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An In Vitro Study on Bacterial Growth Interactions and Intestinal Epithelial Cell Adhesion Characteristics of Probiotic Combinations

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Abstract The aims of this study were to examine long-term growth interactions of five probiotic strains (*Lactobacillus casei* 01, *Lactobacillus plantarum* HA8, *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri* ATCC 55730 and *Bifidobacterium lactis* Bb12) either alone or in combination with *Propionibacterium jensenii* 702 in a co-culture system and to determine their adhesion ability to human colon adenocarcinoma cell line Caco-2. Growth patterns of probiotic *Lactobacillus* strains were not considerably affected by the presence of *P. jensenii* 702, whereas lactobacilli exerted a strong antagonistic action against *P. jensenii* 702. In the co-culture of *Bif. lactis* Bb12 and *P. jensenii* 702, a significant synergistic influence on growth of both bacteria was observed ($P < 0.05$). The results of adhesion assay showed that when probiotic strains were tested in combination, there was evidence of an associated effect on percentage adherence. However, in most cases these differences were not statistically significant ($P < 0.05$). Adhesion percentage of *Lb. casei* 01 and *Lb. rhamnosus* GG both decreased significantly in the presence of *P. jensenii* 702 compared to their adhesion levels when alone ($P < 0.05$). These results show that the survival and percentage adhesion of some probiotic strains may be influenced by the presence of other strains and this should be considered when formulating in the probiotic products.

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Introduction

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [8, 9]. Probiotics primarily belong to the genera *Lactobacillus* and *Bifidobacterium*, however, some strains of propionibacteria have also been considered as probiotics.

There is a diverse range of health benefits reported to be associated with some dairy propionibacteria. These include synthesis of some beneficial substances such as vitamin B12 and folate [15], secretion of antimicrobial compounds (e.g. propionic acid and bacteriocins) [32], production of β-galactosidase which prevents lactose intolerance [48], modulating the host's immune system [36], anti-hyperlipemic effect [36], stimulating the growth of bifidobacteria [18–21, 28, 45, 47], improving colonic inflammation by nitrate reduction [26] and anticarcinogenic effect [16, 22, 23, 35].

In most cases, however, it is recognised that in order to initiate conferring these health promoting properties on the host, the probiotic micro-organisms need to survive at sufficiently high numbers and colonise the gastrointestinal tract. A prerequisite for intestinal colonisation is adherence to intestinal epithelial mucosa [1, 3]. Probiotic adhesion to intestinal epithelial cells using single strains of probiotic propionibacteria has been studied in vitro and in vivo [14, 33, 44, 50]. Few studies have investigated however how strain interaction could affect either individual bacterial viability or adhesion ability.

Ouwehand et al. [33] have previously demonstrated that primarily adhered *Lb. rhamnosus* GG, *Bif. lactis* Bb12 and *Bif. infantis* Bbi significantly enhanced the subsequent adhesion of some propionic acid bacteria to human intestinal mucus in paired-strain combinations, while primarily

adhered propionibacteria did not increase the subsequent adhesion ability of lactobacilli and bifidobacteria to the mucus. Collado et al. [7] further identified positive changes in human intestinal mucosal adhesion rates of *P. freudenreichii* ssp. *shermanii* JS in 2-, 3- and 4-strain combinations with probiotic lactobacilli and bifidobacteria. Mucosal adherence of lactobacilli used in this study improved in all combinations containing *P. freudenreichii* ssp. *shermanii* JS.

In addition to measurable changes in adhesion rate, it has been observed that some propionibacteria can stimulate the growth of bifidobacteria in vivo and in vitro through the production of specific growth stimulating factors [13, 18–21, 27, 28, 42, 45, 47]. A further study has shown that bifidobacteria may also stimulate growth of propionibacteria [11]. An earlier study reported that lactobacilli have different effects on growth of propionibacteria including prevention, stimulation and no effect [34]. It has been reported that selected lactobacilli stimulated the growth of propionibacteria through production of lactic acid serving as energy source for them [32]. However other metabolites produced by lactobacilli may be involved in growth stimulation of propionibacteria. Piveteau et al. [39] reported that short peptides produced by *Lb. helveticus* DPC 4571 in milk stimulate the growth of *P. freudenreichii* DPC 3801. The preceding literature indicates that growth interactions between propionibacteria and lactobacilli or bifidobacteria in probiotic combinations are species- and strain-dependent. Moreover, composition of growth culture media may play an important role.

The aims of this study were to examine long-term growth interactions of five probiotic strains (*Lb. casei* 01, *Lb. plantarum* HA8, *Lb. rhamnosus* GG, *Lb. reuteri* ATCC 55730 and *Bif. lactis* Bb12) either alone or in combination with the novel probiotic *P. jensenii* 702 in a co-culture medium, and to determine their adhesion ability to human colorectal epithelial cell line Caco-2.

Methods and Materials

Bacterial Strains and Growth Conditions

Four commercial probiotic strains *Lb. rhamnosus* GG, *Lb. reuteri* ATCC 55730, *Lb.casei* 01 and *Bif. lactis* Bb12, and two new probiotic strains *Lb. plantarum* HA8 and *P. jensenii* 702 isolated in our laboratory were used in this work. *Lb. reuteri* ATCC 55730 was kindly provided by BioGaia Biologics Inc. (BioGaia Biologics Inc. Raleigh, USA). *Bif. lactis* Bb12 and *Lb. casei* 01 were generous gifts from Chr. Hansen (Chr. Hansen Pty. Ltd. Melbourne, Australia). *Lb. rhamnosus* GG was isolated from CULTURELLE® capsule (a gift from Amerifit Brands Inc., Cromwell, USA). Bacterial identifications were confirmed using 16S rRNA gene targeted species-specific primers. For longer survival and higher quantitative retrieval of the cultures, they were stored at −80°C using Microbank® Bacterial and Fungal Preservation System (Pro-Lab Diagnostics, Richmond Hill, Canada). When needed, recovery of strains was undertaken by two consecutive subcultures in appropriate media prior to use. *Lactobacillus* strains and *Bif. lactis* Bb12 were grown overnight at 37°C, respectively, in MRS and RCM broths (Oxoid Australia Pty Ltd, Adelaide, Australia) under anaerobic conditions. *P. jensenii* 702 was grown anaerobically in yeast extract lactate (YEL) medium [25] at 30°C for 48 h.

Chemicals and Reagents

All chemicals used in this study were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Co-Culture Growth Interactions

Growth interactions of *P. jensenii* 702 with other probiotics were examined in a co-culture system. YEL medium supplemented with 2% glucose (GYEL) was used as the co-culture medium, on the basis of preliminary experiments in which good individual growth of all probiotic strains was observed in this medium (data not shown). The cultures were individually adapted to GYEL medium prior to examining co-culture growth interactions. This adaptation was performed by sub-culturing in GYEL medium and incubation at 33°C overnight (*Lactobacillus* strains and *Bif. lactis* Bb12) or for 48 h (*P. jensenii* 702). Bacterial cells were then harvested from fresh probiotic cultures in their stationary phases by centrifugation at 3000 rpm for 10 min and washed three times with Dulbecco's Phosphate-Buffered Saline (PBS) (Gibco, Invitrogen Corp., Carlsbad, CA, USA) pH 7.0. Bacterial pellets were then resuspended in PBS. 50 ml of the medium dispensed in sterile screw-cap polypropylene containers (Sarstedt Australia Pty Ltd, Mawson Lakes, SA, Australia) was inoculated with an aliquot of 500 µl of each bacterial suspension either alone or in combination with *P. jensenii* 702. Containers were incubated anaerobically at 33°C for 2 weeks. Bacterial counts were determined by plating 100 µl aliquots of decimal dilutions of cultures on agar plates at days 0, 1, 4, 7 and 14. *Lactobacillus* spp and *Bif. lactis* Bb12 were counted, respectively, on Lactobacillus Selective (LBS) agar [41] and Bifidobacterium Iodoacetate (BIM) agar [30] after 3 days of incubation at 37°C under anaerobic conditions. As growth of *P. jensenii* 702 is inhibited on BIM agar, in the co-culture of *P. jensenii* 702 and *Bif. lactis* Bb12, colonies appeared on BIM are considered to be exclusively *Bif. lactis* Bb12. *P. jensenii* 702 can grow on

LBS agar, however its growth rate is very slow and colonies appear after 5–7 days of incubation. Thus, the colonies which appeared on LBS agar following 24–48 h of incubation are considered to be *Lactobacillus* spp. *P. jensenii* 702 was counted on YEL agar [25] following 7 days of incubation at 30°C under anaerobic conditions. In the co-culture of *Lactobacillus* strains and *P. jensenii* 702, *Lactobacillus* strains can also grow on YEL agar but their colonies can be easily differentiated from each other. *P. jensenii* 702 can be differentiated from *Lactobacillus* strains on the basis of its typical colony morphology and colour as well as by its later appearance on the YEL agar. *P. jensenii* colonies appear after 7 days of incubation as drop-like mustard coloured colonies. The results were expressed as Log CFU/ml of bacterial counts. The pH of the culture media was measured by a Cyberscan 510 pH meter (Eutech Instruments Pty Ltd., Singapore) on the same days as the counts performed.

Caco-2 Cell Line

The Caco-2 cell line ATCC HTB-37 (American Type Culture Collection, Rockville, MD, USA) was kindly provided by Dr. Matthias Ernst (Ludwig Institute for Cancer Research, Melbourne, Australia). The cells were cultured in Nunc^{TM} tissue culture flasks (Thermo Fisher Scientific, Rochester, NY, USA) containing RPMI 1640 medium (Gibco, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 20% heat inactivated fetal bovine serum (Gibco, Invitrogen Corp., Carlsbad, CA, USA), 2% HEPES buffer (Gibco, Invitrogen Corp., Carlsbad, CA, USA), 2% sodium bicarbonate (Gibco, Invitrogen Corp., Carlsbad, CA, USA), 1% L-glutamine (Gibco, Invitrogen Corp., Carlsbad, CA, USA) and 2% penicillin/streptomycin (Gibco, Invitrogen Corp., Carlsbad, CA, USA). The cells were grown in this medium at 37°C in a 5% CO_2/95% air atmosphere using a humidified HERAcell 150 CO_2 incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cell-culture medium was replaced with fresh medium every other day.

In Vitro Bacterial Adhesion Assay

The Caco-2 cells were seeded at a concentration of 10^5 cells/well in each well of a Nunc^{TM} 24-well tissue culture plates (Thermo Fisher Scientific, Rochester, NY, USA) and incubated at 37°C in 5% CO_2 atmosphere in a humidified incubator until post-confluence. The cell-culture medium was changed every other day. At least 1 hour before the adhesion assay, the RPMI medium was replaced with the same medium without antibiotic. Prior to the adhesion assay, the monolayers of Caco-2 cells were washed three times with PBS.

A 500 µl aliquot of each bacterial suspension (at concentrations of 10^7–10^8 CFU/ml) was added to post confluent monolayers of Caco-2 cells in each well of the 24-well micro-plates and incubated at 37°C in 5% CO_2/95% air for 3 h. Afterwards, the cells were washed three times with PBS in order to remove non-adherent bacteria. Caco-2 cells were then detached from the plastic surfaces of wells by addition of 500 µl trypsin/EDTA (Gibco, Invitrogen Corp., Carlsbad, CA, USA) and 500 µl PBS followed by incubation at 37°C for 2–3 min. An amount of 1 ml of each suspension was added into a tube containing 9 ml sterile Maximum Recovery Diluent (MRD) (Oxoid Australia Pty Ltd, Adelaide, Australia), and serial decimal dilutions were prepared. 100 µl of each dilution was plated on agar plates. Bacterial counting was performed as described in detail in "Co-Culture Growth Interactions" section. Adhesion was expressed as the percentage of bacteria adhered to Caco-2 cells compared to the initial amount of bacteria added to the Caco-2 cells.

Scanning Electron Microscopy

For the qualitative examination of adhesion by scanning electron microscopy (SEM), 13 mm coverslips (Sarstedt Inc., Newton, NC, USA) were placed in the bottom of tissue culture plate wells before seeding with Caco-2 cells. Preparation stages were the same as those applied for other wells during the growth phase of the Caco-2 cells (see "Caco-2 Cell Line" section). After incubating post-confluent monolayers of Caco-2 cells with each probiotic suspension, coverslips were removed from wells and washed three times with 1 ml pre-warmed (37°C) PBS buffer to remove non-adherent bacteria. Thereafter, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at room temperature and then coverslips washed three times with 0.1 M cacodylate buffer (10 min each time). A second fixation step was performed by exposing the cells to 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, followed by three times washing with cacodylate buffer. The specimens were then dehydrated with a graded series of ethanol solutions (25, 50, 75, 95, and two times 100%, 10 min each session). Coverslips were then air dried at room temperature for 30 min, mounted on stubs and coated with a conductive material (gold particles) using a SPI Sputter Gold Coater (SPI Structure Probe Inc., West Chester, PA, USA). Specimens were then examined with a Philips XL30 scanning electron microscope (Philips, Eindhoven, The Netherlands) equipped with the EDS Link (Isis, Oxford Instruments, Concord, MA, USA).

Statistical Analysis

Statistical analyses were performed using SPSS software Ver. 15 (SPSS Inc., Chicago, IL, USA). Results of

adhesion and bacterial interaction experiments were expressed as averages obtained from two independent experiments each performed in triplicate. Adhesion and bacterial interactions data were analysed using two-tailed *t* test and general linear model (GLM), respectively. A *P* value <0.05 was considered statistically significant for analyses.

Results

Co-Culture Growth Interactions

Growth patterns and pH changes of the mono- and co-cultures in GYEL medium over 14 days incubation are shown in Fig. 1. The growth pattern of each individual culture of *Lb. rhamnosus* GG, *Lb. casei* 01 and *Lb. plantarum* HA8 was overall similar to that of each in the presence of *P. jensenii* 702. In general, after a dramatic increase in viability of these three lactobacilli, either alone or in combination with *P. jensenii* 702, over the first day of incubation, viability was observed to decrease gradually over the reminder of the incubation period. The same trend was also observed for *Lb. reuteri* ATCC 55730. However, after day 7, viable counts of *Lb. reuteri* ATCC 55730 decreased more rapidly in mono-culture than in combination with *P. jensenii* 702, and on day 14, the viable cells of *Lb. reuteri* ATCC 55730 in combination with *P. jensenii* 702 were significantly higher than that of *Lb. reuteri* ATCC 55730 as mono-culture ($P < 0.05$).

pH changes of the culture medium of each single *Lactobacillus* strain were the same as those of the medium containing *Lactobacillus* strains in the presence of *P. jensenii* 702. During the first day of incubation, pH declined rapidly, then steadily decreased over the next 3 days, reaching a plateau on day 4. The pH value of the culture medium inoculated with *P. jensenii* 702 alone decreased by the fourth day of incubation when it started to remain stable for the next 10 days. At all time points, pH values were lower for lactobacilli either alone or in combination with *P. jensenii* 702 than that of *P. jensenii* 702 alone.

One day lag phase was observed for the mono-culture of *Bif. lactis* Bb12. The number of the bacterium then increased sharply, reached a peak on day 4 and declined steeply over the next 10 days. No bacterium was recovered on day 14. The viability of *Bif. lactis* Bb12, in combination with *P. jensenii* 702 however rose rapidly and reached a peak (5.0×10^7 CFU/ ml) on day 1 and remained relatively unchanged by day 14.

Growth of the mono-culture of *P. jensenii* 702 increased gradually, over the first 4 days and reached a peak on day 4, then fell steadily until day 14 when the bacterial count reached 1.1×10^5 CFU/ ml. Viability of *P. jensenii* 702 in combination with *Lactobacillus* strains: *Lb. rhamnosus* GG, *Lb. casei* 01 and *Lb. plantarum* HA8 increased slightly over the first day of incubation, then dramatically decreased until day 4 when no viable cells were recovered. A similar but slower rate of decrease in the viability of *P. jensenii* 702 was observed when incubated in combination with *Lb. reuteri* ATCC 55730. The bacterial count reduced to zero by day 7.

A different growth pattern was found for *P. jensenii* 702 in the presence of *Bif. lactis* Bb12. It grew rapidly by day 1 and then gradually by day 7 when it was at peak. Thereafter, the bacterial count decreased steadily and reached 3.7×10^7 CFU/ ml on day 14 i.e. approximately 2.5 Log CFU/ ml more than that of mono-culture of *P. jensenii* 702 at this time point.

The bacterial counts of both *P. jensenii* 702 and *Bif. lactis* Bb12 in the presence of each other were the highest among all examined bacteria either alone or in combinations with *P. jensenii* 702 at the end of the experiment (day 14).

The pH changes of the culture medium of *P. jensenii* 702 alone were the same as those of the medium containing the combination of *Bif. lactis* Bb12 and *P. jensenii* 702. The pH values declined by day 4 and remained almost stable for the next 10 days. After 1 day incubation, at all other time points, pH values were lower for *P. jensenii* 702 either alone or in combination with *Bif. lactis* Bb12 than those of *Bif. lactis* Bb12 alone.

Adhesion Assay

All examined strains either alone or in combination with *P. jensenii* 702 were able to adhere to Caco-2 human intestinal epithelial cells (Fig. 2). However, adhesion rate varied widely from 5.07% for *Bif. lactis* Bb12 in combination with *P. jensenii* 702 to 83.15% for *Lb. plantarum* HA8 alone. When adhesion ability of probiotic strains was tested in the presence of *P. jensenii* 702, there was evidence of an effect on percentage adherence. Adhesion percentage of *Lb. casei* 01 and *Lb. rhamnosus* GG both decreased significantly in the presence of *P. jensenii* 702 compared to their adhesion levels when alone ($P < 0.05$). Non significant trends were observed for the other combinations. The percentage adhesion of *Lb. reuteri ATCC 55730* improved insignificantly in the presence of *P. jensenii* 702, whereas the adhesion ability of *Lb. plantarum* HA8 and *Bif. lactis* Bb12 decreased in combination with *P. jensenii* 702 however insignificantly. Lactobacilli and *Bif. lactis* Bb12 also had a slight effect on the adhesion ability of *P. jensenii* 702. An insignificant increase in adhesion of *P. jensenii* 702 was observed in combination with *Lb. rhamnosus* GG and *Lb. plantarum* HA8 compared to *P. jensenii* 702 alone. In other combinations adhesion percentage of *P. jensenii* 702 decreased insignificantly.

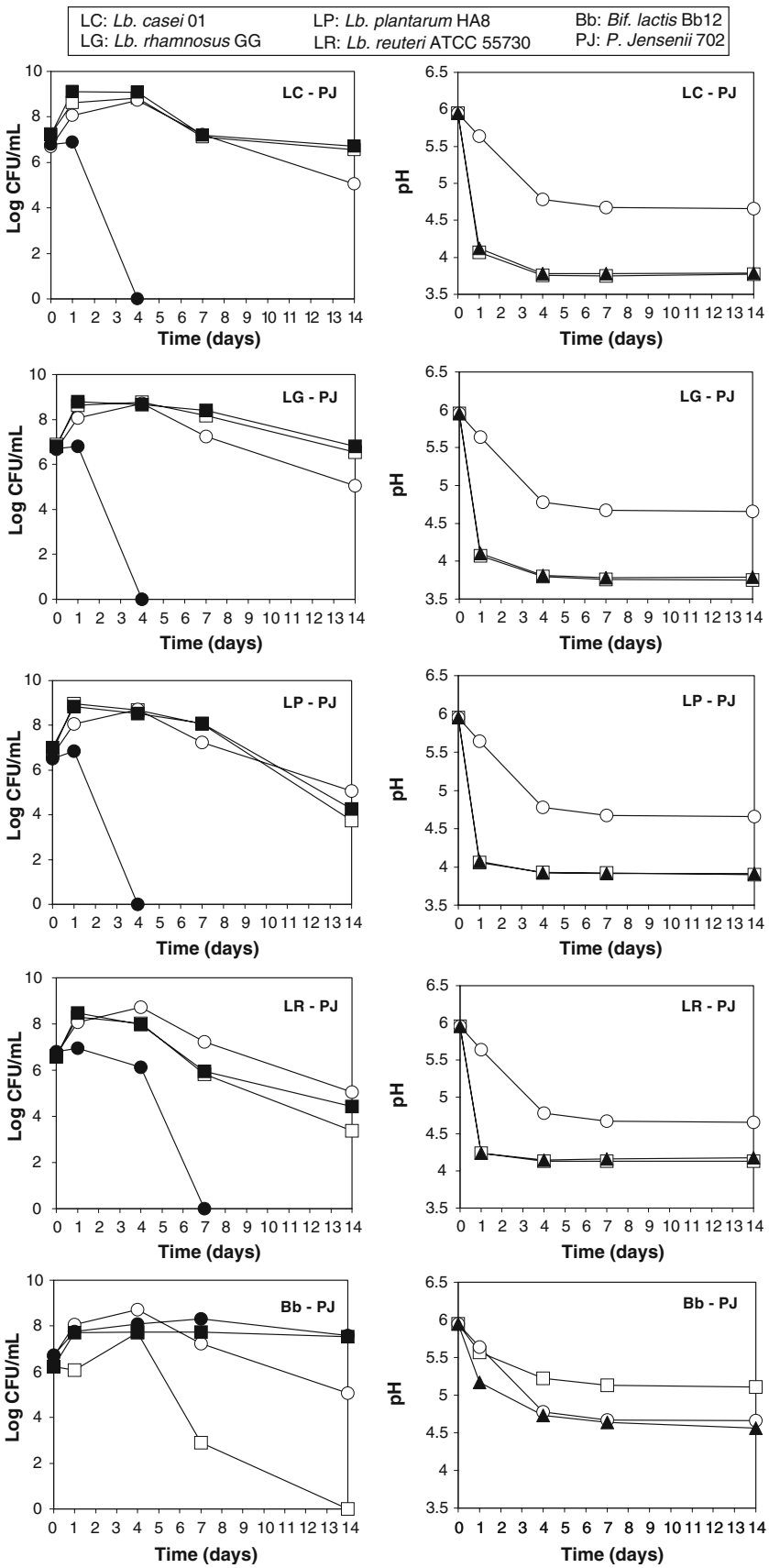

Fig. 1 Growth interactions (*left column*) and pH changes (*right column*) of *Lactobacillus* strains or *Bif. lactis* Bb12 either alone or in combination with *P. jensenii* 702 in GYEL medium at 33°C over 14 days incubation. *open square* viable cell counts and medium pH of monocultures of *Lactobacillus* strains and *Bif. lactis* Bb12, *closed square* viable cell counts of *Lactobacillus* strains and *Bif. lactis* in combination with *P. jensenii* 702, *open circle* viable cell counts and medium pH of *P. jensenii* 702 alone, *closed circle* viable cell counts of *P. jensenii* 702 in combination with Lactobacilli and *Bif. lactis* Bb12, *closed triangle* medium pH of co-cultures

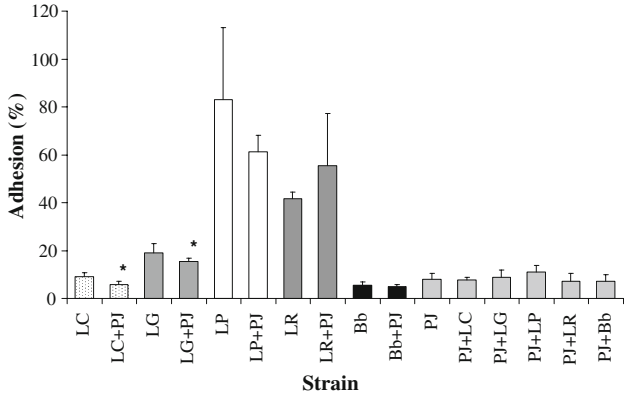

Fig. 2 Percentage adhesion of different probiotic strains: *Lb. casei* 01 (*LC*), *Lb. rhamnosus* GG (*LG*), *Lb. plantarum* HA8 (*LP*), *Lb. reuteri* ATCC 55730 (*LR*) *and Bif. lactis* Bb12 (*Bb*), either alone or in combination with *P. jensenii* 702 (PJ) to Caco-2 human intestinal epithelial cells. In combinations, the first listed bacterium has been counted. Data represent means + standard deviation of two independent experiments, each performed in triplicate. An *asterisk* indicates statistical significance ($P < 0.05$)

Adhesion of single and paired probiotic strains to Caco-2 cells can be seen in the SEM micrographs of Fig. 3.

Discussion

Co-Culture Growth Interactions

The results of co-cultivation of each *Lactobacillus* strain with *P. jensenii* 702 revealed that lactobacilli exerted an antagonistic action on growth of *P. jensenii* 702. However, *Lb. reuteri* ATCC 55730 showed a slower inhibition rate than that of other three *Lactobacillus* strains. Previous studies have reported that lactobacilli have different effects on growth of propionibacteria including inhibition, stimulation and no effect [2, 5, 6, 29, 31, 34, 39, 46].

The pH value of the media in co-cultures of *Lactobacillus* strains and *P. jensenii* 702 dropped quickly (Fig. 1). Obviously this is because of production of copious amounts of organic acids, especially lactic acid, in the media. Lactic acid is known to serve as a suitable energy source for propionibacteria and is catabolised to propionic acid by them [32]. Coincident with a dramatic decrease in pH, was a strong growth inhibition of *P. jensenii* 702, such that no viable cell was recovered after 4 days of incubation in the presence of *Lb. casei* 01, *Lb. rhamnosus* GG *and Lb. plantarum* HA8. Also the viability of *P. jensenii* 702 decreased to zero after 7 days of incubation in combination with *Lb. reuteri* ATCC 55730. Therefore, it could be concluded that low pH is the main responsible factor in growth inhibition of *P. jensenii* 702 in combination with the *Lactobacillus* strains. These results are consistent with previous studies in which rapid decrease in pH by lactobacilli showed a strong growth-inhibitory effect on *Propionibacterium* strains in associative cultures [37, 38]. However other metabolites such as bacteriocins produced by lactobacilli may be involved in growth inhibition of *P. jensenii* 702. *Lb. plantarum* inhibits growth of *Propionibacterium* spp. [5, 29, 47]. Plantaricin, a bacteriocin produced by *Lb. plantarum*, has been reported as an active

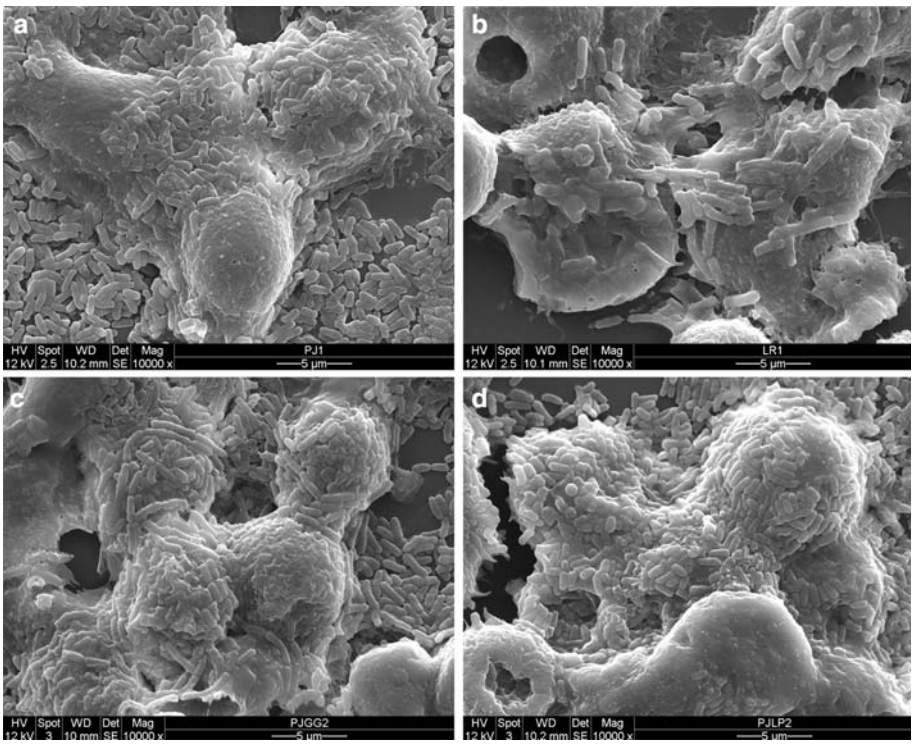

Fig. 3 Scanning electron micrographs of some of the probiotic strains adhered to Caco-2 cells. **a** *P. jensenii* 702, **b** *Lb. reuteri* ATCC 55730, **c** *P. jensenii* 702 + *Lb. rhamnosus* GG, **d** *P. jensenii* 702 + *Lb. plantarum* HA8

antimicrobial agent against *Propionibacterium* spp. [12, 17, 29, 46]. An inhibition activity has also been reported for *Lb. casei* against growth of *P. freudenreichii* spp. *shermanii* in a cheese model [10].

P. jensenii 702 and *Bif. lactis* Bb12 appeared to have a synergistic growth-promoting effect on each other. Growth of bifidobacteria might be stimulated by propionibacteria in two ways: (1) propionate and acetate produced as end products of fermentation of glucose and lactate by propionibacteria [40] enhance the growth of bifidobacteria [19]; (2) some dairy propionibacteria may produce specific growth-stimulating factors for bifidobacteria [13, 18–21, 27, 28, 42, 45, 47]. On the other hand, stimulation of *P. jensenii* 702 by *Bif. lactis* Bb12 is supported by a recent study showing that some strains of bifidobacteria have a growth-promoting effect on propionibacteria [11]. However, to authors' knowledge this is the first report of mutual synergistic growth stimulation of *Propionibacterium* and *Bifidobacterium* in an associative co-culture system.

In this experiment, an in vitro simple model of co-culture bacterial interaction was used to investigate growth interactions of *P. jensenii* 702 and a *Lactobacillus* strain or *Bif. lactis* Bb12. It might be concluded that using *P. jensenii* 702 in the presence of the *Lactobacillus* strains especially in a fermentation process, is not advisable, because these *Lactobacillus* strains prevent the *P. jensenii* 702 growth and final product may not carry efficient amount of *P. jensenii* 702 which is needed to ensure efficacy. On the contrary, our results also revealed that a combination of *P. jensenii* 702 and *Bif. lactis* Bb12 could be used in fermentation processes. However, given that we know that the composition of food may influence the probiotic interactions, this must be tested in a food system, for instance in milk. If the aim is to take advantage of combinations of *P. jensenii* 702 and the *Lactobacillus* strains, alternative strategies could include incorporating potentially active probiotic combinations into chilled or frozen probiotic food products or in supplement forms such as tablets and capsules. Another issue to consider is how *P. jensenii* 702 interacts with intestinal microbiota in vivo especially lactobacilli and bifodobacteria. This may be a possible future research avenue. Previous research on human subjects has shown that consumption of *P. freudenreichii* resulted in a significant increase in bifidobacteria population in their fecal samples [4, 13, 42].

Adhesion Assay

Adhesion of probiotics to intestinal epithelial mucosa is one of the main criteria which a micro-organism should fulfil to be considered as a 'probiotic'. Adhesion is crucial for intestinal colonisation by probiotics which is necessary for efficient conferring of their beneficial effects on the host. Bacterial adhesion to intestinal epithelial mucosa is a complicated process involving contact of the bacteria with the surface and it is influenced by multiple surface biophysical and biochemical properties of both bacteria and epithelial mucosa such as passive forces, electrostatic interactions, hydrophobicity, steric forces and specific cellular surface components [43].

Since the entire intestine is lined by a thin layer of mucus produced by the epithelial cells, the ability of probiotic candidates to adhere to the intestinal mucosa in vitro is tested by performing adhesion assay to intestinal cell lines and/or mucus. Previous studies have shown that some dairy propionibacteria have acceptable adhesion ability to both intestinal mucus and epithelial cell lines [14, 24, 33, 44, 49, 50]. There are also few recent works on the adhesion of probiotic combinations including dairy propionibacteria spp to intestinal mucus [7, 33]. However, to our knowledge, there have been no previous studies examining whether adhesion of probiotics to intestinal epithelial cell lines may be influenced by the presence of other probiotic strains.

In the current study, the initial number of probiotic bacteria inoculated into wells has been much more than that adhered to the Caco-2 cells, therefore it could be concluded that all available binding sites on the epithelial cells have been saturated by probiotic bacteria. Our findings also showed that out of five-paired probiotic combinations, three combinations did not show any significant adverse effect on the adhesion ability of both strains in each combination. This may indicate that different strains used in each combination have different adhesion sites on the intestinal epithelial cells. Adhesion percentage of *Lb. casei* 01 and *Lb. rhamnosus* GG to the intestinal epithelial cell line Caco-2 decreased significantly in the presence of *P. jensenii* 702 (Fig. 2). A possible reason for reduction in adhesion rate of these two *Lactobacillus* strains in the presence of *P. jensenii* 702 is that these two bacteria may compete for the same adhesion sites. However these extrapolations should be further elucidated.

Conclusion

Previous research has demonstrated that not all probiotic micro-organisms are identical and none of them possesses all desirable properties. Probiotics have different promoting health effects based on genus, species and strain. Therefore, using combinations or cocktails of probiotics may be an appropriate strategy to confer a broad range of health beneficial effects on the host. However, in preparing probiotic foods/preparations using combinations of probiotics it is necessary to identify possible occurrence of any

potential interactions including synergy or antagonism within combination of probiotics.

Our findings showed that the survival and percentage adhesion of some strains of probiotic may be influenced by the presence of other strains and this should be considered when formulating in the food product. Moreover, it is possible to utilise combinations of different genus/species/strains of probiotic bacteria which may have different health promoting properties conferring more benefits on the host.

Further studies are needed to elucidate the interaction mechanisms and to examine how the probiotic combinations perform in vivo. Particularly, whether or not strains produce inhibitory or growth-promoting substances that could influence the survival and functionality of the co-administered probiotics in the intestinal tract. It is also valuable to examine how probiotic combinations interact with gut microbiota.

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