlight the potential usefulness of the probiotic strain BR03 in the treatment and prevention of gastrointestinal infections as well as the reduction of bacterial overgrowth in IBS

EXPERIMENTAL SECTION

Bacteria and culture conditions

Bifidobacterium breve BR03 (DSM 16604) was stored in 20 percent glycerol at -20°C. Prior to use in experiments, probiotic strain was grown overnight at 37°C in de Man-Rogosa-Sharpe (MRS, Difco) medium supplemented with 0.05 percent L-cysteine (Sigma Chemical Co) and incubated at 37°C in anaerobic condition. Overnight bacterial culture was subcultured for further 6 hours until mid-log phase. For the enumeration of bacteria, BD Cell Viability Kit with BD Liquid Counting Beads (BD Biosciences) was used as instructed by the manufacturer to accurately quantify the number of live, dead, and total bacteria in a sample.

Bifidobacterium breve BR03 is able to promote the development of a desirable anti-inflammatory cytokines pattern by the PBMCs of healthy subjects

Isolation of PBMCs

PBMCs, from 10 healthy individuals, were obtained by Ficoll-Hypaque (Lymphoprep) density gradient centrifugation from freshly collected, leukocyte-rich buffy coats provided by the local Blood Transfusion Service (Borgomanero, Italy). Cells were washed and suspended in culture medium consisting of RPMI 1640 (Invitrogen) supplemented with 2mM L-glutamine, 5mM Hepes and 10 percent heat-inactivated fetal calf serum.

Activation of mononuclear cells

The effects of probiotic bacteria directly on the different cell populations as well as possible interfering effects of the same on cytokine production were evaluated. To quantify PBMC responses to BR03, $1x10^5$ mononuclear cells were incubated with bacteria at a relative ratio of 1:1. All cultures were performed in triplicate using 200 µl of complete medium in 96 well round-bottom microtiter plates in the absence of any stimulus (negative control) and in the presence either of the polyclonal T cell stimulator phytohaemagglutinin (PHA, 10µg/ml, Sigma Chemicals) (positive control) or the strain *Bifidobacterium breve* BR03 ($1x10^5$/100µl/well). Cultures were performed in triplicate and incubated at 37°C and 5 percent CO_2. After 5 days, the cells were collected and used for subsequent analysis; culture supernatants were collected, filtered and stored at -20°C until use.

For proliferation study, 5-bromo-2'-deoxyuridine (BrdU, 20mM; Sigma Chemicals) was added to each well during the last 16h of culture, and lymphocytic proliferation was assessed by flow cytometry as BrdU incorporation by $CD3^+$ lymphocytes, as described elsewhere (40, 41). Briefly, cells were stained with anti-CD3 monoclonal antibodies (mAb, ImmunoTools, Germany). Following cell fixation, permeabilization and partial DNA denaturation, cells were directly stained with anti-BrdU mAb (Becton Dickinson). Cells were collected and analyzed using a FACScalibur (Becton Dickinson) within 24h. Results are expressed as proliferation index, calculated as the ratio between the mean percentage values of stimulated and unstimulated $CD3^+$ cells. A significant PI was defined as >2. In vitro stimulation of control antigen (PHA) demonstrated a PI >2 in all subjects, confirming that PBMC were viable and able to proliferate.

Cell surface phenotype expression

After 5 days of culture, cells were directly stained with anti-CD4 fluorescein isothiocyanate (FITC), anti-CD8 phycoerythrin (PE), anti-CD3 phycoerythrin-cyanin 5 (PE-Cy5), anti-CD19 FITC and anti-CD20 PE mAb. Corresponding isotype-matched conjugated irrelevant mAb were used as a negative control. All mAb were supplied by ImmunoTools. Staining was performed for 30 min at 4°C, and cells were washed twice in staining buffer and resuspended in PBS. A minimum of 10,000 cells were acquired and analyzed using the CellQuest software (Becton Dickinson).

Cytokine assays

Levels of interferon (IFN)-g, IL-4, IL-10 and IL12p70, from cell culture supernatants, were measured using specific sandwich enzyme-linked immunosorbent assay (E.L.I.S.A.) commercial kit, used according to the manufacturer's instructions (Bender MedSystems). Absorbance was measured at 450nm using a photometric microplate absorbance reader (Thermo Scientific Multiskan EX). For each cytokine a standard curve was constructed in duplicate and used to quantify the cytokines amount.

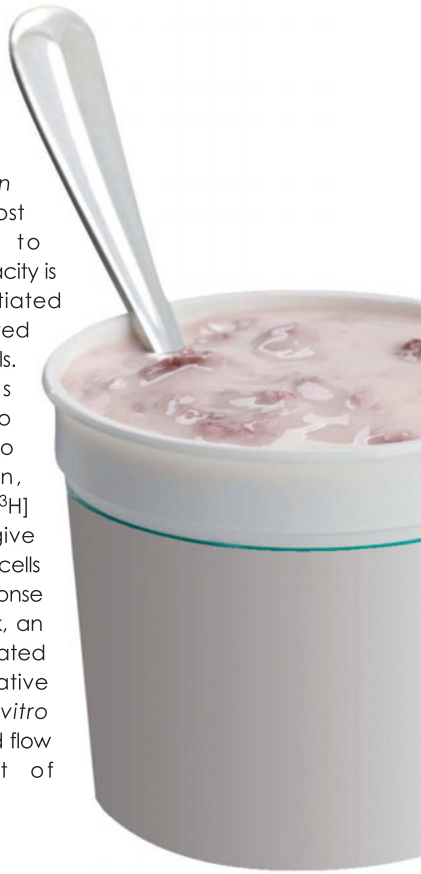
Statistical analysis

Results are expressed as the means ± standard error of the mean (S.E.M.) of 10 independent experiments. Significant differences between unstimulated and stimulated samples were tested using the paired-Student's *t*-test. Values of $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Effects of probiotic bacteria on proliferative response

Proliferation of PBMCs is an important *in vitro* parameter of *in vivo* immune function. The most commonly used method to determine cell proliferative capacity is based on the measure of tritiated thymidine ($[^3H]TdR$) incorporated into the DNA of proliferating cells. Since this method requires radioactive facilities able to manage the waste, it's not so easy to use. In addition, proliferation as measured by $[^3H]$ TdR incorporation does not give information on which subsets of cells are actually proliferating in response to the *in vitro* stimuli. In this work, an alternative method was evaluated for assessment of cell proliferative capacity in response to *in vitro* stimulation. This method involved flow cytometric measurement of



5-bromo-2'-deoxyuridine (BrdU) incorporation into newly synthesized DNA of proliferating cells. BrdU is a pyrimidine analogue which is incorporated in place of thymidine into DNA of proliferating cells.

Most *Bifidobacterium* sp. are commensal microorganisms usually present in the gut of adult individuals and responsible for different interactions with immune cells. Therefore, to elucidate the possible *in vitro* consequences of these interactions, PBMCs were used as a responder population to determine cell proliferation and cytokines production after stimulation with *Bifidobacterium breve* BR03. Based on preliminary experiments, we examined the proliferative response in cultures using a PBMCs:bacteria ratio of 1:1. As showed in Figure 1A, after 5 days of stimulation the proliferative response of $CD3^+$ T cells incubated both with either PHA or with *B. breve* BR03 was significantly higher than those from the control condition ($p<0.0001$ and $p<0.001$, respectively).

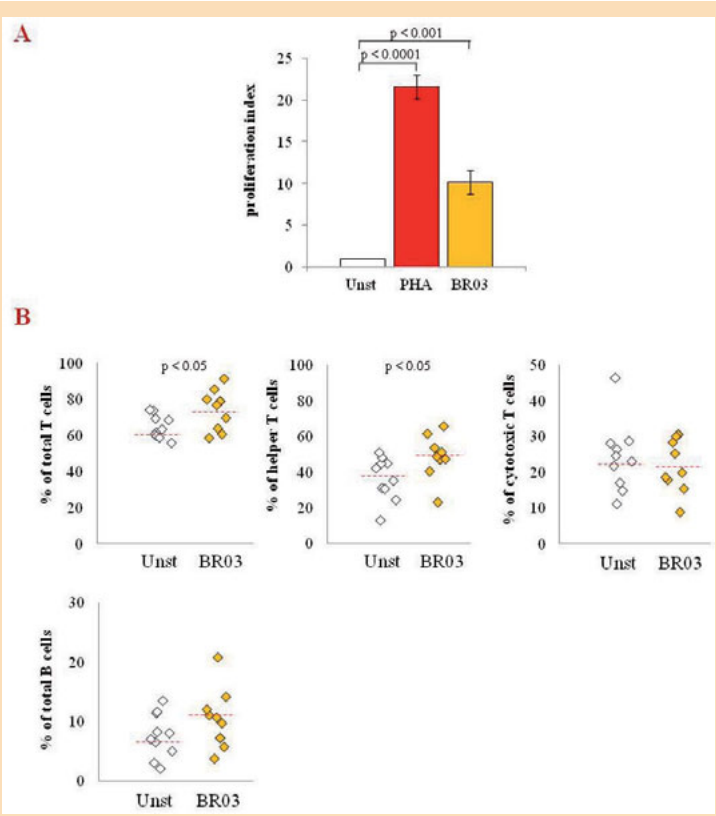


Figure 1. Induction of cell proliferation by the probiotic strain *Bifidobacterium breve* BR03. A) Proliferation of CD3+ total T lymphocytes; B) Proliferation of subpopulations of lymphocytes.

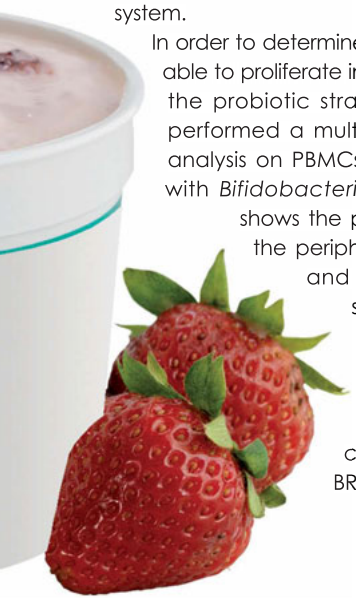
Effects of live bacteria on lymphocyte subsets

PBMCs are the mononuclear cellular component of blood, consisting of T lymphocytes (about 75 percent), B lymphocytes, monocytes and NK cells.

T lymphocytes play a central role in cell-mediated immunity. They can be distinguished from other lymphocyte types, such as B cells and natural killer cells, by the presence of a special receptor on their cell surface called T cell receptor (TCR). Several different subsets of T cells have been discovered, each with a distinct function. T cells which express the CD4 protein on their surface are called T Helper cells (Th) because they assist other leukocytes in immunological processes. Once activated in response to antigen recognition, Th cells rapidly divide and secrete small proteins called cytokines which regulate or assist the immune response. Depending on the size and the cytokine signals received, these cells differentiate into Th1, Th2, Th3, Th17 or one of other subsets, which secrete different cytokines. Cytotoxic T cells (T_C cells, or CTLs) destroy virally infected cells and tumour cells, and are also implicated in transplant rejection. These cells are also known as $CD8^+$ T cells.

B cells are lymphocytes that play a large role in the humoral immune response. The main functions of B cells are the secretion of antibodies against antigens, the role as Antigen Presenting Cells (APCs) and the possible development into memory B cells after activation by antigen interaction. B cells are an essential component of the adaptive immune system.

In order to determine which cellular subsets were able to proliferate in response to stimulation with the probiotic strain under investigation, we performed a multiparameter flow cytometry analysis on PBMCs after 5 days of co-culture with *Bifidobacterium breve* BR03. Figure 1B shows the percentages of lymphocyte subsets in the peripheral blood of control subjects before and after stimulation with the probiotic strain. Stimulation for 5 days with BR03 had no noticeable effect on the populations of $CD8^+$ cells and $CD19^+$/$CD20^+$ B cells. However, a significant increase in the percentage of $CD4^+$ cells was observed in the presence of BR03 ($p<0.05$).



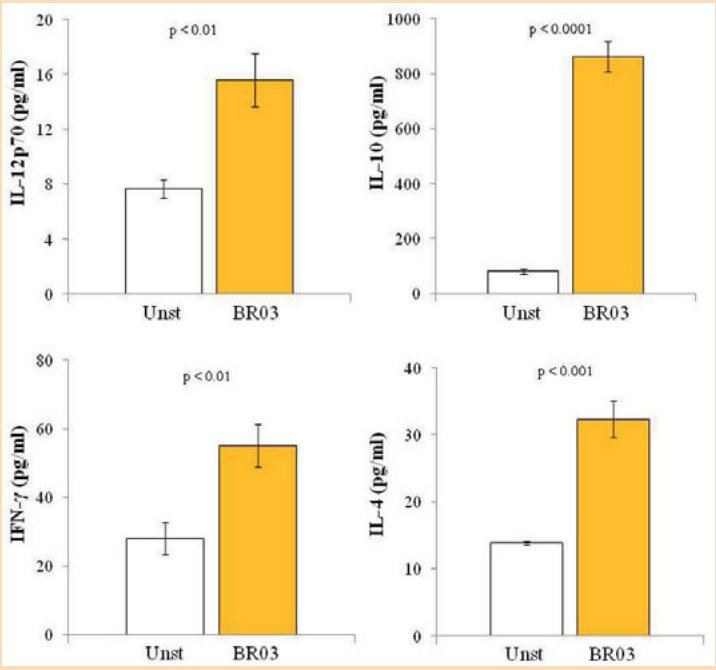


Figure 2. Production of cytokines by peripheral blood mononuclear cells (PBMCs) in response to live probiotic strain *Bifidobacterium breve* BR03. Results are expressed as mean ± S.E.M. of ten independent experiments. Significant differences among samples were calculated with paired T-test.

Effects of live bacteria on cytokines production

T cells are the main effectors and regulators of cell-mediated immunity. In response to an antigen or a pathogen, T cells synthesize and secrete a variety of cytokines that serve as growth, differentiation and activation factors for other immunocompetent cells. The differentiation of naïve helper T cells into effector ones is initiated by the activation of their TCRs (signal 1) and costimulatory molecules (signal 2) in the presence of specific cytokines produced by the innate immune system. IFN-γ and IL-12 initiate the differentiation of

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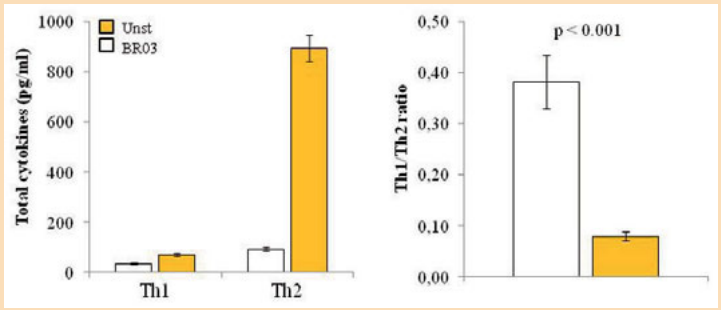

Figure 3. Ratio between Th1 and Th2 cytokines.

T helper lymphocytes in Th1 cells, that are essential for cell-mediated immunity. IL-4, IL-5 and IL-10 trigger the differentiation of Th2 cells, typically associated with humoral immunity and host defense against extracellular pathogens.

To determine the pattern of cytokines expression induced by *Bifidobacterium breve* BR03, the amount IL-12p70, IL-10, IFN-γ and IL-4 has been determined in the supernatants of bacteria-stimulated PBMCs recovered after 5 days of culture. As shown in Figure 2, live cells of *B. breve* BR03 were potent inducers of all tested cytokines. In particular, BR03 promoted the release of significantly higher amounts of the regulatory and Th2 cytokine IL-10 ($p<0.001$), compared with unstimulated cells, while showing the lowest ability to induce the secretion of other cytokines, namely IL-4, IL12p70 and IFN-γ. In this way, the stimulation of production of Th2 cytokines seemed to be inversely related to the production of Th1 cytokines.

Given that several bacteria are strong inducers of both pro-inflammatory and regulatory anti-inflammatory cytokines, in an attempt to predict subsequent Th cell responses, we calculated the ratios between cytokines that are relevant for T cells differentiation. In detail, we used the (IFN-γ + IL-12p70)/(IL-4 + IL-10) ratio as the most reliable parameter to predict the balance between Th1 and Th2 immune responses. As shown in Figure 3, *B. breve* BR03 significantly shifts Th1/Th2 balance towards a Th2 response mediated by an anti-inflammatory cytokines pattern.

Numerous studies support the view that IL-10 exerts a strong suppressive effect on Th1 lymphocytes, antigen presenting cells and the production of inflammatory mediators (42). Thus, IL-10 can counteract the production of other cytokines such as IFN-γ or IL-4. A previous human clinical study by Saggioro (43) demonstrated a great efficacy of *Bifidobacterium breve* BR03 in the relieve of the main symptoms typically associated with Irritable Bowel Syndrome (IBS), thus suggesting its anti-inflammatory activity. The present results further highlight the potential usefulness of the probiotic strain BR03 in the treatment and prevention of gastrointestinal infections as well as the reduction of bacterial overgrowth in IBS by promoting Th2- and counteracting Th1-immune responses (42, 43).

CONCLUSION

Many evidences suggest that probiotic bacteria may have, in a species- or even strain-dependent manner, a potential use as anti-inflammatory agents in some chronic inflammatory diseases (8). The most promising clinical results have been obtained in the prevention and management of atopic eczema, the treatment of inflammatory bowel disease (IBD) and post-operative pouchitis (44). On the basis of experimental data, the anti-inflammatory effects of probiotics may be a consequence of their antagonism of potentially pathogenic/pro-inflammatory endogenous microbiota, the modulation of balance between Th1, Th2 and regulatory T (T_{reg}) cells, the down-regulation of pro-inflammatory (e.g. IL-12, TNF-α) and/or stimulation of anti-inflammatory (e.g. IL-10) cytokines production. Other effects such as enhanced elimination, modified degradation, permeation and presentation of pro-inflammatory antigens can play an important role in this sense, especially in the human gut (44). In the present work, we demonstrate that *Bifidobacterium breve* BR03 is able to promote the development of a desirable anti-inflammatory cytokines pattern by the peripheral blood cells of healthy subjects. This effect was associated with an increase in the amount of T helper cells and in the secretion of the Th2-cytokines, especially IL-10 and IL-4. A previous study by Saggioro (43) has demonstrated the efficacy of *B. breve* BR03, used in association with *Lactobacillus plantarum* LP01 strain (LMG P-21021), in the treatment of Irritable Bowel Syndrome in humans. In particular, the most striking results were the reduction of abdominal pain in different locations, bloating and flatulence. Further clinical studies are now warranted to confirm the immunomodulation activity in humans and assess whether the peripheral effects of BR03 strain are reflective of local beneficial anti-inflammatory action in the intestine, as already suggested in the study by Saggioro.

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