Evaluation of the probiotic properties of new *Lactobacillus* and *Bifidobacterium* strains and their *in vitro* effect

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Abstract

Probiotic ingestion is recommended as a preventive approach to maintain the balance of the intestinal microbiota and to enhance the human well-being. During the whole life of each individual, the gut microbiota composition could be altered by life style, diet, antibiotic therapies and other stress conditions, that may lead to acute and chronic disorders. Hence, probiotics can be administered for the prevention or treatment of some disorders, including lactose malabsorption, acute diarrhoea, irritable bowel syndrome, necrotising enterocolitis, mild forms of inflammatory bowel disease. The probiotic-mediated effect is an important issue that needs to be addressed in relation to strain-specific probiotic properties. In this work, the probiotic properties of new *Lactobacillus* and *Bifidobacterium* strains were screened and their effects *in vitro* were evaluated. They were screened for probiotic properties by determining their tolerance to low pH and to bile salts, antibiotic sensitivity, antimicrobial activity, vitamin B8, B9 and B12 production; and by considering their ability to increase the antioxidant potential and to modulate the inflammatory status of systemic-miming cell lines *in vitro*. Three out of the examined strains presenting the most performant probiotic properties, as *L. plantarum* PBS067, *L. rhamnosus* PBS070 *and B. animalis subsp. lactis* PBSO75, were evaluated for their effects also on human intestinal HT-29 cell line. The obtained results support the possibility to move to another level of study, that is, the oral administration of these probiotical strains to patients with acute and chronic gut disorders, by *in vivo* experiments.

Keywords: Probiotics, *Lactobacillus, Bifidobacterium,* intestinal microbiota, gut disorders

Introduction

Probiotics are recognized as live microorganisms that confer a health benefit to the host when administered in adequate amounts (FAO/WHO, 2001). These bacteria exert health-promoting properties including the maintenance of the gut functions and the local and systemic modulation of the host immune system. Many studies have demonstrated the clinical potential of probiotics against many diseases, such as allergic pathologies, including atopic eczema and rhinitis, diarrhea, necrotising enterocolitis inflammatory bowel disease and viral infection (Robles Alonso and Guarner 2013). For these reasons, there is an increasing interest in the development of adjunct or alternative therapies based on bacterial replacement, by using probiotics isolated and characterized for their specific properties (Kailasapathy and Chin 2000). Probiotics could be used as supplements to improve the functional properties of nutraceutical food products and therapeutical preparations.

Actually, the most known probiotic microorganisms are bacteria belonging to the *Lactobacillus* and *Bifidobacterium* genera (Prasad et al. 2000). However, species belonging to the genera *Lactococcus* (Kimoto et al. 1999), *Enterococcus* (Dunne et al. 1999), *Saccharomyces* (Sanders and Huis in't Veld 1999) and *Propionibacterium* (Mantere-Alhonen 1995) are also considered as probiotic microorganisms. The ability of lactobacilli and bifidobacteria to exert beneficial effects on the human health is a species and strain-specific feature (Ramos et al. 2013). This explains the need for a continuous search for novel strains with probiotic potential, that could be useful to investigate their effect on different target districts or disorders.

Several important mechanisms underlying the beneficial effects of probiotics include the effects of probiotic properties on specific tissues, particularly on the intestine. Most of these mechanisms involve the modification of the gut microbiota, the competitive adherence to the mucosa and epithelium against pathogens, the strengthening of the gut epithelial barrier and the regulation of the immune system and inflammation. For this reason the probiotic-mediated effect is an important issue that needs to be addressed in relation to strain-specific probiotic properties (Fontana et al. 2013; Collado et al. 2009).

The most efficient strains should be robust enough to survive the harsh physico-chemical conditions of the gastrointestinal tract. This includes gastric acid (Corcoran et al. 2005), bile secretion (Begley et al. 2005) and competition with the resident bacteria of the gut microbiota (Fooks and Gibson 2002). As safety requirement, the origin of the strains, their non-pathogenicity and the absence of transferable antibiotic-resistance genes should also be assessed (Saarela et al. 2000). Depite of the increasing awareness of the benefits derived from a regular intake of probiotic bacteria, the knowledge about these mechanisms is still limited. Proven modes of action include the production of substances such as bacteriocins or organic acids with antagonistic activity against potential pathogens (Servin 2004), immunomodulating attitude on the intestinal tissues (Valeur et al. 2004), or the release of essential micronutrients in the gut, such as group B vitamins (LeBlanc et al. 2011). For the gastrointestinal colonization, probiotics need to be ingested as large populations and on a daily basis (Berman et al. 2006).

By contrast, information related to the effects on human intestinal cells mediated by the action of probiotics is scarce. There is a need for further clinical studies that evaluate the mechanism of action of probiotics both in healthy and in patients with chronic diseases. During the whole life of each individual, the gut microbiota composition could be altered by life style, diet, antibiotic therapies and other stress conditions, and this may lead to acute and chronic disorders (Bervoets et al. 2013; Pérez-Cobas et al. 2013).

In this prospective, another level of study is necessary before the *in vivo* experiments, by the evaluation of probiotial properties effects on human intestinal cell lines *in vitro*. In fact, *in vitro* evidences are particularly important considering that the UE directives tend to discourage experiments on animals.

In this work, the probiotic properties of new *Lactobacillus* and *Bifidobacterium* strains were investigated and their biological effects on cell lines by *in vitro* tests were evaluated. The probiotic properties were determined by their tolerance to low pH and to bile salts, antibiotic sensitivity, vitamins B8, B9 and B12 production, antimicrobial activity, as well as their inflammation response

on systemic-miming cell lines *in vitro*. Moreover, the probiotic properties of the most *Lactobacillus* and *Bifidobacterium* performant strains were evaluated on human intestinal cell lines, in order to acquire data to successively move to another level of study involving patients with acute and chronic gut disorders by *in vivo* experiments.

Materials and Methods

Strains and culture conditions

This study comprised seven strains of *Lactobacillus* spp. and *Bifidobacterium* spp. supplied from a private collection. *L. paracasei* ATCC 334 and *Bifidobacterium* sp. (supplied from University of Bologna), known for its antimicrobial properties (Aloisio et al. 2012), were also included in this work as controls.

Unless otherwise specified, *Lactobacillus* spp. strains were cultured in deMan, Rogosa and Sharpe (MRS) medium. For *Bifidobacterium* spp. strains the MRS medium was supplemented with 0.3 g/liter L-cysteine hydrochloride monohydrate (cMRS) (Sigma-Aldrich). The cultures were incubated at 37 °C under microaerophilic or anaerobic conditions using anaerobic atmosphere generation bags (Anaerogen, Oxoid). As antagonistic microorganisms for antimicrobial activity assays *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231 were employed. With the exception of *C. albicans*, maintained in Sabouraud (Oxoid) agar (1.5 % w/v Agar Technical, Oxoid) medium, the antagonists were cultured in Tryptic Soy Agar (TSA; Oxoid) at 37 °C in aerobiosis.

Molecular identification of lactobacilli and bifidobacteria

Genomic DNA from microbial strains was extracted from 2 mL of overnight cultures by using Ultra Clean Microbial DNA Isolation Kit (Mo Bio, Carlsbad, USA). 16S rRNA gene amplification was performed with the universal primers 27F and 1492R (Lane 1991) by a standard PCR reaction. The PCR products were then purified with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) and

sequenced. The 16S rRNA sequences of the isolated strains have been deposited in the GeneBank DataBase (Accession Numbers KM577182, KM577183, KM577184, KM577185, KM577186, KM577187, KM577188).

Resistance at acidic pHs and simulated intestinal fluids

Lactobacilli and bifidobacteria strains were cultured in MRS (or in cMRS) agar at 37 °C for 24 h in anaerobiosis. Cells were collected in 0.9 % (w/v) saline solution, until the optical density at 600 nm ($O.D._{600nm}$) was 2. Cell suspensions were diluted in 50 mM of the following buffers: glycine-HCl pH 2.0, glycine-HCl pH 3.0, sodium acetate pH 4.0, sodium acetate pH 5.0 and sodium phosphate pH 7.0, as control. The residual viability was determined at 0, 30, 60, 120 and 180 min of incubation at 37 °C. Samples were serially diluted and appropriate dilutions were cultured on MRS (or cMRS) agar.

Simulated intestinal fluid (SIF) was prepared as described by Priya et al. (2011). The SIF consisted of 1 g/l of Pancreatin (Sigma-Aldrich), 3 g/l of bovine bile salts (Sigma-Aldrich), 0.835 g/l of KCl (Sigma-Aldrich), 6.5 g/l of NaCl (Sigma-Aldrich), 0.22 g/l of $CaCl_2$ (Sigma-Aldrich), and 1.386 g/l of $NaHCO_3$ (Sigma-Aldrich), with a final pH of 7.5. After that, the solution was filtered through a 0.22 µm membrane. Then, 100 µl of cell suspensions at $O.D._{600nm}$ 2 were diluted in 1.9 ml of SIF and incubated for 180 min at 37 °C. As described above, at regular time intervals (0, 30, 60, 120 and 180 min), the residual viability was determined by the count plate method. Experiments were performed three times with a standard error around ± 10%.

Antibiotic resistance

The antibiotic resistance profiles of lactobacilli and bifidobacteria strains were determined by broth microdilution method in LSM broth (90 % Iso Sensitest Broth Oxoid, 10 % MRS broth) or cLSM (for bifidobacteria), according to ISO 10932:2010. The minimal inhibitory concentrations (MICs) of the following antibiotics were determined: ampicillin (range, 0.032 to 16 µg/mL), vancomycin (range, 0.25 to 128 µg/mL), gentamicin (range, 0.5 to 256 µg/mL), kanamycin (range, 2 to 1024 µg/mL), streptomycin (range, 0.5 to 256 µg/mL), erythromycin (range, 0.016 to 8 µg/mL),

tetracycline (range, 0.125 to 64 μg/mL), chloramphenicol (range, 0.125 to 64 μg/mL), clindamycin (range, 0.032 to 16 μg/mL) (Sigma-Aldrich). As control, the MICs for *L. paracasei* ATCC 334 were also determined and compared with the MIC values resulting from the interlaboratory trials (ISO 10932:2010).

The detection of *tet(W)* gene in the tetracycline-resistant strains was performed. The tetracycline resistance gene *tet(W)* in *B. animalis* subsp. *lactis* PBS075 strain was amplified by PCR with the specific primers TetF and TetR. The presence of the transposase gene *trp* upstream the *tet(W)* gene was investigated by using the primers TrpF and TetR (Gueimonde et al. 2010). The 50 μL reaction mixture consisted of 25 μL of GoTaq®Green (Promega), 1 μM of each primer and 0.5 ng/μL of template. The PCR product obtained by the amplification of *trp*-*tet*(W) region was purified with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) and sequenced.

Assessment of the antimicrobial activity

Well diffusion agar assay using pH neutralized and non-neutralized cell free supernatants

A modification of the protocol of Santini et al. (2010) for lactobacilli and bifidobacteria well diffusion agar assay was used. Over-night MRS (or cMRS) cultures were centrifuged at 12000 × *g* at 4 °C for 10 min. The pHs of the supernatants were recorded and measured. An aliquot of each supernatant was neutralized to pH 7.0 with NaOH 3.0 M. The not-neutralized (NN) and the neutralized (N) supernatant fractions were filtered with 0.22 μm pore filter membranes to remove any residual bacterial cell. A single colony of the selected antagonists *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231, was inoculated into the suitable medium and allowed to grow until O.D. $_{600nm}$ was 0.1, corresponding approximately at 10^6 CFU/mL.

2.5 % (v/v) of each culture was inoculated into 20 mL of melted Tryptic Soy Agar (or Sabouraud agar in the case of *C. albicans*) and the plates were allowed to solidify. Five wells of 8 mm in diameter were made on each agar plate with a sterile cylinder. 100 μL of both NN and N cell culture supernatants, were dispensed into each well. 100 μL of not-inoculated MRS was used as

control. Plates were incubated overnight at 37 °C in aerobiosis. The growth inhibition zones around the wells were measured.

Inhibitory activity of not-neutralized cell-free supernatant on the growth of antagonistic microorganisms

The antimicrobial activities previously detected, were quantified by measuring the growth inhibition of *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231 in liquid cultures in the presence of the NN cell culture supernatants. Single colonies from freshly streaked plates of the antagonists were resuspended in saline solution at a concentration of 10^6 CFU/mL. The assay was performed in 96-well plates. Each well contained: double concentrated TSB, 10 % (v/v) cell suspension of antagonistic strains, 25 % (v/v) of NN cell culture supernatants and distilled water up to 150 µL as final volume.

Positive controls were prepared by substituting to NN cell culture supernatants an equal volume of not-inoculated MRS medium. *Bifidobacterium* sp. strain NN supernatant was used as reference (Aloisio et al. 2012). Plates were incubated at 37 °C in aerobiosis for 24 hours. At regular times, the cultures were sampled and the growth was evaluated by count plate technique. Experiments were performed three times with a standard error around ± 10%.

Antimicrobial activity of lactobacilli and bifidobacteria strains by using living cells

The antimicrobial activity of lactobacilli and bifidobacteria living cells against the antagonists, was evaluated by the overlay method, using a modification of the protocol described by Strus et al. (2005). A single colony of each lactobacilli and bifidobacteria strain was cultured into MRS broth until the $O.D._{600nm}$ was 0.2. Then, 50 µL of each culture was spread by forming a stripe 2 cm wide across the MRS (or cMRS) agar plates. The plates were incubated in anaerobiosis at 37 °C for 24 h. After strain growth, plates were overlaid with 10 mL of melted TSA or MYPG (3 g/L Malt extract, Difco; 3 g/L Yeast extract, Biolife; 3 g/L Bacto Peptone, Difco; 2 g/L glucose, Sigma-Aldrich; pH, 6.2) soft agar (8 g/L) media. *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *P. aeruginosa* ATCC

9027, *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231 colonies from freshly streaked plates were resuspended into 0.9 % (w/v) saline solution at a concentration of 10^8 CFU/mL. Cell suspensions were streaked over TSA (or MYPG in the case of *C. albicans*) surface with a cotton swab. The plates were incubated at 37 °C for 24 h in aerobic conditions. The antimicrobial ability of the examined lactobacilli and bifidobacteria strains was semi-quantitatively evaluated in terms of absent (-), moderate (+) and strong (++) growth inhibition of the antagonist, depending on the dimension of the inhibition halos.

Screening of lactobacilli and bifidobacteria strains for B8, B9 and B12 vitamins production

Biotin (B8 vitamin), folic acid (B9 vitamin), cobalamin (B12 vitamin) production were respectively assayed in the semisynthetic media: Biotin Assay Medium (Difco), Folic Acid Assay Medium (Difco) and B12 Assay Medium (Sigma-Aldrich). They possess all the nutrients for lactobacilli and bifidobacteria growth except for the vitamin of interest. A modification of the protocol described by Pompei et al. (2007) was used. The strains were cultivated in MRS (or cMRS) broth at 37 °C in anaerobiosis for 24 h. Cells were washed two times in saline solution followed by centrifugation at 12000 rpm for 10 min, and the cell pellets were resuspended in saline solution until the $O.D._{600nm}$ was 2. Cell suspensions were inoculated (5 % v/v) into each vitamin assay medium. The cultures were incubated anaerobically at 37 °C for 48 h and sub-cultured for a maximum of 7 times. At every sub-culture the $O.D._{600nm}$ was measured.

Maintenance and growth of cell lines for *in vitro* tests

The experimental model was the murine embryonal fibroblast cell line BALB/c3T3, clone A31 (ATCC-CCL-163). This cell line is largely used for their capability to give standardized responses and to mimic the systemic environment of an organism; these cells are in fact used as biologic matrix alternative to *in vivo* tests according to ECVAM (Kinsner-Ovaskainen et al. 2009; Freire et al. 2009) and ISO guideline (ISO 10993-5). Cells were routinely grown in Dulbecco modified Eagle's medium (DMEM) (Sigma-Aldrich) in 75 cm^2 culture flasks at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air until confluent monolayers were obtained. For biological

assays 3T3 cells were seeded in 12-well plates at a concentration of 10^5cells/well and incubated in 1 mL of DMEM for 24 h at 37 °C in 5 % CO_2.

For human intestinal cell line *in vitro* tests, the HT-29 cell line was used. HT-29 are cells from human colon presenting a complete anatomical structure with microvilli, microfilaments, large vacuolated mitochondria with dark granules and rough endoplasmic reticulum with free ribosomes, lipid droples, few primary and many secondary lysosomes. Cells were routinely grown in McCoy's 5a Medium Modified (Sigma-Aldrich) in 75 cm^2 culture flasks at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air until confluent monolayers were obtained. For biological assays HT-29 cells were seeded in 12-well plates at a concentration of 10^5cells/well and incubated in 1 mL of McCoy's 5a Medium Modified for 24 h at 37 °C in 5 % CO_2.

Resistance to the inflammatory stress

Inflammatory stress was induced by treating the 3T3 or HT-29 cells with Sodium Dodecyl Sulphate (SDS) as pro-inflammatory agent (OECD Test Guideline N.439). SDS was added into the culture medium at a final concentration of 0.05 % (w/v). Cells were incubated in 5 % CO_2 at 37 °C.

Bacterial cultures were routinely grown in MRS agar plates, then the cells were resuspended in DMEM at a final concentration of 10^7 CFU/mL. These suspensions were added into each well. The residual cell viability at 24 hours and at 5 days was evaluated by MTT assay. The cell culture supernatants were removed and collected for cytokines determination. Cells were washed with Dulbecco's Phosphate Buffered Saline. Then, 0.05 % (w/v) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in DMEM was added into each well and incubated for 4 hours at 37 °C. At the end of the incubation, the MTT-medium was removed and replaced by an equal volume of MTT solubilization solution (10 % v/v Triton X-100, 0.1 N HCl in isopropanol) in order to dissolve the formazan produced by MTT reduction. After 30 min incubation on a rotary shaker, the produced formazan was measured at 570 nm with a microplate reader. The results are expressed as A_{570} of treated cells – A_{570} untreated cells/A_{570} untreated cells × 100.

Cytokines production determination

The concentrations of interleukine 4 (IL-4) and interleukine 10 (IL-10) as anti-inflammatory cytokines and of tumor necrosis factor alpha (TNF-α) as pro-inflammatory cytokine on cell culture supernatants, collected at 24 h and 5 days, were determined by enzyme-linked immune-sorbent assay (ELISA) commercial kits (Boster Biological Technology), according to manufacturer's instructions. The sensitivity of each cytokine assay was as follows: 7.8 pg/mL for IL-4 and IL-10, and 1 pg/mL for TNF-α. The results are expressed as pg/mL and compared with a negative control, not subjected to any treatment, and a positive control, subjected to the SDS treatment previously described.

Determination of the antioxidant potential

For biological assays 3T3 or HT-29 cells were seeded in 12-well plates at a concentration of 10^5 cells/well and incubated in 1 mL of DMEM for 24 h at 37 °C in 5 % CO_2.

Bacterial cultures were routinely grown in MRS agar plates, then the cells were resuspended in DMEM at a final concentration of 10^7 CFU/mL. These suspensions were added into each well. The negative control was carried out with an equal volume of DMEM without bacterial cultures. The improvement of the antioxidant ability of 3T3 or HT-29 cells in the presence of bacterial strains was evaluated by Ferric Reducing Antioxidant Parameter (FRAP) assay, as described by Benzie et al. 1996. Ferric to ferrous ion reduction at pH 3.6 causes a colored ferrous-2,4,6-tripyridyl-s-triazine (TPTZ) complex to. The absorbance at 595 nm of each sample was recorded after 30 min. The absorbance values are compared to a Fe(II) standard curve. Final results are expressed as Fe(II) μM.

Statistical analysis

Experiments were performed in triplicate and results elaborated as the mean ± standard error of the mean of three experiments. The statistical significance was assessed by Student's *t*-test. Differences were considered significant at p value < 0.05.

Results

Molecular identification of the strains

The microbial strains were taxonomically characterized by 16S rRNA sequence analysis. Five *Lactobacillus* strains, *L. acidophilus* PBS066, *L. fermentum* PBS073, *L. plantarum* PBS067, *L. reuteri* PBS072, *L. rhamnosus* PBS070 and two *Bifidobacterium* strains, *B. animalis* subsp. *lactis* PBS075, *B. longum* subsp. *longum* PBS108 were identified at the species level and deposited to the DSMZ culture collection (Table 1).

Resistance of lactobacilli and bifidobacteria strains at acidic pHs and simulated intestinal fluid (SIF)

In order to evaluate the survival of lactobacilli and bifidobacteria strains at the acidic conditions, the viability of the seven strains at different acidic pHs was determined (Fig. 1). All the strains do not lose their viability when exposed at pH 3.0, 4.0, 5.0 for 180 min. At pH 2.0, *L. reuteri* PBS072 and *L. rhamnosus* PBS070, showed the best survival rate over the 180 min of exposure. *B. lactis* PBS075 and *B. longum* PBS108 viability was slightly affected after 180 min of exposure. *L. acidophilus* PBS066, *L. plantarum* PBS067 and *L. fermentum* PBS073 viability declined at undetectable levels just after 60 min and the latter one after 120 min of exposure.

All the strains were also exposed for 180 min to the simulated intestinal fluid, containing a standard concentration of bile salts. The examined strains showed a good resistance to bile salts. The less resistant strain, *L. fermentum* PBS073, exhibited a viability drop comprised within three order of magnitude (data not shown).

Antibiotic resistance of lactobacilli and bifidobacteria strains

The antibiotic resistance profiles of the strains to the nine antibiotics suggested in the most recent European Food Safety Authority (EFSA) guidelines was determined. The sensitivity or resistance of the strains was assessed by comparing the obtained MIC values for each antibiotic to the reference breakpoints described for the corresponding genus or species (EFSA 2012). All the strains displayed MIC values behind or close to the reference breakpoints with the exception of *B. animalis* subsp. *lactis* PBS075, whose MIC value for tetracycline was above the breakpoint established for the *Bifidobacterium* genus. The results are shown in Table 2.

The *tet(W)* gene, the most common tetracycline resistance determinant in bifidobacteria, was amplified by PCR. A second amplification with the forward primer specific for the transposase *trp* and the reverse primer specific for *tet(W)*, confirmed that *tet(W)* is flanked upstream by the putative transposase gene *trp*.

Antimicrobial activity by cell culture supernatants and by cells of lactobacilli and bifidobacteria strains

Complementary approaches were combined in order to define the antimicrobial activity of lactobacilli and bifidobacteria strains against pathogens. We have chosen *E. coli* ATCC 29522, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538, and *C. albicans* ATCC 10231, as the most representative pathogens able to colonize different districts of the organism.

The well diffusion agar assay provided preliminary information about the inhibitory action exerted by microbial culture supernatants.

Most of not-neutralized culture supernatants of the examined strains showed an inhibition grade on the pathogen growth, measured by the inhibition halo diameters (data not shown). Results are compared with a positive control consisting in a *Bifidobacterium* sp. strain, whose antimicrobial properties have been already described (Aloisio et al. 2012). The only exception was for *C. albicans* ATCC 10231, whose growth seemed not to be influenced by the culture supernatants. The neutralized culture supernatants did not exert any antimicrobial activity.

The not-neutralized cellular supernatants of the strains, that positively responded to the preliminary assay, were subjected to further investigations. Their antimicrobial properties were evaluated by comparing the growth of the antagonists within 24 hours in the presence and in the absence of culture supernatants. Figure 2 showed the inhibition rate of the different microbial culture supernatants towards the antagonist. Some of them were more sensitive to others to the microbial culture supernatants, whereas some culture supernatants were more effective in their antimicrobial activity. *L. acidophilus* PBS066 and *L. plantarum* PBS067 strongly inhibited *E. faecalis*, *S. aureus*, *P. aeruginosa* and *E. coli*.

L. rhamnosus PBS070 and *Bifidobacterium* strains PBS075 and PBS108 inhibited *P. aeruginosa* and *E. coli. L. fermentum* PBS073 exerted an antimicrobial activity mainly towards *S. aureus, E. coli* and *P. aeruginosa,* whereas *L. reuteri* PBS072 inhibited *S. aureus* and *P. aeruginosa*.

In order to verify the antimicrobial potential of cells *in vivo*, cultures of lactobacilli and bifidobacteria strains, were overlaid by the antagonists *E. coli*, *E. faecalis*, *P. aeruginosa*, *S. aureus* and *C. albicans* cultures. As shown in Figure 3, the growth inhibition halos around the colonies confirmed the antimicrobial inhibition profile reported by the analysis of the culture supernatants. In particular, a strong growth inhibition of *C. albicans* exerted by *L. acidophilus* PBS066, *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. longum* subsp. *longum* PBS108 could be observed.

Screening for vitamin B8, B9, B12 producers

All the strains were screened for their ability to grow on biotin (vitamin B8), folic acid (vitamin B9) or cobalamin (vitamin B12) assay media, containing all the nutrients except for biotin or folic acid or cobalamin. The examined strains were able to grow when the absent vitamin was supplemented into each medium at a final concentration of 10 µg/L. This indicates that the media composition was able to support the nutritional requirements of lactobacilli and bifidobacteria strains, with the vitamin of interest being the limiting factor. *L. plantarum* PBS067 was able to grow on all the 3 vitamin-assay media, while *L. fermentum* PBS073 and *L. reuteri* PBS072 were able to grow on folic acid or cobalamin vitamin-assay media for at least seven sub-cultures. In the case of *L. rhamnosus* PBS070 and *B. animalis* subsp. *lactis* PBS075 the grow was observed only on B12 assay medium. *L. acidophilus* PBS066 and *B. longum* subsp. *longum* PBS108 died within four passages (data not shown).

Resistance and modulation of inflammatory stress by lactobacilli and bifidobacteria strains on a systemic-miming cell line *in vitro*

The model fibroblast cell line 3T3 was treated with SDS as irritant agent for 24 hours and for 5 days, in order to induce an acute and a chronic stress, respectively. The protective effect of the

lactobacilli and bifidobacteria strains against the inflammatory damage was evaluated by co-culturing the cell line with the bacterial strains. The residual cell viability and inflammatory response in the presence and in the absence of microbial strains were determined. In the sole presence of SDS, the cell viability was reduced up to 50 % with respect to the negative control. The cultures of *L. plantarum* PBS067, *L. reuteri* PBS072, and *L. rhamnosus* PBS070 increased the cell viability more than 90 % both in acute and in chronic stress conditions. The beneficial effect of *B. longum* subsp. *longum* PBS108 on cell viability was more evident in acute stress condition, with an increase up to 90 %, than in chronic stress conditions. *L. fermentum* PBS073 slightly favored cell viability just up to 60 %. In contrast, *B. animalis* subsp. *lactis* PBS075 did not exert a strong protective effect on the cells (Fig. 4).

The modulation of the anti-inflammatory response by the microbial strains on the fibroblast cells was evaluated by determining the concentration of IL-4 and TNF-α released into the culture medium after SDS treatment. In an acute stress condition (24 h treatment with SDS), the inflammatory state determined a slightly increase of IL-4 and a four-fold increase of TNF-α with respect to the untreated cells. In a chronic inflammation state (5 days treatment with SDS), the level of IL-4 was constant, whereas the TNF-α concentration further increased.

In acute inflammation condition, the cultures of *L. plantarum* PBS067, *L. reuteri* PBS072, *L. rhamnosus* PBS070, and *B. longum* subsp. *longum* PBS108 were able to further stimulate the IL-4 release. At the same time, there was a reduction of the TNF-α, whose concentration was similar to those recorded in the negative controls. *L. fermentum* PBS073 and *B. animalis* subsp. *lactis* PBS075 exerted a less significant modulatory effect (Fig. 5a). In chronic inflammation condition, *L. plantarum* PBS067, *L. reuteri* PBS072, *L. rhamnosus* PBS070 were still able to stimulate the IL-4 release and to reduce the TNF-α concentration, at levels comparable to the negative control (Fig. 5b).

Antioxidant potential of lactobacilli and bifidobacteria strains

The antioxidant potential of 3T3 cell line was determined by FRAP assay in the presence and in the absence of microbial strains both at 24 hours and 5 days of co-culture. The results reported in figure 6 showed that after 24 hours, *L. plantarum* PBS067*, L. reuteri* PBS072*, L. rhamnosus* PBS070 and *B. animalis* subsp. *lactis* PBS075 were able to improve the antioxidant potential of the cell culture. After 5 days, only *L. plantarum* PBS067 and *L. rhamnosus* PBS070 were still able to significant increase the antioxidant potential of the cell line.

Resistance and modulation of inflammatory stress by lactobacilli and bifidobacteria strains on the intestinal HT-29 cell line *in vitro*

On the basis of the obtained results in the preliminary study on 3T3 cells, the most performant strains, *L. plantarum* PBS067, *L. rhamnosus* PBS070 *and B. animalis subsp. lactis* PBSO75 were selected for *in vitro* tests with the human intestinal HT-29 cell line.

Despite the presence of the bacterial strains doesn't protect the cell viability from the irritating agent, they showed a positive effect on the antioxidant potential and inflammatory status of the biological system. In an acute stress condition, the inflammatory state determined a slightly increase of IL-4 and IL-10 and an increase of TNF-α with respect to the untreated cells. In a chronic inflammation state, the level of IL-4 and IL-10 increased in a significant manner, whereas the TNF-α concentration further increased. Also the antioxidant potential showed a significant increase especially in chronic conditions. Results are reported in the Figure 7a, 7b, and Figure 8.

Discussion

The ability of lactobacilli and bifidobacteria to exert beneficial effects on the human health is a species and strain-specific feature (Ramos et al. 2013). This explains the continuously search for novel strains with probiotic potential.

In this work, the probiotic properties of new *Lactobacillus* and *Bifidobacterium* strains were screened and the effects of the strains on cell lines by *in vitro* tests were evaluated. Moreover, the probiotic properties effects of the most *Lactobacillus* and *Bifidobacterium* performant strains were

evaluated on human intestinal cell lines, in order to successively move to another level of study towards patients with acute and chronic disorders of the gut by *in vivo* experiments.

As fundamental requirement a probiotic strain should survive through the passage of the stomach and the duodenum. Our results showed that the viability of the examined lactobacilli and bifidobacteria strains was not affected at pH 3.0 and in presence of a standard concentration of bile salts. On the other hand, the survival at pH 2.0 was revealed to be a species-specific feature. *L. reuteri* PBS072 and *L. rhamnosus* PBS070 evidenced the highest survival rate, whereas *L. acidophilus* PBS066, *L. plantarum* PBS067 and *L. fermentum* PBS073 were the most sensitive strains. These observations are in agreement with literature data (Santini et al. 2010; Maragkoudakis et al. 2006) that evidenced a good survival of these bacteria at pH 3.0 and in presence of bile salts (Charteris et al. 1998; Jacobsen et al. 1999), but a great sensitivity to lower pH.

The consumption of probiotic containing antibiotic resistance strains may pose the risk of the transfer of antibiotic resistance genes to the resident microbial community and hence to pathogenic bacteria. For this reason, the assessment of the susceptibility to antimicrobials of the strains with a standardized method was strongly required. The examined strains were sensitive to all the selected antibiotics, in agreement with the most recent EFSA guidelines (EFSA 2012). The only exception was represented by *B. animalis* subsp. *lactis* PBS075, which exhibited a MIC value of tetracycline above its breakpoint. The *tet*(W) gene, the most widespread in tetracycline resistance determinant in *B. animalis* subsp. *lactis* strains, was found to be also present in PBS075. *tet*(W) is flanked upstream by the transposase *trp* gene, which is co-trancribed in tandem (Gueimonde et al. 2010). Altough the evidence that *tet*(W) is transmissible in these bacteria have been not documented so far (Moubareck et al. 2005) (Ammor et al. 2008) (Gueimonde et al. 2010), our results suggest that *B. animalis* subsp. *lactis* PBS075 strain could not be considered an appropriate strain for future *in vivo* trials. As functional requirement, a probiotic strain should exert antagonistic activity against potential pathogenic bacteria. In this work, a representative selection of bacteria analogous to common human pathogens were included as antagonists. All the examined strains were capable of

contrasting at different extent the growth of the antagonists. *P. aeruginosa* and *E. coli* were the most sensitive microorganisms. The antibacterial activity of cell cultural supernatatants is conventionally attributed to the production of organic acids, diacetyl, bacteriocins or hydrogen peroxide (Servin 2004). Preliminary investigations about the nature of the antimicrobial activities of the examined strains were performed. The lack of pathogen inhibition of the pH neutralized culture supernatants, revealed that the antimicrobial activity mainly resulted from the production of acidic metabolites, such as lactic acid, rather than excreted bacteriocins. The higher amount of lactic acid produced by the homofermentative bacteria with respect to the heterofermentative ones could explain why *L. acidophilus* PBS066 and *L. plantarum* PBS067, exhibited a broader inhibition activity than all the other examined strains. None of the not-neutralized culture supernatants was able to inhibit the growth of *C. albicans*. Interestingly, when *L. acidophilus* PBS066, *L. fermentum* PBS073, *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. longum* subsp. *longum* PBS108 cells were exposed to *C. albicans*, a remarkable inhibition occurred. This effect could be may be due to an inducible production of some antimicrobial metabolites with antifungal properties.

Vitamin production is not a common characteristic of the species, but a single probiotic strain property. For example, as observed by Pompei et al. (2007) despite the presence in *B. longum* NCC2705 genome of the entire pathway for folic acid production, only few *B. longum* strains were able to grow in the absence of this vitamin. A screening for biotin, folic acid and cobalamin producers by our strains revealed that only *L. plantarum* PBS067 was able to synthesize the three examined vitamins; while *L. fermentum* PBS073 and *L. reuteri* PBS072 folic acid and cobalamin. It was already demonstrated that some strains of *L. plantarum* (Sybesma et al. 2003) and *L. reuteri* (Santos et al. 2008) are able to produce significant amount of folate. To our knowledge, no *L. fermentum* strains have been characterized as folic acid producers so far, but the completely sequenced *L. fermentum* IFO 3956 possessed the entire biosynthetic cluster (Rossi et al. 2011). Cobalamin is the only vitamin that is exclusively synthetized by microorganisms. The analysis of recently sequenced lactobacilli genomes, suggested that cobalamin biosynthesis is common among

different *Lactobacillus* species (Capozzi et al. 2012). This observation is in agreement with our screening, which showed the ability of five strains out of seven to grow in the absence of B12 vitamin.

Our strains were also characterized for their ability to interact with the host's cells by *in vitro* experiments. First, a systemic model as the murine fibroblast BALB/c 3T3a cell line was used in this study in order to evaluate the possible different effects. The examined strains were able to modulate the release of IL-4 and TNF-α by the 3T3 cells *in vitro*. Three of the seven strains revealed to have the potential to neutralize the response caused by an irritant agent, by inducing higher production of the anti-inflammatory cytokine IL-4, thus lowering the TNF-α production. For instance, a comparative study of the immunomodulatory properties of *B. longum* evidenced a different ability of the various strains to diverge the immune response towards a Th1 or a Th2 pathway (Medina et al. 2007). The dendritic cells differently responded to *L. plantarum* strains inducing the release of different amounts of IL-10 and IL-12 (Meijerink et al. 2010). It should be noted that the immune profile obtained in co-culture assays with bacteria and immune cells was demonstrated to be predictive of their *in vivo* immunomodulatory activities (Foligne et al. 2007). Moreover, it is demonstrated that probiotic bacteria contribute in the reduction of oxidative damage in humans (Amaretti et al. 2013; Lin and Yen 1999; Spyropoulos et al. 2011). The oxidation in living organisms, essential for energy production, lead to the accumulation of harmful reactive species, that induce oxidative damage. This plays a significant role in the pathogenesis of several human diseases such as cancer, emphysema, cirrhosis, atherosclerosis (Kryston et al. 2011). Results on the examined strains showed that some of them, such as *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. longum* subsp. *longum* PBS108 seemed to have a stronger effect on the antioxidative potential of the cell line. FRAP assay employed in this study provided information on the efficacy of the antioxidants present in the sample (Benzie and Strain 1996; Lin and Yen 1999).

Many studies have demonstrated the clinical potential of probiotics against many diseases, such as necrotising enterocolitis, inflammatory bowel disease and colon cancer (Robles Alonso and Guarner

2013). In this study, three most performant strains, *L. plantarum* PBS067, *L. rhamnosus* PBS070 *and B. animalis subsp. lactis* PBSO75 were selected for *in vitro* tests with a human intestinal cell line. Data showed that the trend of action of HT-29 cells was quite similar to the 3T3 cell one and the examined strains were able to modulate the release of IL-4, IL-10 and TNF-α and to increase the antioxidant potential. This allows to move to another level of study, that is, the administration of the most probiotical performant strains to patients with acute and chronic disorders of the gut by *in vivo* experiments.

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Legends

Figure 1. Effect of pH on the survival of lactobacilli and bifidobacteria strains. Cells were incubated for three hours at 37°C in the following buffers: 50 mM glycine buffer pH 2.0 (♦), pH 3.0 (■), 50 mM sodium acetate buffer pH 4.0 (▲) and 5.0 (×) and sodium phosphate buffer pH 7.0 (●) as controls. The residual viability was determined at regular time intervalsof 0, 30, 60, 120 and 180 min by the count plate method.

Figure 2. Effect of not-neutralized culture supernatants of *L. acidophilus* PBS066 (♦), *L. fermentum* PBS073(■), *L. plantarum* PBS067 (▲), *L. reuteri* PBS072 (◊♦), *L. rhamnosus* PBS070 (■), *B. animalis* subsp. *lactis* PBS075 (×), *B. longum* subsp. *longum* PBS108 (●) on the growth of *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC6538, *C. albicans* ATCC 10231 as antagonistic strians. The not-neutralized culture supernatant of *Bifidobacterium* sp.(○), known for its antimicrobial properties (Aloisio et al. 2012) was also included. A positive growth control of each antagonist in the absence of culture supernatants was included. Samples were taken at regular time intervals of 0, 3, 6 and 24 hours and the viability was determined by the count plate method.

Figure 3. Example of agar plate method showing strong inhibition of *Candida albicans* growth over *Lactobacillus* cultures.

Figure 4. Protective effect of lactobacilli and bifidobacteria strains on BALB/c3T3 cells in the presence of SDS as irritant agent after 24 hours (■) and at 5 days (□) of exposure. The negative control was represented by not-treated cells. Residual cell viability was measured by means of MTT assay. The results, expressed as percentage (%), were calculated.

Figure 5. Effect of lactobacilli and bifidobacteria strains on the cytokine secretion by BALB/c3T3 cells in the presence of SDS as irritant agent. The not-treated sample was included as negative control. The concentration of TNF-α (■) and IL-4 (■) at 24 hours (panel a) and at 5 days (panel b) was monitored by ELISA test. The experiments were performed three times in triplicate. The results

are expressed as the means ± standard deviations, * p-value < 0.05, ** p-value < 0.01 as compared to SDS-treated samples.

Figure 6. Effect of lactobacilli and bifidobacteria strains on the antioxidant potential of BALB/c3T3 cells. The cell culture at 24 hours (■) and at 5 days (□) was monitored by FRAP assay. The results, expressed as μM of Fe(II), are the means ± standard deviations, * p-value < 0.05, ** p-value < 0.01 as compared to the negative control.

Figure 7. Protective effect of lactobacilli and bifidobacteria strains on HT-29 cells in the presence of SDS as irritant agent after 24 hours (■) and at 5 days (□) of exposure (panel a). The negative control was represented by not-treated cells. Residual cell viability was measured by means of MTT assay. The results, expressed as percentage (%), were calculated.

Effect of lactobacilli and bifidobacteria strains on the antioxidant potential of HT-29 cells (panel b). The cell culture at 24 hours (■) and at 5 days (□) was monitored by FRAP assay. The results, expressed as μM of Fe(II), are the means ± standard deviations, * p-value < 0.05, ** p-value < 0.01 as compared to the negative control.

Figure 8. Effect of lactobacilli and bifidobacteria strains on the cytokine secretion by HT-29 cells in the presence of SDS as irritant agent. The not-treated sample was included as negative control. The concentration of TNF-α (■), IL-4 (■) and IL-10 (□) at 24 hours (panel a) and at 5 days (panel b) was monitored by ELISA test. The experiments were performed three times in triplicate. The results are expressed as the means ± standard deviations, * p-value < 0.05, ** p-value < 0.01 as compared to SDS-treated samples.